INVESTIGATION OF TOXIN PRODUCTION, STRESS TOLERANCE AND COMPATIBLE SOLUTE UTILISATION BY BENTHIC CYANOBACTERIA ISOLATED FROM ANTARCTICA

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
Bp	Base pair
°C	Degree Celsius
BI	Bayesian Inference
BLAST	Basic local alignment search tool.
Chl-a	Chlorophyll a
cells mL^{-1}	Cells per millilitre
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleotide acid
D2O	Deuterium Oxide
ELISA	Enzyme-linked immunosorbent assay
d	Day
g	Gram
GG	Glucosylglycerol
h	Hour
MC	Microcystins
Min	Minute
mg	Milligram
ML	Maximum Likelihood
NaCl	Sodium Chloride
nm	Nanometer
NMR	Magnetic Resonance Spectroscopy
NOD	Nodularins
PCR	Polymerase chain reaction
ppt	Parts per thousand
OTU	Operational Taxonomic Units
µg	Microgram
µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer

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KAJIAN PENGHASILAN TOKSIN, TOLERANSI TEKANAN DAN PENGGUNAAN BAHAN LARUT SERASI OLEH SIANOBAKTERIA BENTIK YANG DIPENCIL DARI ANTARTIKA

ABSTRAK

Sianobakteria mendominasi ekosistem Antartika kerana keupayaan penyesuaian luar biasa mereka terhadap pelbagai keadaan, termasuk perubahan saliniti. Walau bagaimanapun, maklumat mengenai tindak balas mereka terhadap tekanan garam masih terhad. Kajian ini bertujuan untuk mengenal pasti strain sianobakteria, menilai penghasilan sianotoksin, dan mengkaji tindak balas mereka terhadap tekanan garam. Sebanyak 17 strain sianobakteria unialgal telah dipencilkan daripada sampel sejuk beku yang diambil dari kolam air lebur, tasik, dan persekitaran hidro-terestrial di Cape Royds, Scott Base, Victoria Valley, dan McMurdo Ice Shelf. Pengenalpastian morfospecies dilakukan dengan memerhati ciri fenotip utama dalam kultur. Di samping itu, analisis molekul gen 16S rRNA menyediakan maklumat tambahan untuk memperkukuh proses pengenalpastian morfologi. Semua strain diperiksa untuk kehadiran gen yang terlibat dalam biosintesis Anatoksin (ATX), cylindrospermopsin (CYN), mikrosistin (MC), dan saxitoksin (STX) menggunakan kaedah PCR. Dua strain, *Phormidesmis priestleyi* (CR147_USMFM) dan *Microcoleus autumnalis* (SB19_USMFM), didapati positif untuk gen mikrosistin (mcyE). Analisis ELISA terhadap ekstrak daripada strain ini mengesahkan kehadiran mikrosistin pada kepekatan <0.15 μg/L dan 0.35 μg/L dalam *P. priestleyi* dan *M. autumnalis*, masing-masing. Sebanyak dua puluh (20) strain, termasuk tujuh belas (17) strain dan tiga (3) strain Antartika tambahan yang disimpan dalam koleksi kultur makmal plankton USM, dinilai untuk toleransi mereka terhadap tekanan garam. Kesemua strain ini

mempamerkan pelbagai toleransi saliniti, dengan kebanyakkan menunjukkan sifat eurihalin. Antara strain tersebut, strain *Phormidesmis* CR147_USMFM menunjukkan toleransi saliniti tertinggi (105 ppt). Sebaliknya, satu strain *Wilmottia* (MIS54_USMFM) menunjukkan sensitiviti garam, yang hanya tumbuh dalam keadaan kawalan (0 ppt). Kehadiran sebatian osmo-perlindungan dalam semua strain ini telah disiasat menggunakan Spektroskopi Resonans Magnet Nuklear (NMR). Analisis menunjukkan bahawa semua strain yang dikaji menggunakan pelbagai osmolit. Menariknya, glisin betain, yang biasanya dikaitkan dengan persekitaran hipersalin, tidak dikesan. Sebaliknya, sukrosa, trehalosa, glukosilgliserol, dan trimetilamina N-oksida (TMAO) dikenal pasti sebagai osmolit utama dalam strain ini. Analisis terhadap dua strain *Phormidesmis* dengan toleransi garam yang berbeza menunjukkan perbezaan jelas dalam komposisi biokimia dan tindak balas terhadap tekanan garam. Penjujukan genom lengkap memberikan maklumat tentang ciri genomik dan gen yang berkaitan dengan penyesuaian terhadap garam. Strain CR11a_USMFM mempunyai saiz genom 5.2 Mb, kandungan G+C sebanyak 50.47%, dan 4,838 CDS. Strain CR147_USMFM pula mempunyai saiz genom 5.7 Mb, kandungan G+C sebanyak 52.58%, dan 5,448 CDS. Analisis genom menunjukkan bahawa CR11a_USMFM mempunyai gen (treY dan treZ) yang terlibat dalam sintesis trehalosa, manakala CR147_USMFM memiliki gen (stpA, ggpS, TreY, dan TreZ) yang bertanggungjawab untuk biosintesis glukosilgliserol dan trehalosa. Strain CR11a_USMFM mempunyai gen yang terlibat dalam pengangkutan dan pengawalan kation (aqpZ, kdpA-D, nhaP, dan trkA), manakala strain CR147_USMFM menunjukkan gen trkA/G dan nhaP tanpa sistem kdp. Penemuan ini menyumbang kepada pemahaman tentang adaptasi fisiologi dan genomik sianobakteria Antartik terhadap tekanan garam.

INVESTIGATION OF TOXIN PRODUCTION, STRESS TOLERANCE AND COMPATIBLE SOLUTE UTILIZATION BY BENTHIC CYANOBACTERIA ISOLATED FROM ANTARCTICA

ABSTRACT

Cyanobacteria dominate the Antarctic ecosystem due to their remarkable adaptability to diverse conditions, including changing salinity. However, limited information exists about their response to salt stress. This study aimed to identify cyanobacterial strains, assess cyanotoxin production, and examine their salt stress response. A total of 17 unialgal cyanobacteria strains were isolated from deep freeze mat samples collected from meltwater ponds, lakes, and hydro-terrestrial environments in Cape Royds, Scott Base, Victoria Valley, and McMurdo Ice Shelf. Morphospecies identification was performed by observing major phenotypic features in the culture. In addition, molecular analysis of 16S rRNA genes provided complementary information to enhance the morphological identification process. All strains were examined for the presence of genes involved in the biosynthesis of Anatoxins (ATXs), cylindrospermopsins (CYNs), microcystins (MCs), and saxitoxins (STXs) using PCR-based methods. Two strains, *Phormidesmis priestleyi* (CR147_USMFM) and *Microcoleus autumnalis* (SB19_USMFM), tested positive for microcystin gene (mcyE). ELISA analysis of extracts from these strains confirmed the presence of microcystin at concentrations at <0.15 μg/L and 0.35 μg/L in *P. priestleyi* and *M. autumnalis*, respectively. A total of twenty (20) strains, consisting of seventeen (17) strains and three (3) additional Antarctic strains deposited in the USM Plankton laboratory culture collections, were evaluated for their tolerance to salt stress. The

strains exhibited a wide range of salinity tolerance, with many displaying euryhaline. Among the strains, *Phormidesmis* strain CR147_USMFM demonstrated the highest salinity tolerance (105 ppt). Conversely, a strain of *Wilmottia* (MIS54_USMFM) showed salt sensitivity, growing only under control conditions (0 ppt). The presence of osmoprotective compounds in these strains was investigated using Nuclear Magnetic Resonance Spectroscopy (NMR). The analysis revealed that the studied strains utilized various osmolytes. Interestingly, glycine betaine, typically associated with hypersaline environments, was not detected. Instead, sucrose, trehalose, glucosylglycerol, and trimethylamine *N*-oxide (TMAO) were identified as the predominant solutes in these strains. The analysis of two Phormidesmis strains with varying salt tolerance revealed distinct differences in biochemical composition and contrasting responses to salt stress. Whole genome sequencing provided insights into their genomic characteristics and salt acclimation-related genes. Strain CR11a_USMFM had a genome size of 5.2 Mb, G+C content of 50.47%, and 4,838 CDS. Strain CR147_USMFM had a genome size of 5.7 Mb, G+C content of 52.58%, and 5,448 CDS. Genomic analysis showed that CR11a_USMFM possessed genes (*treY* and *treZ*) involved in trehalose synthesis, while CR147_USMFM harboured genes (*stpA, ggpS, TreY*, and *TreZ*) responsible for glucosylglycerol and trehalose biosynthesis. Strain CR11a_USMFM possessed genes involved in cation transport and regulation (*aqpZ, kdpA-D, nhaP*, and *trkA*), while strain CR147_USMFM exhibited *trkA*/*G* and *nhaP* genes without the *kdp* systems. These findings contribute to our understanding of the physiological and genomic adaptations of Antarctic cyanobacteria to salt stress.

CHAPTER 1

INTRODUCTION

1.1 The context of this study

Cyanobacteria, commonly referred to as blue-green algae, are ancient photosynthetic prokaryotes that have significantly influenced Earth's biosphere. Fossil evidence suggests their existence for over 3.5 billion years (Oliver *et al.,* 2023), during which they played an important role in the oxygenation of Earth's atmosphere, an event known as the Great Oxygenation Event (Schirrmeister *et al.,* 2013). This process transformed the planet's biochemistry, enabling the emergence of aerobic life forms and fostering biodiversity (Lewis, 2017).

Cyanobacteria exhibit unique metabolic versatility, enabling them to thrive in diverse environments, from freshwater and marine ecosystems to extreme habitats such as deserts, geothermal springs, and Polar regions (Pelaez *et al.,* 2010; Pathak *et al.,* 2022). This adaptability is largely attributed to their ability to utilize various survival mechanisms, including nitrogen fixation, synthesis of secondary metabolites, and production of osmoprotective compounds. Their ecological significance extends to their role as primary producers and contributors to biogeochemical cycles, particularly carbon and nitrogen cycling.

Cyanobacteria are prevalent in Polar regions, where they can be found within ice, on snow surfaces, in freshwater and saline lakes and streams, as well as in soils, and even within rocks (Taton *et al.,* 2003; Zakhia *et al.,* 2008). In Antarctic ecosystems, cyanobacteria dominate microbial communities, forming extensive benthic mats in mats in lakes, streams, and hydro-terrestrial habitats (Vincent &

Quesada, 2012). These mats are critical to the region's primary productivity, supporting local food webs and biogeochemical processes in environments where higher plants are largely absent. Despite extreme abiotic stressors such as sub-zero temperatures, prolonged darkness, desiccation, limited nutrient availability and fluctuating salinity, cyanobacteria demonstrate remarkable resilience through the production of antifreeze proteins, compatible solutes, and robust photosynthetic mechanism (Pandey *et al.,* 2004).

Antarctic cyanobacteria are promising candidates for biotechnological innovation due to their ability to produce a diverse array of bioactive secondary metabolites (Singh *et al.,* 2005; Cornet *et al.,* 2018). These compounds contribute to their survival in extreme environments and hold potential for pharmaceutical and industrial applications (Velichko *et al.,* 2021). Studies have demonstrated that several strains isolated from Antarctic microbial mats exhibit antimicrobial, antifungal, and antitumor properties (Biondi *et al.,* 2008). Moreover, certain strains produce cyanotoxins, including microcystins and cylindrospermopsins, which may influence their interactions within microbial communities and contribute to their ecological success (Kleinteich *et al.,* 2014).

Despite advances in understanding Antarctic cyanobacteria, several challenges continue to hinder comprehensive insights into their biology and ecological roles. Research has predominantly focused on diversity assessments, employing morphological and metagenomic approaches (Velichko *et al.,* 2021). Although these methods have provided valuable data, inconsistencies between morphology-based classifications and molecular phylogenies have frequently been reported. This divergence complicates taxonomic resolution and highlights the limitations of relying solely on either morphological or genetic approaches.

A significant constraint is the limited availability of cultured Antarctic cyanobacterial strains. Culturing these organisms under laboratory conditions remains a considerable challenge, with only a small proportion successfully maintained for experimental studies. This limitation impedes investigations into physiological responses, biochemical adaptations, and genomic traits. Furthermore, the logistical difficulties of conducting fieldwork in Antarctica, including extreme environmental conditions and restricted accessibility, significantly limit sample collection efforts and the establishment of diverse culture collections.

The physiological and genomic mechanisms underlying cyanobacterial tolerance to salinity in Antarctic ecosystems remain poorly understood. Salinity, a key abiotic factor, plays a critical role in shaping the distribution and activity of microbial communities in these environments (Jungblut *et al.,* 2005). However, the adaptations that enable cyanobacteria to survive and thrive under variable salinity conditions are largely unexplored.

Overcoming these limitations requires integrative methodologies that combine morphological, molecular, physiological, and genomic approaches. By addressing these gaps, this study seeks to provide novel insights into the ecological and biochemical and genomic adaptations of Antarctic cyanobacteria and could contribute to the discovery of bioactive compounds with potential therapeutic and industrial applications. The establishment of a unialgal culture collection from the cryopreserved samples is a significant outcome of this study, offering a robust platform for detailed investigations into cyanotoxin biosynthesis, salinity tolerance, and osmoprotective solute production.

1.2 Project aims and an overview of the structure of this thesis.

This study aims to investigate toxin production, the salinity stress tolerance and accumulation of compatible solutes in benthic cyanobacteria isolated from Antarctica. The specific objectives are

1. To establish unialgal cultures of cyanobacteria and identify them using morphological characteristics and molecular techniques, specifically employing the 16S rRNA gene as the molecular marker.

2. To screen the established cultures for the presence of cyanotoxin biosynthesis genes.

3. To assess the physiological responses of cyanobacterial strains to varying salinity levels.

4. To explore the genomic characteristics of selected strains related to salt acclimation.

Chapter 2 describes the isolation and establishment of cyanobacteria unialgal cultures. The isolated strains are characterised and precisely identified using a combination of morphological and molecular approaches, including 16S rDNA gene analysis.

Chapter 3 describes the screening procedure used to identify the presence of genes related to cyanotoxin production on the isolated cyanobacterial strains.

Chapter 4 investigates the effect of different levels of salt stress on the growth of cyanobacteria. The stress tolerance limits and the analyses of the compatible solutes accumulated by each strain were carried out using NMR.

Chapter 5 explores the impact of salinity stress on growth, biochemical composition, and accumulation of compatible solutes in two halotolerant *Phormidesmis* strains. The whole genome sequencing was carried out to examine the molecular mechanisms underlying salt adaptation in these strains.

Chapter 6 briefly summarises and discusses the main findings from the study, and potential future research directions.

This research has increased our knowledge of the adaptation mechanisms of cyanobacteria to salt stress and their ecological significance in extreme environments.

CHAPTER 2

IDENTIFICATION OF BENTHIC CYANOBACTERIA ISOLATED IN THIS STUDY

2.1 Introduction to cyanobacteria

Cyanobacteria commonly known as the blue green algae constitute a group of diverse morphological forms, from unicellular to colonial, simple filaments, and differentiated multicellular filamentous forms (Schirrmeister *et al.,* 2013). Morphologically, unicellular cyanobacteria can occur as a single cell or in aggregations, free in their medium or attached to substrata (De Los Ríos *et al.,* 2007). Filamentous forms may exist as a single trichome, often a thick or thin sheath could surround a bundle of trichomes. Some filamentous cyanobacteria form branches and produced specialised cells; akinetes and heterocytes (Schirrmeister *et al.,* 2013).

Ecologically, cyanobacteria can survive exposure to an extremely wide range of environmental conditions, including temperature (Zakhia *et al.,* 2008; Vincent & Quesada, 2012), light intensity (Śliwińska-Wilczewska, *et al.,* 2019), pH (Steinberg *et al.,* 1998; Lopez-Archilla *et al.,* 2004) and salinity (Mackay *et al.,* 1984; Jungblut *et al.,* 2005; Regueiras *et al.,* 2018; Olofsson *et al.,* 2020). This versatility underlines the group's cosmopolitan distribution, allowing them to inhabit almost all types of habitats, ranging from hydrothermal lakes (Momper *et al.,* 2019), to deserts (Lacap-Bugler *et al.,* 2017), acidic tropical soils (Seiderer *et al.,* 2017), Antarctic lakes (Taton *et al.,* 2003; Pessi *et al.,* 2023) and hydro-terrestrial environments (Richter, 2018; Hirose *et al.,* 2020).

Cyanobacteria are the leading primary producers in aquatic and hydro-terrestrial ecosystems (Richter, 2018; Hirose *et al.,* 2020) and play a significant role in nutrient cycling (Kumar *et al.,* 2015; Van Goethem & Cowan, 2019). Some cyanobacteria, such as members of the order Nostocales, Stigonematales and some non-heterocystous taxa (e.g., the filamentous genera *Trichodesmium* and *Lyngbya* and the unicellular *Gloeothece* and *Cyanothece*) can fix atmospheric nitrogen (Fujita & Uesaka, 2022).

Apart from their key role as primary producers, cyanobacteria produce a wide variety of bioactive secondary metabolites (Regueiras *et al.,* 2018; Velichko *et al.,* 2021). These metabolites are vital for their survival, enabling them to adapt to various environmental conditions (Regueiras *et al.,* 2018). These compounds include photoprotective metabolites such as scytonemin (Wada *et al.,* 2013), enzymatic antioxidants (such as superoxide dismutases (SOD), catalases and peroxidases), non-enzymatic antioxidants (such as carotenoids, phycobiliproteins, tocopherols and ascorbic acid) (Kultschar & Llewellyn, 2018) and toxic metabolites known as cyanotoxins (Chorus & Bartram, 1999;). Many of these compounds are of interest in terms of their potential use in cosmeceutical and pharmaceutical applications, for instance through their antifungal, antibacterial, antiviral, and anticancer properties (Singh *et al.,* 2017; Divyashree *et al.,* 2019).

2.2 Taxonomy of Cyanobacteria

The taxonomy of cyanobacteria has long been a subject of complexity and on-going revision (Komárek, 2018). Traditionally, cyanobacteria have been classified based on morphological traits, such as cell shape, polarity, division patterns, color, and the presence of specialized cells like heterocysts and akinetes (Valerio *et al.,* 2009). Historically grouped as algae, cyanobacteria were classified according to the

International Code of Botanical Nomenclature (ICBN), now known as the International Code of Nomenclature for Algae, Fungi, and Plants (ICN) (Oren & Ventura, 2017).

This morphology-based classification placed species with similar features into the same taxonomic group (Komárek & Anagnostidis, 2005). However, many characteristics such as cell size, color, and sheath presence can vary significantly under different environmental conditions, making identification challenging (Rippka *et al.,* 1979). Additionally, subjective assessments of these traits often lead to inconsistent classifications (Sciuto & Moro, 2015), necessitating frequent re-evaluation of taxonomic groups.

In 1925, Geitler proposed a classification system that divided cyanobacteria into seven orders: Chroococcales, Entophysalidales, Pleurocapsales, Dermocarpales, Siphononematales, Nostocales, and Stigonematales. Subsequent revisions, including those by Desikachary (1959) and Drouet (1968, 1978), reshuffled many taxa, reducing them to four orders: Synechococcales, Oscillatoriales, Spirulinales, and Nostocales (Komárek & Johansen, 2015).

In 1978, Stanier introduced a bacteriological approach, classifying cyanobacteria based on their prokaryotic nature, considering morphological, physiological, and genetic traits of axenic and clonal strains (Waterbury & Stanier, 1977; Rippka *et al.,* 1979). However, replacing the ICBN with the International Code of Nomenclature of Bacteria (ICNB), now the International Code of Nomenclature of Prokaryotes (ICNP), proved challenging (Stanier *et al.,* 1978).

While the bacteriological approach provided valuable insights, it often underestimated cyanobacterial diversity, limiting its use in ecological studies (Whitton, 2011; Palinska & Surosz, 2014). The five-section classification system recommended

by *Bergey's Manual of Systematic Bacteriology* divides cyanobacteria into the following: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonematales (Rippka *et al.*, 1979; Komárek *et al.*, 2014).

The coexistence of two nomenclatural systems—botanical and bacteriological—further complicates cyanobacterial taxonomy (Oren & Garrity, 2014; Oren, 2014). Currently, names listed under the ICBN for blue-green algae are not recognized under the Bacteriological Code (Palinska & Surosz, 2014). The differences between the two systems create difficulties in integrating them for consistent classification (Johansen & Casamatta, 2005; Komárek, 2016).

To address these challenges, a polyphasic approach has been proposed. Polyphasic approach integrates morphological, molecular, and ecological data to achieve robust cyanobacterial identification. (Komárek, 2018). This comprehensive approach, championed by Komárek & Anagnostidis (1999, 2005) and later Komárek (2013, 2016, 2018), integrates both botanical and bacteriological data to create a more accurate classification system. It has been widely adopted in modern cyanobacterial taxonomy (Bagchi *et al.*, 2017; Radzi *et al.*, 2021; Strunecký *et al.*, 2023). The use of molecular data, particularly phylogenetics, alongside traditional morphology has helped redefine taxon boundaries, leading to the discovery of numerous new species (Whitton, 2008; Komárek, 2018). In this classification scheme, the phylum cyanobacteria are currently divided into eight orders (Table 2.1).

Table 2. 1 Cyanobacteria classification system for Orders and Families based on Komárek, 2014, 2016, 2018.

No.	Order	Families
I	Gloeobacterales	Gloeobacteraceae

No.	Order	Families
II	Synechococcales	Acaryochloridaceae, Coelosphaeriaceae, Chamaesiphonaceae, Heteroleibleiniaceae, Leptolyngbyaceae, Merismopediaceae Prochloraceae, Pseudanabaenaceae, Romeriaceae, Schizotrichaceae, Synechococcaceae
III	Spirulinales	Spirulinaceae
IV	Chroococcales	Aphanothecaceae, Chroococcaceae, Cyanobacteriaceae, Cyanothrichaceae, Entophysalidaceae, Gomphosphaeriaceae, Microcystaceae, Stichosiphonaceae
V	Pleurocapsales	Dermocarpellaceae, Hydrococcaceae, Pleurocapsaceae, Xenococcaceae
VI	Oscillatoriales	Borziaceae, Coleofasciculaceae, Cyanothecaceae, Gomontiellaceae, Homoeotrichaceae,

No.	Order	Families
		Homoeotrichaceae, Microcoleaceae, Oscillatoriaceae
VII	Chroococcidiopsidales	Chroococcidiopsidaceae
VIII	Nostocales	Aphanizomenonaceae, Capsosiraceae, Chlorogloeopsidaceae, Godleyaceae, Gloeotrichiaceae, Hapalosiphonaceae, Nostocaceae, Rivulariaceae, Scytonemataceae, Stigonemataceae, Symphyonemataceae, Tolypothrichaceae

2.3 Cyanobacteria in Antarctica

Cyanobacteria are the dominant phototroph in Antarctic freshwater and terrestrial environments. They form extensive biomass of microbial communities in benthic lakes, ponds, ice-free surfaces, and other hydro-terrestrial habitats (Taton *et al.,* 2003; Rego *et al.,* 2019). Their presence contributed significantly to primary productivity by supplying enough carbon through photosynthesis, which enhanced biodiversity and supported carbon-nitrogen cycles (Van Goethem & Cowan, 2019).

Antarctica is characterised by extreme environmental conditions including low temperature, freeze and melting cycle, desiccation salinity, limited organic nutrients, low humidity, and extended period of darkness (Pandey *et al.,* 2004; Kvíderová *et al.,*

2019). Despite these harsh conditions, cyanobacteria exhibit remarkable dominance and adaptive success in the Antarctic ecosystem (Rego *et al.,* 2019).

Identification of Antarctic cyanobacteria has previously been based on observation from field specimens, this is partly due to the lack of sufficient data from culture isolates attributed to the difficulties of cultivating them (Broady, 1996). Attempts were made by Broady & Kibblewhite (1991) to apply the traditional identification method to study the Antarctic cyanobacteria using field specimens and cultured isolates. Several culture-dependent studies have been conducted since, applying traditional identification methods on Antarctic cyanobacterial (Radzi *et al.,* 2019; Rego *et al.,* 2019; Zaki *et al.,* 2020).

While molecular techniques such as metagenomics analysis have been employed to investigate the genotypic diversity of Antarctic cyanobacteria, these studies present several Operational Taxonomic Units (OTU) but lack comprehensive morphological and ecological data (Taton *et al.,* 2003; Taton *et al.,* 2006; De los Rios *et al.,* 2007; Komárek, 2010). Moreover, many studies have relied heavily on field observations, with limited use of cultured isolates, hindering a deeper understanding of these microorganisms.

To address these challenges, a comprehensive Antarctic cyanobacteria collection is needed. The collection of Antarctic cyanobacteria is crucial due to the unique environmental conditions of the continent, which contribute to the evolution of distinct microbial communities. These organisms play a key role in primary productivity and nutrient cycling in extreme environments, where they are often the dominant phototrophs (Taton *et al.,* 2003; Rego *et al.,* 2019). By establishing a comprehensive collection of Antarctic cyanobacteria, researchers can further study their adaptations, ecological functions, and potential biotechnological applications.

Furthermore, understanding their taxonomy and evolution can inform broader studies on microbial life in extreme environments globally.

In this study, a polyphasic approach was adopted to overcome the limitations of relying solely on morphology for cyanobacterial identification. This method integrates morphological assessments with molecular techniques, specifically targeting the 16S rRNA gene, a well-established marker in microbial taxonomy (Strunecký *et al.,* 2013). The use of 16S rDNA is justified by its ubiquity in cyanobacteria and its capacity to provide phylogenetic resolution, making it a cornerstone for understanding microbial diversity. Morphological traits alone are often insufficient due to the plasticity of cyanobacterial characteristics, which can vary significantly with environmental conditions. By combining these methods, this study ensures robust identification and classification, particularly for Antarctic cyanobacteria, whose unique habitats contribute to their diversity and potential adaptations.

The decision to focus on 16S rDNA was informed by its widespread acceptance and its ability to delineate species boundaries effectively in cyanobacteria. For example, studies such as Casamatta *et al.,* (2005), Taton *et al.,* (2003), Jungblut *et al.,* (2010), Komárek (2010), Komárek *et al.,* (2014), and Strunecký *et al.,* (2023) demonstrated how 16S rDNA analysis, integrated with morphological observations, enhances the resolution of taxonomic ambiguities. While alternative molecular markers, like *rpo*B, offer complementary insights, 16S rDNA remains the primary tool for microbial phylogenetics due to its extensive reference database and evolutionary stability.

This study seeks to address the challenges of isolation and accurately identify and characterize the diversity of cyanobacteria within Antarctic benthic mat samples.

The current reliance on morphological characteristics alone limits our ability to fully understand these important microorganisms, particularly in extreme environments.

Cyanobacterial taxonomy, particularly the reliance on solely morphological characteristics, hinder our understanding of these crucial microorganisms in extreme environments.

The samples used in this study were collected from various Antarctic freshwater and hydro-terrestrial environments. These cyanobacterial mat samples, originally gathered by Dr. Paul A. Broady from the University of Canterbury, New Zealand, were stored and supplied by Dr. P. Novis (Landcare Research Limited, New Zealand). The collection spanned multiple expeditions between 1984 and 1991, focusing on Ross Island (e.g., Cape Royds and Scott Base), Victoria Valley, and the McMurdo Ice Shelf. Each sample was cryopreserved at -20°C until used in this study. These preserved samples are essential for exploring cyanobacterial diversity over time, especially considering changes in environmental conditions and potential ecological shifts.

Specifically, the mats were collected from nine freshwater bodies on Ross Island, three hydro-terrestrial mats in Victoria Land, and two ponds on the McMurdo Ice Shelf. Despite the long-term storage, cyanobacteria's resilience allows for successful isolation, contributing valuable data to the understanding of Antarctic microbial ecosystems.

2.4 Aims and Objectives

1) To establish unialgal cultures of cyanobacteria from cryopreserved benthic environmental mat samples

2) To identify cyanobacteria encountered in the field samples and culture isolates based on morphology and molecular phylogenetic analysis using 16S rDNA.

2.5 Materials and Methods

2.5.1 Samples origin

The Antarctic cyanobacteria samples used in this study originate from a series of expeditions between 1984 and 1991(Figure. 2.1). These mats were collected from diverse environments, including Cape Royds (77°33' S, 166°09' E) on Ross Island and Victoria Valley in Victoria Land (77°23' S, 162°00' E) (Table 2.2). A total of nine samples were obtained from lakes and ponds on Ross Island, including the Coast Lake and Snow Pond, while other samples came from ponds on the McMurdo Ice Shelf and Scott Base. These samples have been cryopreserved at -20°C, which, combined with cyanobacteria's inherent dormancy mechanisms, has preserved their viability for decades. This long-term storage offers a unique opportunity to study microbial life in extreme conditions across both time and space.

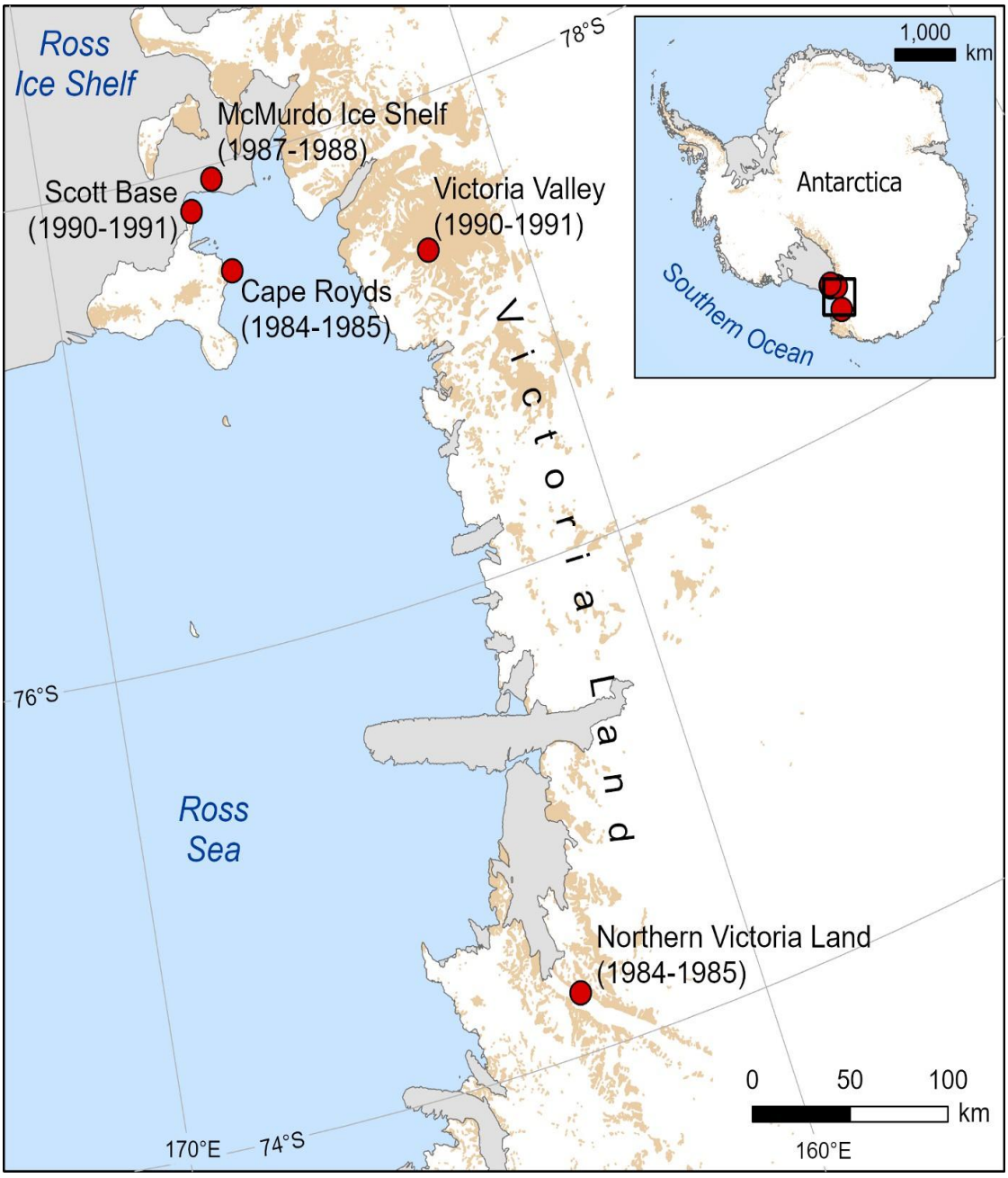

Figure 2. 1 Location of the study site. Sampling localities and the year of collection are shown by red circles.

Table 2. 2 Sampling localities, date of collection, mat sample ID sample descriptions and coordinates

Sample location and year of collection	Sample ID	Original description of field-collected material	Latitude/longitude
Ross Island			
Cape Royds (1984-1985)	10,11	Cyanobacterial mat in Coast Lake	77° 33' 00.0" S, 166° 09' 00.0" E
	46,47	Cyanobacterial mat in "Snow Pond"	
Cape Royds (1986-1987)	147	Brown cyanobacterial mat in trickle stream from a snowdrift	
	175	Black crusts on moss, green filaments, thin grey-brown mats	
	181	Red-brown cyanobacterial mat in a large pond	
Scott Base (1990-1991)	19	Cyanobacterial mats in Pond by "Cos-Ray" huts.	77° 50' 00.0" S, 166° 45' 00.0" E
	30	Grey-brown cyanobacterial mat in melt trickle at the north end of Geophysics Lab.	
Victoria Valley (1990-1991)	62	Purple-red cyanobacterial mat in southernmost pools, Upper Victoria Lake.	77° 23' 00.0" S, 162° 00' 00.0" E
	141, 142	Thin cyanobacterial mats amongst stones	
	144	Cyanobacterial mats, downslope from small snowfields, dry rivulet	
McMurdo Ice Shelf			

Sample location and year of collection	Sample ID	Original description of field-collected material	Latitude/longitude
McMurdo Ice Shelf (1987-1988)	38	Red-brown cyanobacterial mats in "*Nostoc* Pond"	77° 56' 29.0" S, 166° 09' 45.3" E
	54	Thin greyish, shoreline cyanobacterial mat in Pond "P70",	

2.5.2 Media preparation

The general medium used for cultivation in this study is BG-11, also known as blue-green medium (Rippka *et al.,* 1979). BG-11 and BG-11_o (lacking chemically combined nitrogen) were prepared by mixing distilled water and nutrients important for cyanobacterial growth (Table 2.3). The media was supplemented with 100 µg/ml cycloheximide to prevent the growth of eukaryotes (Bolch & Blackburn, 1996). Both liquid and agarose media were prepared as described below (Rippka *et al.,* 1979).

Table 2. 3 Composition of BG-11 Medium for blue-green algae.

Component	Stock solution ($g \cdot L^{-1}$ dH2O)	Quantity Used
$NaNO_3$	150	10 mL
$K_2HPO_4 \cdot 3H_2O$	40	1 mL
$MgSO_4 \cdot 7H_2O$	75	1 mL
$CaCl_2 \cdot 2H_2O$	36	1 mL
Citric Acid	6	1 mL
Ferric Ammonium Citrate	6	1 mL
$MgNa_2EDTA \cdot H_2O$	1	1 mL
Na_2CO_3	20	1 mL
Trace Metals Solution		1 mL
H_2BO_3	2.86 g	

Component	Stock solution ($g \cdot L^{-1}$ dH2O)	Quantity Used
MnCl2 · 4H2O	1.81 g	
$ZnSO_4 \cdot 7H_2O$	0.22 g	
$NaMoO_4 \cdot 5H_2O$	0.39 g	
$CUSO_4 \cdot 5H_2O$	0.079 g	
Co $(NO_3)_2 \cdot 6H_2O$	0.049 g	

For 1-liter agarose media, the stock solution listed in Table 3 above was mixed in Schott bottles following the volume in the recipe. Distilled water was added to the mixture to get a final volume of 500 mL. In a separate Schott bottle, 1% agar was prepared by adding 10 g of agar powder to 250 mL of distilled water. The two solutions were autoclaved separately at 121 ℃ for 15 min. After cooling to about 50ºC-60ºC, the autoclaved solutions were then mixed. Cycloheximide solution was then prepared by dissolving 0.1 g in 250 mL distilled water (100 µg/l). The cycloheximide solution was filter-sterilised using a 0.25 µm pore filter and then added to the agar mixture solution, giving the final volume to a 1 L medium. The media was then poured onto sterile 90 mm Petri dishes and allowed to solidify. The preparation was carried out in the lamina flow cabinet. Following solidification, the agar plates were inverted, sealed in airtight plastic bags, and stored at 4°C.

Liquid media was prepared in a Schott bottle by adding the stock solutions (Table 2.3) and the mixture was then topped up with distilled water to 750 mL (Table 2.1). The solution was then autoclaved as described above. After cooling, 250 mL of filter-sterilised cycloheximide solution at a concentration of 100µg/mL was added, making the final volume of 1 L. The pH of the medium was adjusted to pH 7. To prevent contamination, the media were tightly sealed until future usage.

2.5.3 Culture establishment

Frozen mat samples were thawed overnight in an incubator at 18°C. A portion of the mats was retrieved and then placed onto the slide using sterile loops before covering it with coverslips. Samples were observed under a compound microscope (Olympus BX53) at 100–2000X magnification. All the mat samples were thoroughly screened for the presence of cyanobacteria. Selection of the mat samples was conducted based on the abundance and diversity of cyanobacteria observed from the initial screening.

A sterile streaking loop was used for inoculation of the sample material by placing a small amount of the samples onto solidified agar and streaking thoroughly. Triplicate plates were prepared and sealed using Parafilm to avoid contamination and evaporation. The plates were then incubated for 3-4 weeks under 24 h light at 18°C. Illumination was provided by white fluorescent lamps with 27.03µmol photon m^{-2} s^{-1} light intensity. The plates were checked regularly for visible growth colonies. The streaking procedure was repeatedly applied until unialgal cultures were obtained. Unialgal cultures were then utilised for morphological description as well as DNA extraction for molecular analysis of 16S rDNA. Due to the poor conditions of cells in most of the field samples, the descriptions presented here are based on cultures.

2.5.4 Morphological identification

Morphological examination of isolated strains was conducted using an Olympus BX53 microscope at 100–2000× magnifications. Important morphological features were recorded, including trichome colour and shape, cell shape, length, width, and granulation, apical cell shape, presence or absence of calyptra, necridic cells and sheath. For each morphospecies, photomicrographs and descriptions, including size

measurements of 30 specimens, were recorded. Identifications were made following the keys by Komárek & Anagnostidis (2005) Komárek *et al.,* (2009) and Komárek, 2013).

2.5.5 DNA extraction, PCR amplification and sequencing of 16S rDNA

A subsample of 0.1 g biomass harvested from a culture of each strain was placed in a 1.5 mL microcentrifuge tube. Genomic DNA was extracted from this using the Intron G-spin™ Total DNA Extraction Mini Kit for bacteria (iNtRON Biotechnology, Inc., Korea) following the manufacturer's protocol. DNA was stored frozen at -20°C before PCR. Its quantity and purity were determined by spectrophotometry using a Nanodrop Quawell UV Spectrophotometer Q3000.

Amplification of the 16SrRNA gene was by the polymerase chain reaction (PCR) using generic primers for cyanobacteria. A combination of primers 2 (5'- GGG GGA TTT TCC GCA ATG GG - 3') and 3 (5'- CGC TCT ACC AAC TGA GCT A - 3') of Boyer *et al.* (2001) was used to amplify approximately 1300 bp of the 16S rRNA gene segment. All PCR reactions were carried out in a 200 µL reaction tube containing 25 µL of MyTaq™ Red Mix, a pre-prepared mixture of buffer, dNTPs and Taq polymerase (Bioline, United Kingdom), 2 µL of each forward and reverse primer, 2 µL of the template DNA and 19 µL of Milli-Q water, giving a total volume of 50 µL. A control tube containing the reaction mixture without a DNA template was included as a negative control.

PCR was carried out using a BioRad T100 Thermal Cycler at 94°C for 4 min for pre-denaturation, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 20 s, and a final extension at 72°C for 7 min. PCR products were then visualized using 1% agarose gel electrophoresis, stained with 1 µL RedSafe Nucleic Acid Staining Solution. The size of the DNA fragments was

assessed using Syngene GeneFlash Bio-Imaging Gel Documentation UV/VVIS using a commercial ready-to-load 100 bp DNA Ladder (Bioneer). PCR products of the expected length were then purified using the MEGAquick-spinTM Total Fragment DNA Purification Kit. The purified PCR product was sequenced commercially by BIONEER Corporation (Daejeon, South Korea). Sequences obtained were compared with other sequences available in the NCBI GenBank database via BLAST to aid identification (Basic Local Alignment Search Tool; Altschul *et al.*, 1990). Following Schmidt *et al.*, (2014), we accepted the frequently used similarity threshold of 97.5% for assigning sequences into Operational Taxonomic Units (OTUs). Relevant sequences from GenBank which had high similarity (97.5%) were downloaded and used in phylogenetic analysis.

The downloaded sequences together with the sequences obtained in this study were edited and assembled using the software package Geneious 11.0 (Biomatters, http://www.geneious.com). Sequences were prepared using the MUSCLE algorithm in Geneious 11.0 and then manually verified by eye.

The phylogenetic tree was constructed and analysed using the Maximum likelihood (ML) and Bayesian inference (BI) methods. Kakusan4 program (Kenehisa *et al.*, 2011) was employed to determine the best-fit model of DNA substitution before running ML and BI analyses. ML analyses were carried out with RaxML v7 (Stamatakis, 2006) in Geneious 11.0 using the general time-reversible invariant-sites (GTRI) nucleotide substitution model with the default parameters. The probability of each branch was calculated via bootstrapping using 1,000 replications. Additionally, the program MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) was used to perform BI analyses. Two independent analyses, each consisting of four Markov chains were run

concurrently for 2,000,000 generations, sampling every 100 generations. Tracers ver. 1.5 (Rambaut & Drummond, 2009) program was used to evaluate the Log-likelihood and parameter values. A burn-in of 25% of saved trees was removed, and the remaining trees were used to calculate the Bayesian posterior probability values. The program FigTree v1.3.1 (Ronquist & Huelsenbeck, 2003) was used in editing both the ML and BI trees.