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Metagenomic analyses of pristine, Arctic environments for the risk assessment – evaluation of antibiotic resistance genes presence

by
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Thesis presented to Higher School of Biotechnology of the Catholic University of Portugal to fulfil the requirements of Master of Science degree in

Applied Microbiology

by
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RESUMO

A resistência antimicrobiana é um dos principais desafios globais para a saúde pública, sendo essencial compreender sua origem e disseminação em diferentes ecossistemas. Este estudo investigou a diversidade microbiana e os genes de resistência a antibióticos em solos do Ártico, um ambiente extremo e pouco explorado. Foram utilizadas abordagens microbiológicas clássicas e metagenômicas para caracterizar a microbiota e avaliar a presença de genes de resistência a antibióticos. Os resultados revelaram uma diversidade microbiana significativa e a presença de genes de resistência, mesmo em áreas sem impacto antrópico aparente. A análise metagenômica indicou que a resistência pode estar associada a mecanismos naturais de adaptação microbiana, reforçando a necessidade de monitoramento ambiental para compreender melhor os riscos da disseminação de resistência antimicrobiana. Estes achados contribuem para a avaliação do risco ecológico da resistência antimicrobiana e a bioprospecção de novos compostos bioativos.

Palavras-chave: Antibiótico, bactéria, metagenômica, solos árticos, genes de resistência, microbiota.

ABSTRACT

Antimicrobial resistance is a major global public health challenge, and it is essential to understand its origin and spread in different ecosystems. This study investigated microbial diversity and antibiotic resistance genes in soils from the Arctic, an extreme and underexplored environment. Classical microbiological and metagenomic approaches were used to characterize the microbiota and assess the presence of antibiotic resistance genes. The results revealed significant microbial diversity and the presence of resistance genes, even in areas with no apparent anthropogenic impact. The metagenomic analysis indicated that resistance may be associated with natural mechanisms of microbial adaptation, reinforcing the need for environmental monitoring to better understand the risks of antimicrobial resistance spread. These findings contribute to the assessment of the ecological risk of antimicrobial resistance.

Keywords: Antibiotics, bacteria, metagenomics, arctic soils, resistance genes.

INDEX

ABBREVIATIONS & SYMBOLS

AMR - Antimicrobial Resistance

ARG - Antibiotic resistance genes

bp - base pairs

CFU - Colony-forming unit

dd water - double-distilled water

EPS - Extracellular polymeric substances

IC - Initial Concentration

MRG - Metal resistance genes

MDR - Multidrug-resistant(ce)

MGE - Mobile Genetic Elements

MIC - Minimal inhibitory concentration

NGS - Next generation sequencing

PCR - Polymerase chain reaction

qPCR - Quantitative pcr

RPKM - Reads Per Kilobase per Million

TOC - Total organic carbon

UV - Ultra Violet

WHO - World health organization

µ - micro

1. INTRODUCTION

Bacterial resistance represents one of the greatest threats to contemporary global health, directly impacting the ability to treat infections and compromising the effectiveness of modern medical procedures. With the emergence of common infections that can once again become lethal, there is an urgent need to understand the mechanisms that underpin microbial resistance and to develop new therapeutic approaches. This scenario highlights the relevance of exploring unexplored and isolated environments, such as polar regions, deep caves and other extreme ecosystems, which have proven to be true repositories of bacterial genetic diversity.

Recent studies have shown that these ecosystems, often overgrown, harbour a unique genetic wealth, including new antimicrobial resistance (AMR) mechanisms and potential producers of as yet unknown antimicrobial compounds. Furthermore, investigating the origin and evolution of resistance mechanisms in extreme environments can provide valuable insights to better understand how these mechanisms emerge, spread and adapt. Among these environments, Arctic soils stand out for their ecological uniqueness and the extreme conditions that shape microbial diversity, making them promising targets for studies on AMR.

1.2. OBJECTIVES

The primary objective of this study is to investigate the diversity and characterization of antibiotic resistance in Arctic soils by employing an integrative methodological approach that combines classical microbiological techniques (e.g., bacterial plating and isolation) with advanced metagenomic analysis. Specifically, the study aims to:

Identify the prevalence and types of antibiotic-resistant bacteria present in Arctic soil environments.

Characterize the genetic mechanisms underlying antibiotic resistance within these bacterial populations.

Examine bacterial hosts diversity and taxonomic composition harboring resistance genes in these unique and understudied ecosystems.

This research seeks to contribute to our understanding of the spread and evolution of antibiotic resistance in extreme environments, providing insights into the potential ecological and public health implications of such resistance reservoirs.

2. STATE OF THE ART

Resistance to antibiotics is increasing at an alarming rate, resulting in infections that are more difficult and expensive to treat, as well as increasing mortality rates. According to the World Health Organization (WHO), AMR is one of the top ten threats to global health, with the expectation that by 2050, it could cause 10 million deaths annually if adequate solutions are not adopted (World Health Organization, 2019; O'Neill, 2020; Centers for Disease Control and Prevention, 2019).

Discovering new antibiotics has become increasingly important. First, new antibiotics are needed to replace those that have become ineffective due to bacterial resistance. Furthermore, research into new antimicrobial compounds may reveal innovative mechanisms of action that can overcome current bacterial defenses.

In addition to discovering new strategies, it is crucial to implement complementary strategies to address resistance. These strategies include the rational use of antibiotics in human and veterinary medicine, improving diagnostics to ensure appropriate treatments, and investing in research to develop antibiotic alternatives, such as bacteriophage therapies and microbiome modulation. Implementing global infection surveillance and control policies, such as those promoted by the WHO Global Action Plan on AMR, is also critical to mitigating the spread of resistance.

Bacterial resistance to antibiotics has intensified as a critical global public health challenge in recent years. Multidrug-resistant (MDR) bacteria are responsible for a growing number of infections that do not respond to conventional treatments, significantly increasing the morbidity and mortality associated with infectious diseases. According to a 2022 WHO report, AMR is one of the leading global threats to health, sustainable development, and food security.

The problem of bacterial resistance is worsened by the indiscriminate and inappropriate use of antibiotics in humans and animals, as well as the lack of new antibiotics on the market. Recent studies indicate that, in many countries, up to 80% of antibiotics are used in agriculture and livestock, contributing to the selection of resistant strains (Van Boeckel et al., 2021). Furthermore, the COVID-19 pandemic has exacerbated the situation, with an increase in the use of antibiotics to treat secondary infections, often without proven clinical need, as reported in a 2021 study published in the Journal of Global AMR (Rawson et al ., 2021).

The impacts of bacterial resistance are particularly severe in regions with weak healthcare systems, where limited access to accurate diagnoses and appropriate treatments intensifies the spread of resistant infections. In low- and middle-income countries, the lack of infrastructure for infection control and AMR surveillance further worsens the crisis. A 2022 study in The Lancet estimated that in 2019, more than 1.2 million deaths were directly attributed to AMR, highlighting the urgent need for coordinated, global action (Murray et al., 2022).

Given this scenario, it is necessary to reinforce policies for the rational use of antibiotics, invest in research and development of new antimicrobials, and implement effective infection surveillance and control programs. International collaboration, such as that promoted by the WHO Global Action Plan on AMR, is essential to address this challenge effectively and sustainably (World Health Organization, 2022).

Bacterial antibiotic resistance is a global phenomenon affecting all regions of the world, including extreme environments such as the Arctic, deserts, and other isolated ecosystems. Globally, AMR has been recognized as a growing crisis, with the WHO highlighting it as a significant threat to public health. In extreme environments, such as the Arctic, bacterial resistance has been documented at alarming levels. Studies have revealed that even in remote areas, where human activity is minimal, bacteria carry antibiotic resistance genes (ARGs). Furthermore, resistance has also been observed in microorganisms isolated from deep caves, deserts, and other extreme environments, indicating the ubiquity and persistence of resistance genes (Hernando-Amado et al., 2022).

In this perspective, 2022 research found ARGs in soil and water samples in the Arctic, suggesting that these regions may act as reservoirs of resistance due to the spread of pollutants and microorganisms through ocean currents and migrants (McCann et al., 2022).

The impact of bacterial resistance in extreme environments is significant because these ecosystems often harbor organisms that may be sources of new antibiotics (McCann et al., 2022). However, the presence of resistance genes in these locations may compromise the discovery and effectiveness of new antimicrobial compounds. Studies suggest that natural selection and evolutionary pressure in extreme environments may favor the retention and spread of resistance genes, further complicating efforts to control the spread of resistance (Silva, 2022).

Globally, bacterial resistance is exacerbated by human and animal mobility, which facilitates the spread of resistant pathogens across continents and ecosystems. Integrating global data on bacterial resistance, including those from extreme environments, is crucial to developing effective monitoring and intervention strategies. International collaboration, as promoted by the WHO Global Action Plan on AMR, is essential to address this challenge holistically and sustainably (World Health Organization, 2022).

1.2. ANTIBIOTICS

Antibiotics are natural, semi-synthetic, or synthetic chemical substances that inhibit the growth or cause the death of microorganisms. Since the discovery of the first antibiotic at the beginning of the 20th century, the medical field has gained a valuable weapon in treating bacterial infections. Antibiotic use has increased as the global healthcare system has improved over the last century. There has been a growing trend of inappropriate use of antimicrobial agents in human medicine due to a lack of education about antibiotic use, the regulation of prescription drugs, and over-the-counter sales.

Systematic data collection on the distribution of clinical and personal use of antimicrobial agents on a global scale has been scarce, especially in developing countries. A study on global human consumption in 2010 showed more than 70 billion standard units from 2000 to 2010, representing a 35% increase compared to the previous decade.

The consumption of antibiotics in animals in recent years is twice that of humans. Advances in optimizing the agricultural industry have been attributed in part to the extensive use of antibiotics to prevent diseases and promote the growth of plants and animals. This overuse has resulted in the cumulative release of antibiotics into the environment, challenging environmental microbial populations (Kurt et al., 2019). Furthermore, antibiotics have been classified as emerging pollutants due to bioaccumulation in the environment and their non-biodegradability (Pazda et al., 2019).

Currently, the most common types of antibiotics used in clinical settings and agriculture are β-lactams (such as penicillins and cephalosporins), macrolides, quinolones, sulfonamides, trimethoprim, and tetracyclines (Al-Riyami et al., 2020). Furthermore, in

Europe, there are groups of antibiotics used exclusively for personal medical uses (Graham et al., 2021).

Growing concern about antibiotic resistance has significantly challenged global public health. The indiscriminate and excessive use of these medicines, both in clinical and agricultural contexts, has contributed to the emergence of resistant bacteria, complicating the treatment of common infections and increasing the risks of outbreaks of multidrug-resistant diseases. In response to this threat, researchers have been investigating the impact of antibiotics in different environments and the effectiveness of wastewater treatment processes in removing antibiotic resistance. In this context, understanding the differences between broad-spectrum and narrow-spectrum antibiotics and early detection of resistance is fundamental to developing effective mitigation and prevention strategies.

Broad-spectrum antibiotics have broad applicability, being effective against different types of bacterial infections, while narrow-spectrum antibiotics are targeted at specific bacterial infections. Historically, broad-spectrum antibiotics have been more widely used than narrow-spectrum antibiotics. As a result, resistance associated with broad-spectrum antibiotics detected in conventional wastewater treatment plants has been more prevalent than resistance to narrow-spectrum antibiotics (Hiller, Hübner, Fajnorova, Schwartz, & Drewes, 2019).

Resistance to new antibiotics is commonly identified during the period approximately a decade after the drug’s launch. The excessive and indiscriminate use of antibiotics in medical practice has contributed to the worrying increase in resistant pathogenic bacteria and MDR in hospital environments. To deal with serious infections caused by MDR, the WHO has designated a list of last-line and emergency antibiotics. Studies demonstrate that implementing national regulations for the disposal and clinical and agricultural use of antibiotics effectively mitigates the global problem of AMR (Pazda et al., 2019).

1.2.1. MODE OF ACTION OF ANTIBIOTICS

Antibiotics can stop bacterial reproduction by attacking the cell wall or membrane of bacterial cells. Other targets include the synthesis of nucleic acids and proteins. Protein synthesis is carried out by ribosomes, structures composed of a smaller and a larger subunit (30S and 50S in bacteria). Furthermore, antibiotics can act as substances that interfere with metabolism, blocking the production of folic acid and DNA synthesis. They can also inhibit the activity of DNA gyrase, an enzyme crucial in DNA replication and transcription. The antibiotic classes associated with each mechanism are indicated in gray.

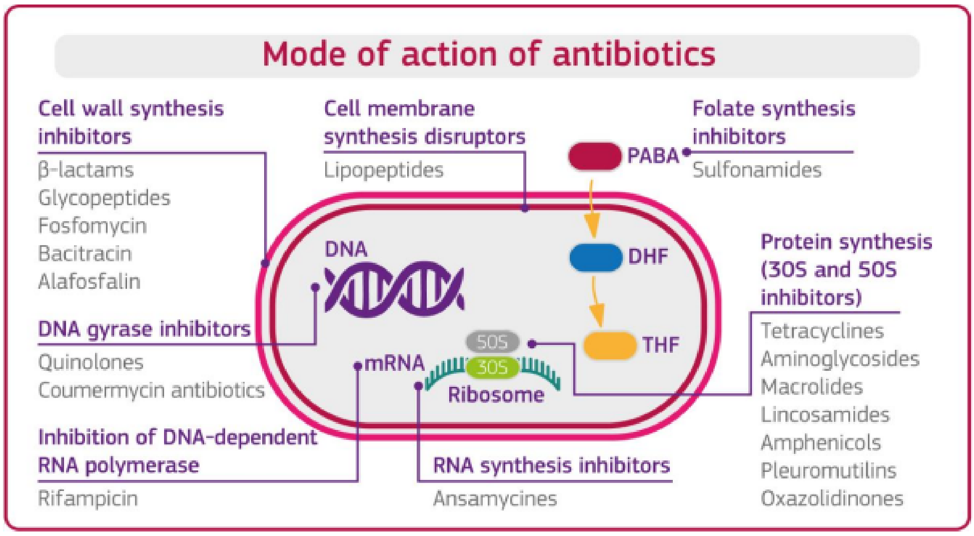


Fig. 1: Mode of action of antibiotics (Sanseverino, et. al, 2018).

From this perspective, some antibiotics interfere with the synthesis of the bacterial cell wall, while others interfere with protein synthesis or bacterial DNA metabolism. This diversity of mechanisms of action allows for a more precise approach to treating bacterial infections, minimizing side effects on patients and reducing the chance of bacterial resistance (Hiller et al., 2019; Pazda et al., 2019; Wright, 2021).

2.2.1.1. INHIBITION OF CELL WALL SYNTHESIS

Inhibition of cell wall synthesis is a key mechanism of action of antibiotics to stop bacterial growth. This process is essential in the development of new therapeutic strategies for the treatment of bacterial infections. Authors such as Oliveira et al. (2021) investigated the effectiveness of antibiotics that inhibit cell wall synthesis in animal models of bacterial infections. Furthermore, Smith et al. (2020) analyzed bacterial resistance associated with inhibiting cell wall synthesis in hospital settings, highlighting the importance of infection control strategies to combat this emerging problem.

Furthermore, the horizontal transfer of resistance genes between different bacterial strains may contribute to the spread of resistance to antibiotics that act in this specific mechanism. Understanding these resistance mechanisms is crucial for developing new therapeutic strategies and infection control policies.

2.2.1.2. INHIBITION OF NUCLEIC ACID SYNTHESIS

Inhibition of nucleic acid synthesis is a vital mechanism of action of antibiotics that aims to interrupt the replication and transcription of bacterial genetic material. This process has been necessary in searching for new therapeutic approaches for treating bacterial infections. For example, Silva et al. (2020) investigated the effectiveness of antibiotics that inhibit nucleic acid synthesis in experimental models of MDR infections.

Furthermore, Santos et al. (2019) analyzed the mechanisms of bacterial resistance associated with the inhibition of nucleic acid synthesis in clinical settings, providing crucial insights for developing strategies to combat this emerging resistance.

2.2.1.3. INHIBITION OF PROTEIN SYNTHESIS

In addition to inhibition of cell wall synthesis, inhibition of protein synthesis also represents a crucial mechanism in the action of antibiotics, interrupting the production of proteins essential for bacterial growth and survival. This process plays a fundamental role in the search for effective treatments against bacterial infections. Rodrigues et al. (2021) investigated the effectiveness of antibiotics that act by inhibiting protein synthesis in experimental models of infections resistant to multiple drugs, seeking to understand better how these drugs can be used against resistant bacteria.

In this sense, the research by Oliveira et al. (2019) analyzed bacterial resistance mechanisms related to the inhibition of protein synthesis in clinical settings. These studies increasingly provide valuable insights for developing more effective therapeutic strategies by identifying how bacteria can become resistant to antibiotics that act on this specific mechanism.

2.2.1.4. INHIBITION OF FOLIC ACID SYNTHESIS

Inhibition of folic acid synthesis is a crucial mechanism of action of antibiotics, aiming to interrupt the production of folic acid, which is essential for the metabolism of bacterial cells. Folic acid plays a fundamental role in synthesizing DNA, RNA, and amino acids, which are necessary for the growth and reproduction of bacteria. From this perspective, Souza et al. (2020) investigated the efficacy of antibiotics that inhibit folic acid synthesis in experimental models of MDR bacterial infections.

Still, in this vein, Pereira et al. (2019) analyzed the mechanisms of bacterial resistance associated with inhibiting folic acid synthesis in clinical settings, providing valuable insights for developing more effective therapeutic strategies to act against bacterial cells.

2.2.1.5. CELL MEMBRANE INHIBITION

Inhibition of cell membrane synthesis is a fundamental mechanism of action of antibiotics to interrupt the membrane formation surrounding bacterial cells. This membrane is essential for cells' structural and functional integrity and is crucial for nutrient transport, cellular communication, and protection against external agents.

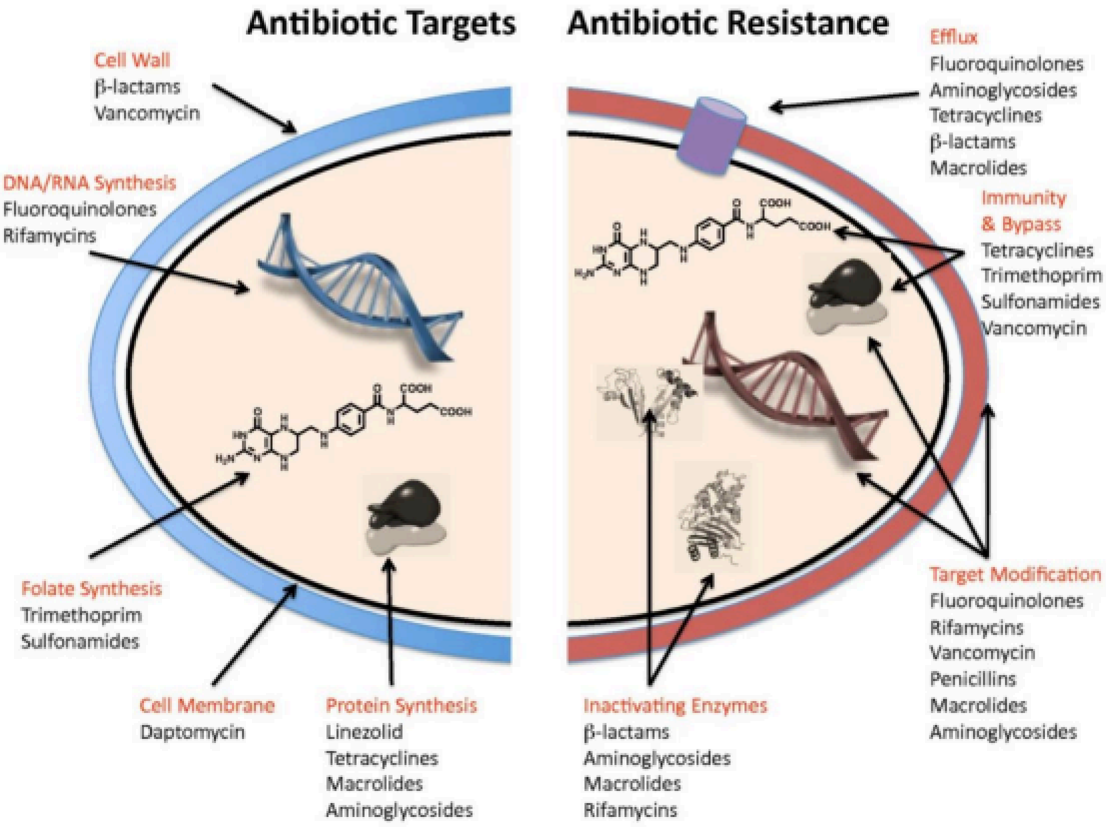


Fig. 2: Antibiotic targets and mechanisms of bacterial resistance (Wright, 2010).

Some authors have emphasized the importance of this process in searching for new therapeutic strategies for treating bacterial infections, such as Santos et al. (2020), who investigated the effectiveness of antibiotics that inhibit cell membrane synthesis in experimental models of MDR infections.

Furthermore, Oliveira et al. (2019) analyzed the mechanisms of bacterial resistance associated with inhibiting cell membrane synthesis in clinical settings, providing valuable insights for developing more effective therapeutic strategies.

2.3. RESISTANCE TO ANTIBIOTICS IN BACTERIA

Antibiotic resistance in bacteria is a global public health problem that has grown significantly in recent decades. Recent studies have highlighted the emergence and spread of resistance mechanisms in several bacterial pathogens, representing a significant challenge for treating infections. For example, a study by Silva et al. (2021) investigated antibiotic resistance prevalence and patterns in different bacterial strains isolated from hospitalized patients. The results revealed a high rate of resistance across several classes of antibiotics, indicating the urgent need for more effective strategies to control and prevent bacterial resistance.

Thus, antibiotic resistance in bacteria is not only limited to the clinical setting but is also observed in communities and the environment. A study carried out by Oliveira et al. (2020) investigated the presence of antibiotic-resistance genes in environmental water and soil samples. The results demonstrated the widespread dissemination of resistance genes in different environments, suggesting the significant contribution of the environment to the spread of bacterial resistance. These findings highlight the importance of integrated approaches to mitigating antibiotic resistance in different contexts.

Furthermore, antibiotic resistance in bacteria represents not only a clinical challenge but also an economic and social one. A recent study by Santos et al. (2022) analyzed the financial impact of antibiotic resistance in different sectors of society. The results highlighted the substantial costs of treating infections caused by resistant bacteria and the adverse effects on patients' productivity and quality of life. These findings reinforce the urgency of effective measures to combat antibiotic resistance and preserve the effectiveness of these important therapeutic agents.

From this perspective, Johan Bengtsson-Palme and D.G. Joakim Larsson (2016), in their article "Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation," elucidate the issue of concentrations of antibiotics in the environment and their potential to select resistant bacteria. The authors highlight the critical need to establish regulatory limits for these concentrations to mitigate the development and spread of bacterial resistance. The authors further state that even low levels of antibiotics can promote bacterial resistance over time. Therefore, it is necessary to propose specific guidelines for antibiotic concentration limits at the level of environmental regulations.

2.4. ARCTIC SOIL

The Arctic tundra is a unique and underexplored environment home to microbial diversity that is still poorly understood. Arctic soils have increasingly been considered unique ecosystems and potential sources of valuable microbial resources. For example, research conducted by Smith et al. (2021) investigated the microbial diversity of Arctic soils and discovered a surprising abundance of microorganisms adapted to extreme temperature and humidity conditions. These microorganisms can produce bioactive compounds with therapeutic, biotechnological, and industrial applications.

Additionally, Arctic soils play a key role in global nutrient cycling and carbon storage. Recent studies have shown that climate change significantly impacts Arctic soil microbiota, with potential consequences for the carbon cycle and global warming. Johnson et al. (2020) investigated the effects of climate change on microbial activity and organic matter decomposition in Arctic soils. Their findings highlighted the need to understand better the microbiological dynamics of these soils to predict and mitigate the effects of climate change in the Arctic region.

Furthermore, Arctic soils are considered important reservoirs of microbial genes with biotechnological potential. Some authors have explored the genetic diversity of microorganisms from Arctic soils in search of new genes and enzymes with industrial and environmental applications, such as Oliveira et al. (2019), who analyzed the metagenome of Arctic soils in search of genes involved in the production of cellulose-degrading enzymes. Their findings suggest that Arctic soils could be promising sources of new enzymes for the biofuel industry and lignocellulosic biomass bioprocessing.

2.4.1 ANTIBIOTICS IN ARCTIC SOIL

Arctic soils have been identified as essential reservoirs of natural antibiotics, offering a wide range of bioactive compounds with therapeutic potential. Some studies have explored the diversity of antibiotic-producing microorganisms in these extreme environments, revealing a wealth that has not yet been fully explored. For example, Silva et al. (2021) research investigated the presence and diversity of genes related to antibiotic biosynthesis in Arctic soil samples. The results highlighted the presence of a wide variety of antibiotic biosynthesis gene clusters, suggesting significant potential for discovering new antimicrobial compounds.

Furthermore, the characterization of metabolites produced by microorganisms from Arctic soils has revealed an impressive diversity of bioactive molecules, many exhibiting antibacterial activity. Metagenomics and metabolomics studies have provided insights into the biosynthesis and functionality of these compounds, paving the way for identifying new therapeutic agents. Santos et al. (2020), who isolated and characterized a new class of antimicrobial peptides from Arctic soil microorganisms, showed that these peptides demonstrated activity against various bacterial pathogens, highlighting the potential of Arctic soils as sources of new antibiotics.

2.4.2. RESISTANCE TO ANTIBIOTICS IN ARCTIC SOIL

Recent research has studied the microbial diversity in the active permafrost layer on Spitsbergen Island in the Arctic. The study by Dziurzynski et al. (2023) revealed a remarkable richness of bacteria and fungi using a combined approach of classical microbiology and metabarcoding. The results highlighted the presence of a wide variety of microorganisms adapted to the extreme conditions of the Arctic environment, including potentially antibiotic-producing species. This unique microbial diversity suggests that Arctic soils may serve as reservoirs of antibiotic-resistance genes, representing an important area of research to explore AMR in natural environments.

Furthermore, applying the metabarcoding technique allowed a more comprehensive analysis of microbial diversity, identifying species not detected by traditional microbiology methods. This innovative approach has revealed even greater complexity in the microbial composition of Arctic soils, providing valuable insights for future studies on antibiotic resistance in these environments. The results of this study highlight the importance of understanding the microbial diversity of Arctic soils and their potential as a source of new microbiological resources while also highlighting the challenges associated with conserving biodiversity in such a sensitive environment as the Arctic.

2.4.3. TOOLS TO ASSESS ANTIBIOTICS RESISTANCE IN ARCTIC SOIL

In the context of growing concern about antibiotic resistance in Arctic soils, developing practical tools to assess this resistance is of paramount importance, and several approaches have been explored to evaluate antibiotic resistance in these unique environments. In this sense, Silva et al. (2020) investigated the application of metagenomics techniques to analyze the genetic diversity of Arctic soil microorganisms and identify antibiotic-resistance genes. This approach has enabled a more comprehensive understanding of antibiotic resistance in Arctic soils, highlighting the importance of monitoring and understanding resistance mechanisms in these microbial communities.

Furthermore, studies such as that by Santos et al. (2021) have explored bioprospecting methods to identify new antimicrobial compounds in Arctic soils, offering promising prospects for developing new strategies to combat antibiotic resistance. This research represents significant advances in the search for practical tools to assess and address the challenge of antibiotic resistance in Arctic ecosystems.

The study carried out by Gorecki et al. 2023 offers essential insights into the tools available to assess antibiotic resistance in Arctic soils. Researchers investigated the functions encoded in the meta plasmidome of low-central Siberian polygonal tundra soils, revealing an impressive diversity of genes related to antibiotic resistance. These metaplasmidomes, composed of mobile genetic elements such as plasmids and integrons, play a key role in the horizontal spread of ARGs among soil microorganisms. This finding highlights the importance of considering individual microorganisms and mobile genetic elements as potential sources of antibiotic resistance in Arctic soils.

Furthermore, the study identified specific genes associated with resistance to different classes of antibiotics, providing valuable information for developing methods for detecting resistance in soil samples. Analysis of the metaplasmidome has enabled a more comprehensive understanding of the genetic diversity of Arctic soil microorganisms and the strategies they have developed to survive in a challenging environment. These findings highlight the importance of developing antibiotic resistance assessment tools adapted to the unique conditions of Arctic soils to monitor and mitigate the potential impact of AMR in these sensitive ecosystems.

2.5. CULTURE-BASED METHODS

Cultivation-based methods are traditional techniques widely used for detecting and quantifying microorganisms in environmental samples, including Arctic soils. These methods involve the cultivation of microorganisms in specific culture media, allowing the identification and characterization of the species present. Recent studies, such as that by Oliveira et al. (2020), have demonstrated the effectiveness of these methods in detecting antibiotic-resistant bacteria in Arctic environments. The application of cultivation-based methods is essential to isolate and study viable microorganisms, in addition to allowing the assessment of susceptibility to antibiotics through sensitivity tests.

While crop-based methods are valuable, they have limitations, especially in extreme environments like the Arctic. Many microorganisms present in these soils are difficult to cultivate under standard laboratory conditions, which can lead to an underestimation of microbial diversity and antibiotic resistance rates. Santos et al. (2021) highlighted that despite these limitations, cultivation-based approaches remain essential for studying microbial ecology and antibiotic resistance, especially when combined with advanced molecular techniques.

The integration of cultivation-based methods with molecular biology approaches has recently shown promising results. Silva et al. (2019) used a combination of cultivation and next-generation sequencing (NGS) techniques to explore microbial diversity in Arctic soils and identify antibiotic-resistance genes. This hybrid approach allows the identification of viable microorganisms and the characterization of mobile genetic elements associated with resistance, providing a more comprehensive view of the dynamics of antibiotic resistance in Arctic environments. These advances underscore the continued importance of cultivation-based methods, especially when integrated with new technologies, to address the challenges of antibiotic resistance in unique ecosystems.

2.5.1.DILUTION IN AGAR

The agar dilution method is a widely used reference for determining antibiotics' minimum inhibitory concentration (MIC) against bacteria. This method involves incorporating different concentrations of an antibiotic onto agar plates, where a standardized suspension of bacteria is then inoculated. Authors such as Oliveira et al. (2020) demonstrated the effectiveness of the agar dilution method in evaluating antibiotic susceptibility in bacterial strains isolated from Arctic soils. This method is considered a gold standard because it provides accurate and reproducible results.

Although the agar dilution method is highly reliable, it has some limitations, especially in research contexts in extreme environments such as the Arctic. Santos et al. (2019) highlighted that harsh environmental conditions can influence the viability and growth of bacteria on agar, which can affect the MIC results. Furthermore, the method is laborious and requires significant materials and time, which can be a challenge in large-scale studies or studies with multiple isolates. However, the accuracy and reproducibility of this method still make it a preferred choice for antimicrobial susceptibility testing.

Recently, technological advances have improved the application of the agar dilution method. Silva et al. (2021) used an automated version of the technique to assess antibiotic resistance in bacteria isolated from Arctic permafrost soils. This computerized approach reduced the time and effort required and increased the accuracy of results. Furthermore, integrating molecular techniques, such as Polymerase Chain Reaction (PCR) and NGS, with the agar dilution method has allowed a more detailed analysis of resistance mechanisms. These advances underscore the continued importance of the agar dilution method in antibiotic resistance research, especially in unique and understudied ecosystems like Arctic soils.

2.6. CULTURE-INDEPENDENT METHODS

Culture-independent methods have revolutionized antibiotic research, enabling the analysis of microbial communities directly from environmental samples without the need for cultivation. These approaches are particularly valuable for investigating antibiotic resistance in environments such as Arctic soils, where many bacteria are not culturable with traditional techniques. Silva et al. (2020) highlighted the use of metagenomics to study antibiotic resistance in microbial communities from Arctic permafrost, revealing the presence of antibiotic-resistance genes that culture-based methods could not detect.

In addition to metagenomics, quantitative PCR (qPCR) and NGS have been widely used to quantify and characterize ARGs in environmental samples. Santos et al. (2021) demonstrated that qPCR can quantify specific ARGs in Arctic soils, offering a detailed view of the prevalence and distribution of these genes. NGS, in turn, allows a more comprehensive and thorough analysis of microbial communities, identifying the presence of resistance genes and the microorganisms that harbor them.

Applying culture-independent methods also facilitates understanding the mechanisms of resistance gene transfer between microorganisms in natural environments. Oliveira et al. (2019) used metatranscriptomic techniques to investigate the expression of ARGs in Arctic soil samples, providing insights into how these genes are activated and transferred in response to environmental pressures. These methodological advances are significant because they expand our knowledge about microbial ecology in extreme environments and help develop strategies to mitigate antibiotic resistance on a global scale.

2.6.1. POLYMERASE CHAIN REACTION (PCR)

PCR is a technique widely used in molecular biology to amplify specific DNA fragments. Since its invention, PCR has been a fundamental tool in several fields, including antibiotic research and the detection of resistance genes. Recently, advances in PCR technology have allowed more sensitive and specific detection of ARGs in environmental samples without the need for microbial cultivation. According to Dziurzynski et al. (2019), developing databases such as LCPDb-MET facilitates the selection of PCR primers to detect genes related to metal metabolism and resistance in bacteria, increasing the precision of analyses.

The application of PCR to detect ARGs in natural environments, such as Arctic soils, has provided significant insights into the spread and prevalence of these genes. Techniques such as qPCR allow the detection and quantification of resistance genes, offering a more detailed view of the resistance load present in samples. For Silva et al. (2021), qPCR can effectively monitor the presence of resistance genes in Arctic permafrost microbiomes.

Furthermore, PCR can be combined with other molecular techniques for a more comprehensive analysis. Multiplex PCR, for example, allows the simultaneous amplification of multiple DNA targets in a single reaction, increasing the efficiency and scope of analysis. Santos et al. (2020) used multiplex PCR to detect several ARGs in soil samples, demonstrating the usefulness of this approach in characterizing environmental resistomes. The integration of PCR with NGS techniques has also shown promise, allowing validation of sequencing results and providing additional confirmation of the presence of resistance genes.

2.7. METAGENOMICS

Metagenomics is a revolutionary approach that allows the analysis of the genetic material of microbial communities directly from environmental samples without the need for prior cultivation. This technique has been widely applied in antibiotic research, offering a comprehensive view of resistomes, which are the sets of ARGs in a given environment.

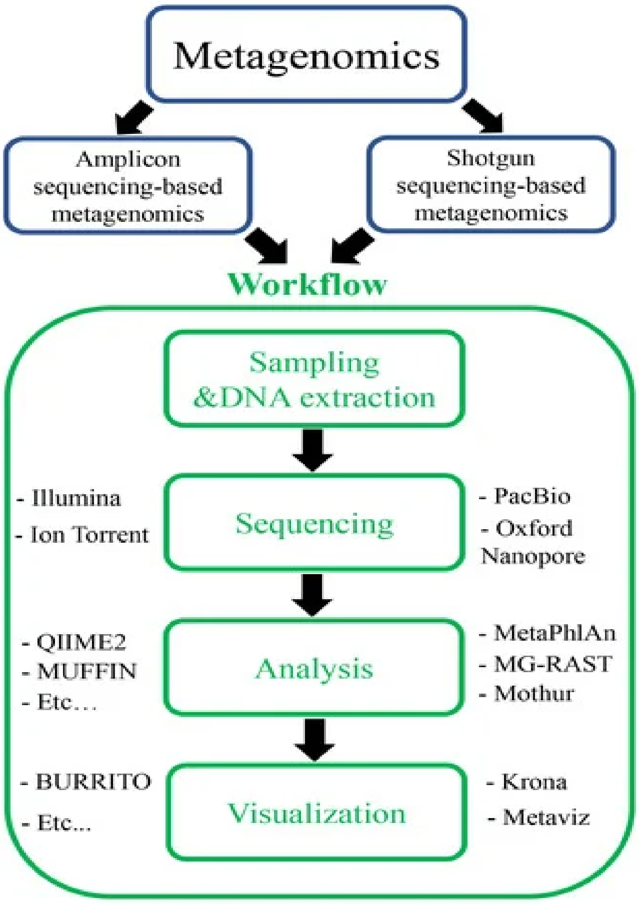


Fig. 3: Metagenomics (Nam et al., 2023).

Metagenomics enables the identification and quantification of resistance genes in complex samples such as soils, waters, and sediments, providing valuable insights into the spread and evolution of antibiotic resistance. For Oliveira et al. (2020), the effectiveness of metagenomics in characterizing resistomes in aquatic environments reveals a significant diversity of resistance genes.

In addition to identifying resistance genes, metagenomics also allows the exploration of new genes that may be associated with antibiotic resistance and the discovery of new antimicrobial compounds. This ability is critical in extreme environments, such as arctic soils and permafrost, where organisms adapted to harsh conditions may possess unique resistance mechanisms. Johnson et al. (2020) used metagenomics to investigate the microbiomes of Arctic soil and identified new resistance genes, highlighting the importance of these ecosystems as potential sources of new microbial resources. Metagenomics facilitates functional analysis and potential bioprospecting of antimicrobial compounds that could be developed as new medicines.

The application of metagenomics techniques, including NGS, has enabled unprecedented resolution in analyzing microbial communities and their resistomes. Recent studies have used metagenomics to monitor the effectiveness of environmental interventions and remediation strategies in reducing the burden of resistance genes. Santos et al. (2021) conducted a metagenomic analysis of soils contaminated with antibiotics and demonstrated a significant decrease in resistance genes after the application of bioremediation. These approaches provide detailed data on the presence of resistance genes and assess the impact of environmental management practices on mitigating antibiotic resistance.

2.7.1. SHOTGUN ILLUMINA

The Illumina shotgun sequencing technique is a powerful approach in metagenomics, enabling detailed and comprehensive analysis of microbial communities directly from environmental samples. This technique involves fragmenting the total DNA extracted from the sample and randomly sequencing all fragments, providing a complete view of the resistome present. Johnson et al. (2020) highlight the efficiency of Shotgun Illumina in identifying a wide range of ARGs in diverse environments, including agricultural and aquatic soils. The depth of coverage achieved by this technique allows the detection of rare and previously uncharacterized genes, offering valuable insights into the diversity and abundance of AMR.

In addition to identifying resistance genes, Shotgun Illumina sequencing enables the functional analysis of microbial communities. This includes the detection of complete metabolic pathways and the identification of genes associated with other relevant ecological functions. Silva et al. (2021) used this approach to map resistomes in urban and rural environments, revealing significant differences in the composition and abundance of resistance genes. These data are essential for understanding how different land use and environmental management practices influence the spread of ARGs.

The application of Shotgun Illumina in metagenomics also facilitates the assessment of the impact of environmental interventions, such as the remediation of contaminated soils and the implementation of sustainable agricultural practices. Santos et al. (2022) demonstrated the use of Shotgun Illumina to monitor the effectiveness of bioremediation in antibiotic-contaminated soils, showing a significant reduction in the abundance of resistance genes following treatment. Furthermore, this technique allows the comparison of resistomes in different ecosystems and under diverse environmental pressures, providing a solid basis for developing management strategies to reduce the spread of AMR.

3. MATERIAL & METHODS

3.1. INITIAL SAMPLE COLLECTION AND ANALYSIS

The soil samples used in this study were previously described by (Dziurzynski et al. 2022). The field campaign was carried out in the summer of 2017. Topsoil samples were collected during the summer of 2017 from six ice-free areas near the Polish Polar Station Hornsund (coordinates: N 77.00278, E 15.54722), located in Spitsbergen, Svalbard, Norway (fig. 4). Sampling sites were selected based on different environmental influences: S1 – station yard (anthropogenic influence); S2 – coastal area (maritime influence); S3 – occasionally flooded tundra (influenced by freshwater flooding, vegetation and animals); S4 – "dry" tundra (with evident impact of vegetation and animals); S5 – glacial moraine (limited vegetation, influenced by the proximity of a glacier); and S6 – breeding colony of lesser puffins (zoogenic and maritime influence) (fig. 4).

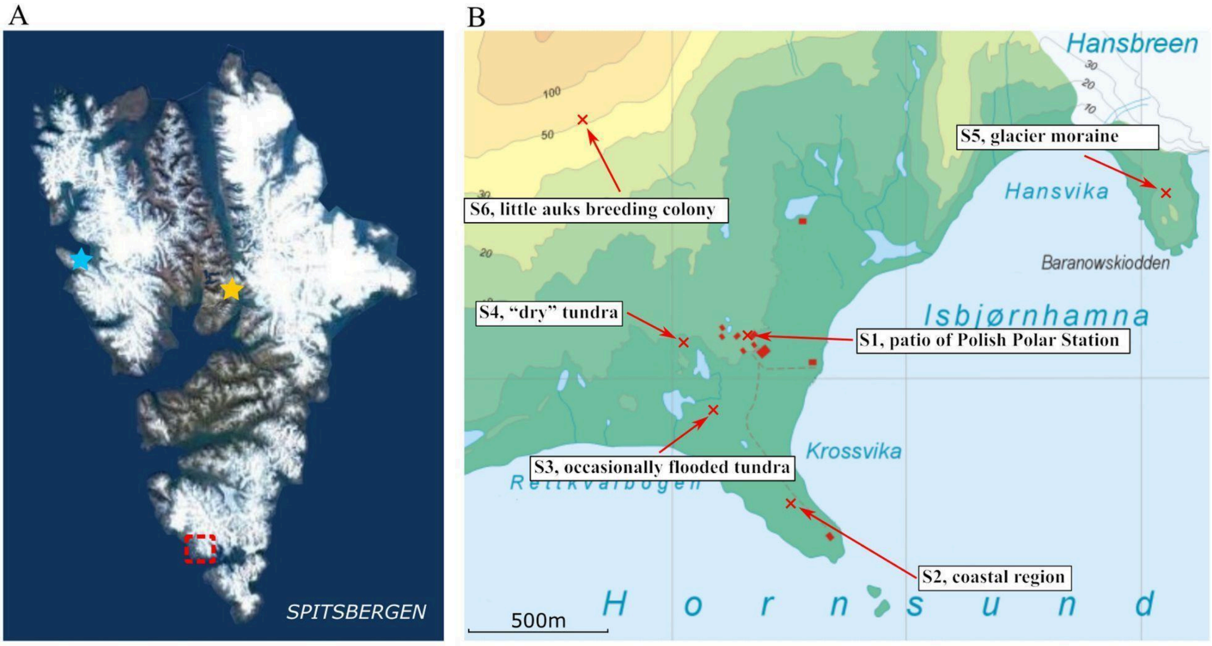
At each site, four soil subsamples of 250 g each were collected from a 1 m^2 area at a depth of 0-10 cm. The samples were packaged in 1627 mL Whirl-Pak polyethylene stereo bags (Nasco, Madison, USA), ensuring contamination-free handling. During collection, temperatures ranged from 4.0 to 5.6 °C. The samples were stored at -20 °C and transported to the Department of Environmental Microbiology and Biotechnology, University of Warsaw, Poland, for further analysis (Dziurzynski et al. 2022).

3.1.1 PHYSICAL-CHEMICAL ANALYSIS OF SOIL

The carbon, hydrogen, nitrogen, and sulfur concentration was determined in each sample using CHNS elemental analysis. The study used combustion with a LECO TruSpec® elemental determinant (LECO Corporation, St. Joseph, MO, USA). The concentrations of ammonium, chloride, nitrate, and nitrite ions were measured with Quantofix strips (Sigma-Aldrich, Saint Louis, MI, USA) and NanoColor tests (Macherey-Nagel, Duren, Germany). In addition, NanoColor was used to quantify TOC.

Soil pH was measured in suspensions of soil and deionized water in a 5:1 (volume: volume) ratio, according to the method described in the standard “Soil, sludge and treated biowaste. Determination of pH”, BS EN ISO 10390.

The characterization of the sample collection site described above is based entirely on the analyses performed by Dziurzynski et al. 2022.



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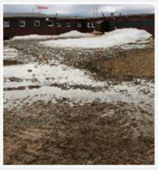
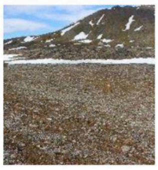
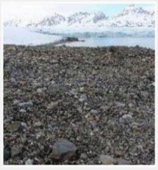
	S1 patio of Polish Polar Station	**S2** coastal region	**S3** occasionally flooded tundra	**S4** "dry" tundra	**S5** glacier moraine	**S6** little auks breeding colony
Sampling site						
Coordinates	N 77.02028 E 15.61278	N 77.13278 E 15.59972	N 77.00000 E 15.54111	N 77.12056 E 15.64083	N 77.12056 E 15.70861	N 77.08222 E 15.64611
Site characteristics	anthropogenic influence	maritime influence	freshwater, animals and plants influence	animals and plants influence	glacier influence limited vegetation	animals and maritime influence
Air temp. [°C]	4.00	4.00	4.50	4.50	5.60	5.40
Soil temp. [°C]	8.00	8.10	10.50	9.80	11.30	10.00
Altitude	24.00	10.00	0.00	5.00	17.00	59.00
pH (2.5 h)	8.57	7.22	6.98	7.23	8.44	7.07
pH (18 h)	8.25	6.95	6.51	7.03	8.29	6.60
Density [kg/dm^3]	1.53	1.68	1.00	1.62	1.57	0.67

Fig. 4: taken from the work of (Dziurzynski et al. 2022), illustrates the composition and characteristics of the soil previously described. Localization and physicochemical properties of the sampling sites. A) Satellite image of Spitsbergen island with the localization of the Polish Polar Station Hornsund indicated with a red dashed rectangle; B) Map showing

localization of six sampling sites; CMDR Basic physicochemical properties of the sampling sites. Blue star – Ny-Alesund, yellow star – Pyramiden. Map source: Google Earth.

3.2. SAMPLE CULTURING

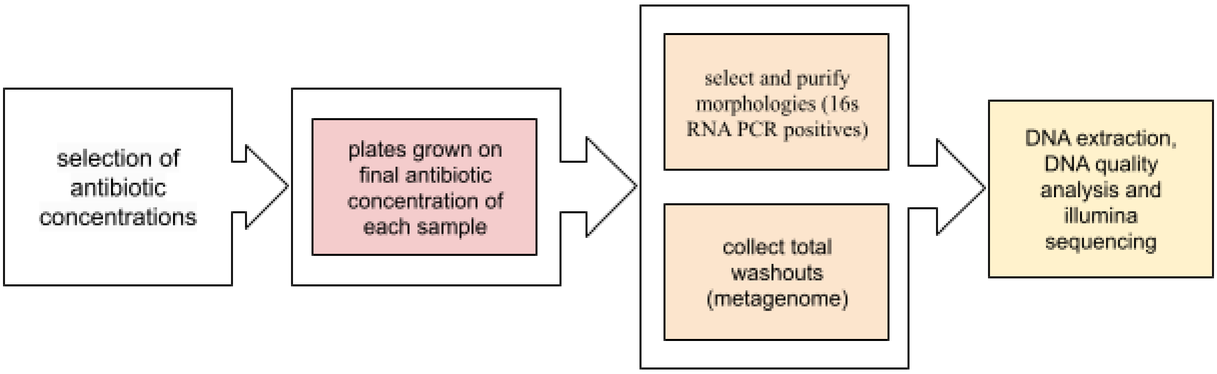


Fig. 5: Classical microbiology methods flowchart.

Before preparation, each sealed sample was thawed for approximately 2 hours. Under a Bunsen burner flame, 10 g of each sample was weighed and separated into 50 ml Falcon tubes. This procedure was intended to avoid the need to thaw all samples for experimental repetitions, allowing the use of only the necessary quantity at a time.

Each 10 g sample was transferred to a 300 mL Erlenmeyer flask with 90 mL of a sterilized 0.1% g/L sodium phosphate solution containing 5 g of glass beads. The samples in the Erlenmeyer flasks were then placed in a shaker with the temperature maintained at 16ºC and homogenized for approximately 16 hours at 250 rpm. This process aimed to release the bacteria present in the soil into the liquid medium, minimizing their retention in the sediment particles.

After shaking, the samples were left to rest, allowing the solid particles to settle at the bottom of the beakers. Then, in a laminar flow hood, the supernatant was removed with a pipette and subjected to serial dilutions (1:10) for five consecutive steps in a sterilized saline solution. All six resulting dilutions (10^{-1} to 10^{-6}) were inoculated in control Petri dishes to determine the samples' standard Colony-Forming Unit (CFU) in the R2A culture medium.

3.2.1 CULTURE MEDIUM: PREPARATION AND INOCULATION

The R2A culture medium was used to grow microorganisms to prevent fast-growing bacterial populations from predominating over slow-growing ones, thus favoring greater genetic diversity. The culture medium was melted and cooled to approximately 55°C and only then, in a laminar flow hood, were the antibiotics added to their respective flasks to maintain the integrity of the antibiotics and the sterility of the media during the preparation of the plates (Wiegand, I. et al. 2008). The antibiotics were incorporated using graduated pipettes. The stock solutions of the antibiotics were prepared and filtered with a 22 µm pore size, at least 24 hours in advance. and their concentrations were adjusted just before inoculation, in dilution scales of 1:10, in dd water (double-distilled water).

- Ampicillin: 6 concentrations, ranging from 4 µg/L to 4 x 10^5 µg/L.
- Azithromycin: 5 concentrations, ranging from 16 µg/L to 16 x 10^4 µg/L.
- Ciprofloxacin: 5 concentrations, ranging from 2 µg/L to 2 x 10^4 µg/L.
- Phosphomycin: 5 concentrations, ranging from 125 µg/L to 125 x 10^4 µg/L.
- Kanamycin: 5 concentrations, ranging from 125 µg/L to 125 x 10^4 µg/L.
- Tetracycline: 5 concentrations, ranging from 16 µg/L to 16 x 10^4 µg/L.

After adding the antibiotics, the plates were left in the hood to solidify so they could be used immediately or stored at 4ºC for a maximum of 4 days until inoculated to avoid dried plates or plates with degraded antibiotics. Then, the samples were inoculated using disposable plastic T-loops and the Spread Plate technique, with 100 µl of soil samples in dilutions from 10^1 to 10^3, and incubated at 16ºC for 7 days to analyze bacterial growth in different concentrations of antibiotics.

3.2.2 COLONIES COUNTS & ISOLATION

The number of colonies on the plates was counted to determine which concentration of antibiotic reduces the number of colonies significantly. For each site and each antibiotic, the plates with the lowest concentrations of antibiotics significantly inhibiting bacterial growth were selected, and the bacteria of each different morphology were isolated based on phenotypic characteristics (pigmentation, surface, edge, texture) by the streak depletion technique (Pereira, J. E. S. et al. 2003), with disposable inoculation loops, on new plates containing the same concentration of the antibiotic from which they were isolated—maintaining the same concentration of antibiotic aimed to ensure the maintenance of possible genetic characteristics of resistance, especially in the case of genes associated with mobile genetic elements.

After selection for purification of colonies with different morphologies present in the samples, the bacteria from the plates containing all the dilutions of the same site and the selected concentration were washed with 2 mL of distilled water and sterilized on each plate, using disposable T-loops. The washed volume containing the colonies was transferred to 1.5 mL Eppendorf tubes, which were centrifuged, and the supernatants discarded to store the volume of all three plates corresponding to each dilution of the same site in the same Eppendorf, ensuring the complete collection of bacterial material from all dilutions, from each site, of the selected concentration. The samples were stored at -20°C for a maximum of 72h before the DNA extraction process, which was performed using the EURx GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (v. 1.2, June 2023)

3.2.3 PCR SELECTION

From the pure colonies, a PCR was performed to amplify the 16S gene. A solution containing 12 µL of 20% Chelex and 38 µL of distilled water was used for DNA extraction. A single purified colony was added to the solution in Eppendorf tubes, heated at 95°C for 15 minutes to promote cell lysis, and, immediately after, cooled on ice to stop the reaction. The tubes were then centrifuged at 13,400 rpm for 2 minutes, and the supernatants containing the DNA were transferred to clean and sterilized tubes and stored at 4°C until the preparation of the PCR master solution.

The PCR master solution consisted of 9.5 µL of nuclease-free water, 12.5 µL of Taq polymerase, 1 µL of 16S forward primer, and 1 µL of 16S reverse primer, totaling 24 µL per reaction. To each PCR microtube containing the master solution, 1 µL of the extracted DNA was added. Positive and negative control samples were included in each run. The samples were subjected to an initial cycle of denaturation at 95°C for 4 minutes, followed by 27 cycles of denaturation at 95°C for 30 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 90 seconds. After the cycles, a final extension at 72°C for 5 minutes was performed, and the samples were stored at 4°C overnight.

To evaluate the amplified products, electrophoresis was performed on a 1.5% (w/v) agarose gel, with each well-containing 5µL, allowing visualization of the bands corresponding to the 16S gene and confirmation of the expected size.

3.2.4 PURIFIED BACTERIAL COLONIES ENRICHMENT

The purified colonies confirmed to contain the 16S gene were subsequently transferred to plastic sealable test tubes containing R2A liquid medium with the same antibiotic concentration as the colony's source plate. Using a plastic inoculation loop, isolated single colonies were inoculated into their respective liquid medium. The tubes were incubated at 16°C at 150 rpm until there was visual growth of bacteria in the medium, typically checked after each overnight incubation.

After visual growth was confirmed, the tubes were centrifuged at 13,400 rpm to collect the pellet. The obtained pellet was resuspended in 1.5 mL of sterile distilled water for subsequent DNA extraction, which was done using the EURx GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (v. 1.2, June 2023)

3.3. DNA EXTRACTION AND QUALITY ANALYSIS

Extraction was performed using the EURx GeneMATRIX Bacterial & Yeast Genomic Purification Kit (v. 1.2, June 2023), aiming to isolate the total DNA content only from the bacteria in the soil samples. After extraction, measurements were performed to assess the quality and integrity of the DNA obtained:

Quantification: DNA concentration was measured using the Qubit™ 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), providing an accurate analysis of the total amount of DNA in each sample.

Purity Ratios: DNA purity was assessed by the Nanodrop Spectrophotometer, checking the 260/280 and 260/230 ratios. These ratios help to identify possible contaminations, such as proteins or chemical reagents remaining from the extraction.

DNA Integrity: A 0.8% (w/v) agarose gel electrophoresis was performed to confirm DNA integrity and identify possible signs of degradation. The gel was stained with loading dye and visualized under UV light.

DNA of adequate quality was sent to Eurofins Genomics for NGS using the Illumina method, resulting in the production of metagenomes for each soil sample and full genomes for the purified strains.

3.4. CRYOPRESERVATION

The Cryopreservation of morphologically distinct colonies from selected plates for further analysis, was given in a solution of 50% glycerin filtreted in 22 µm PES bottle filter from VWR, diluted in dd water, then the samples was added 900 µl of the diluted glycerol solution with 900 µl of enriched bacterial sample in a 2000 µl cryotube, and refrigerated at -80ºC.

3.5. BIOINFORMATICS

The Bioinformatic analysis followed a structured workflow specified on fig. 6 ensuring high-quality data processing, assembly, and annotation. First, raw sequencing reads underwent quality control using FastQC and fastp, with stringent filtering criteria to retain high-quality data. The processed reads were assembled into contigs using MegaHIT, applying optimized k-mer selection for complex microbial communities. Assembly quality was assessed using QUAST, evaluating key metrics like N50 and misassembly rates. Contig abundance was estimated via BWA-MEM2 and CoverM, using RPKM (Reads Per Kilobase per Million mapped reads) for quantification. Functional annotation involved Prokka for gene predictions, AMRFinder for antimicrobial resistance genes, and METGeneDb with MMseqs2 for metal resistance genes. Taxonomic classification was performed using Kraken2 with a protein reference database, while mobile genetic elements (MGEs) were identified using geNomad to detect plasmids, viruses, and integrative elements. Finally, data interpretation and visualization were conducted using Python, employing numpy, pandas, matplotlib, and seaborn to generate insights into microbial community structure and resistance gene distribution.

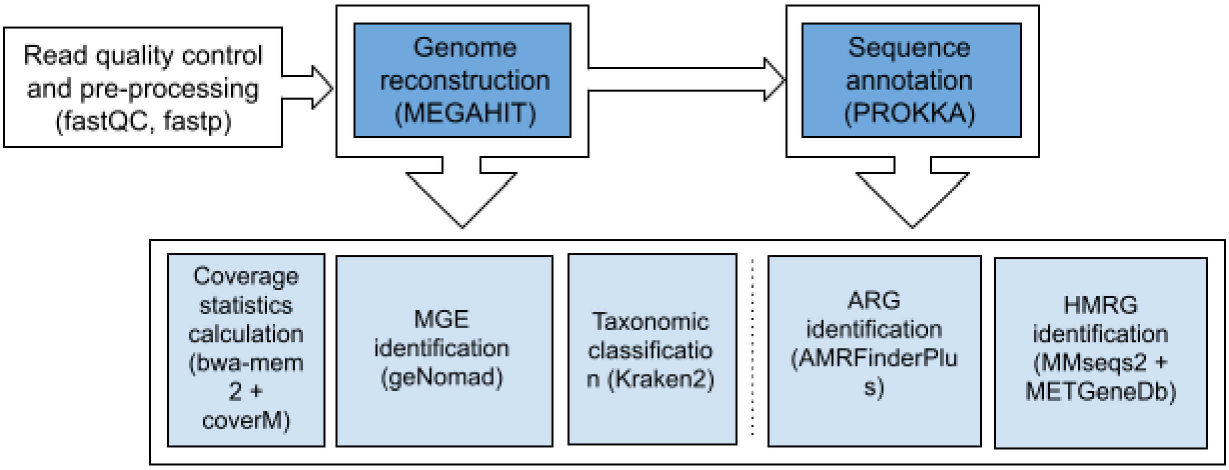


Fig. 6: sequenced data analysis method flowchart.

3.5.1. QUALITY CONTROL OF RAW DATASETS

To ensure high-quality of input data, the raw sequencing reads were evaluated using FastQC (v. 0.12.1, Andrews, n.d.) and then processed with fastp (fastp v. 0.23.4, Andrews et al., 2018). FastQC provided a detailed summary of sequence quality metrics, including per-base quality scores, GC content distribution, sequence duplication levels, and overrepresented sequences. After that, fastp was employed for quality filtering, adapter trimming, and removal of low-quality reads to enhance dataset reliability for downstream analysis. Reads shorter than 50 bases (--length_required 50) were discarded. Bases were considered qualified if they had a Phred score of at least 15 (--qualified_quality_phred 15), and reads were retained if at least 60% of their bases met this quality threshold (--unqualified_percent_limit 40). Base correction was enabled for paired-end reads (--correction). Adapter sequences were automatically detected and trimmed from paired-end reads (--detect_adapter_for_pe). Additionally, bases at the 3' end of reads were trimmed using a sliding window approach: if the average quality within a 10-base window fell below a Phred score of 30, bases within that window and downstream were removed (--cut_tail).

3.5.2. METAGENOME ASSEMBLY

The quality-filtered reads were assembled using MegaHIT (v.1.2.9, Krueger et al., 2015) a *de novo* metagenomic assembler designed for large-scale sequencing data with the meta-sensitive preset (--min-count 1 --k-list 21,29,39,49,...,129,141). This setting optimizes k-mer selection for improved reconstruction of metagenomic contigs, particularly for complex microbial communities. During this process, only contigs longer than 500 bp (--min-contig-len) were kept and considered informative.

3.5.3. ASSESSMENT OF ASSEMBLY QUALITY

The quality of the assembled genomes was evaluated using QUAST (v.5.2.0, Gurevich et al., 2013), which provided key assembly statistics, including total assembly length, number of contigs, N50/L50 values, and misassembly rates. These metrics were used to compare different assembly iterations and ensure that the final assembly met high-quality standards.

3.5.4. CONTIG ABUNDANCE ESTIMATION

To estimate contig abundance, quality-controlled reads were mapped back to the assembled contigs using BWA-MEM2 (v.2.2.1, Li, 2013), a fast and memory-efficient aligner optimized for large datasets. Then, CoverM (v.0.7.0, Aroney et al., 2025) was employed to calculate coverage statistics, with the primary focus on RPKM (Reads Per Kilobase per Million mapped reads) to ensure accurate quantification of contig representation in the dataset.

3.5.5. FUNCTIONAL ANNOTATION

Gene annotation was performed using Prokka (v.1.14.6, Seemann, 2014), with the --metagenome option enabled to improve gene predictions for highly fragmented genomes. Additionally, ARGs were identified using AMRFinder (v.3.12.8, Feltwell et al., 2021), which relies on NCBI's curated Reference Gene Database (v.2024-01-31.1), with the --plus option enable detection of not only AMR genes but also virulence factors, stress-response genes, and other functionally relevant sequences. The identified genes were then filtered by coverage relative to reference sequences with 90% being set as threshold. Metal resistance genes (MRGs) were identified by searching the METGeneDb database (Dziurzynski et al., 2022) using MMseqs2 easy-search (Steinegger & Söding, 2017). The search was performed with a minimum sequence identity of 30% (--min-seq-id 0.3) and required 75% coverage of both query and target sequences (--cov-mode 0, -c 0.75). The sensitivity of the MMseqs2 prefiltering module was set to 7 (-s 7).

3.5.6. TAXONOMIC CLASSIFICATION AND MGE IDENTIFICATION

Taxonomic classification of assembled contigs was conducted using Kraken2 (v.2.1.3, Wood et al., 2019), which assigns taxonomic labels based on k-mer matching to a reference database. In this case Kraken2 classification was based on the protein reference database constructed with non-redundant NCBI protein database (nr) downloaded on 2024.06.13 (Sayers et al., 2022).

MGEs were identified using geNomad (v.1.7.4, Camargo et al., 2023), a sequence-similarity- and machine-learning-based approach to distinguish MGEs such as plasmids, viruses, and integrative elements from chromosomal sequences based on genomic features and protein composition. This allowed for high-confidence identification of horizontally transferred elements, providing insights into the potential location of ARGs, MRGs and other genes of interest. A minimum threshold of 0.7 was applied to guarantee the quality of the classification, based on geNomad's aggregated classification score.

3.5.7. DATA INTERPRETATION AND VISUALIZATION

The results from the quality control, assembly, and annotation steps were integrated and visualized using Python scripts to generate charts for better overall comprehension of the data. This included the use of packages such as numpy (Harris et al., 2020), pandas (McKinney, W., et al., 2010). Data structures for statistical computing in python. In Proceedings of the 9th Python in Science Conference (Vol. 445, pp. 51–56)], matplotlib (Hunter, 2007) and seaborn (Waskom, 2021). Their application facilitated the interpretation of findings and ensured reproducibility in data analysis, providing a comprehensive approach to metagenomic analysis, ensuring high-quality data processing, reliable assembly, and meaningful functional characterization of antibiotic and heavy metal resistance in microbial communities.

4. RESULTS AND DISCUSSION

4.1. MICROBIAL IN ARCTIC SOIL: CLASSIC MICROBIOLOGY APPROACH

4.1.1. CHARACTERIZATION OF THE SAMPLE COLLECTION SITE

The soil used in this study was previously described by Dziurzynski et al. (2022). According to the author, the field campaign was carried out in the summer of 2017. During the sampling period, soil temperature ranged from 8.0 °C to -11.3 °C, with a mean of 10.4 °C ± 1.3 °C and a median of 9.9 °C, being, on average, 5.4 °C higher than the air temperature. The sampling points were selected based on the influence of different environmental factors, namely anthropogenic (S1), marine (S2, S6), plant and/or zoogenic (S3, S4, S6), and glacial

(S5), previously proven to be factors with significant impact on the Arctic soil ecosystem (Zwolicki et al., 2013).

More significant variability was observed in soil characteristics, such as structure, moisture, and vegetation (fig. 4). Soil bulk density ranged from 0.67 kg/dm³ (S6) to 1.62 kg/dm³ (S4) (fig. 4). Soil pH ranged from 6.98 to 8.57, with a mean of 7.4 ± 0.7 and a median of 7.2, similar to soils near Pyramiden (Krajcarová et al., 2016), but more alkaline compared to soils in Ny-Ålesund (Halbach et al., 2017). These differences can be attributed to topological variations since Pyramiden and Hornsund are located inland, covered by typical tundra, while Ny-Ålesund is surrounded by glaciers (fig. 4) (Halbach et al., 2017; Krajcarová et al., 2016).

The carbon, hydrogen, nitrogen, and sulfur concentrations were analyzed in each sample using CHNS analysis. Carbon contents ranged from 2.0 to 33.21 mg/g, significantly increasing in S3 (22.04 mg/g) and S6 (33.21 mg/g). The concentrations of the other elements ranged from 0.11 to 5.1 mg/g, although hydrogen in sites S2, S4, and S5, as well as sulfur in sites S1, S2, S3, and S6, were below the detection limit.

The C/N ratio was determined to estimate nutrient cycling, presenting values from 3:1 (S1) to 14:1 (S6), with an overall average of 10:1, suggesting a strong influence of soil microorganisms in the structuring of the ecosystem (Weil and Brady, 2016). Total organic carbon (TOC) contents ranged from 50 mg/L in S4 to 107 mg/L in S3. NO_2^- and NO_3^- concentrations remained below 1 mg/L and 10 mg/L, respectively, except in S3 and S4, where NO_3^- values reached 10 mg/L and 25 mg/L, respectively. At all sites, Cl^- concentrations ranged between 500 and 1000 mg/L, while ammonium ion levels did not exceed 8.4 mg/L in any sample.

4.1.2. ANTIBIOTIC CONCENTRATIONS

R2A culture media supplemented with antibiotic concentrations described by Bengtsson-Palme, J. Larsson, D. J. (2016) were initially prepared, initially using only tetracycline and kanamycin. After the bacterial growth phase, it was observed that the antibiotic concentration tested did not result in a significant reduction in CFU compared to the control plates. Given this result, an investigation was initiated to determine the MIC for all available antibiotics: ampicillin, azithromycin, ciprofloxacin, phosphomycin, kanamycin, and tetracycline.

This study had an Initial Concentration (IC), the values described in the article by Bengtsson-Palme, J. Larsson, D. J. (2016), with a concentration value ten times higher (1:10) being made to determine the specific MIC for each antibiotic (table 1). The concentrations marked in table 1 in green are the concentrations that demonstrated the first significant reduction in the number of CFUs concerning the control plates of their respective soils.

Table 1. Concentrations were used to verify the minimum inhibitory concentration.

	IC	10x IC	10^2x IC	10^3x IC	10^4x IC	10^5x IC
Ampicillin	4 ug/L	4x10 ug/L	4x10^2 ug/L	4x10^3 ug/L	4x10^4 ug/L	4x10^5 ug/L
Azithromycin	16 ug/L	16x10 ug/L	16x10^2 ug/L	16x10^3 ug/L	16x10^4 ug/L	
Ciprofloxacin	2 ug/L	2x10 ug/L	2x10^2 ug/L	2x10^3 ug/L	2x10^4 ug/L	
Phosphomycin	125 ug/L	125x10 ug/L	125 x 10^2 ug/L	125x10^3 ug/L	125x10^4 ug/L	
Kanamycin	125 ug/L	125x10 ug/L	125 x 10^2 ug/L	125x10^3 ug/L	125x10^4 ug/L	
Tetracycline	16 ug/L	16x10 ug/L	16x10^2 ug/L	16x10^3 ug/L	16x10^4 ug/L	

The bacteria in these antibiotic concentrations were selected to continue the purification process of colonies with different morphologies and extract the total DNA from the plate wash-outs to obtain metagenomic sequences.

4.1.3. ANALYSIS OF THE SAMPLES MICROBIOTA

The inoculations of the collected samples generated a similar microbiological profile between the locations and respective antibiotic concentrations since there was a sizable microbiological growth of several different types of bacteria, as shown in fig 6.

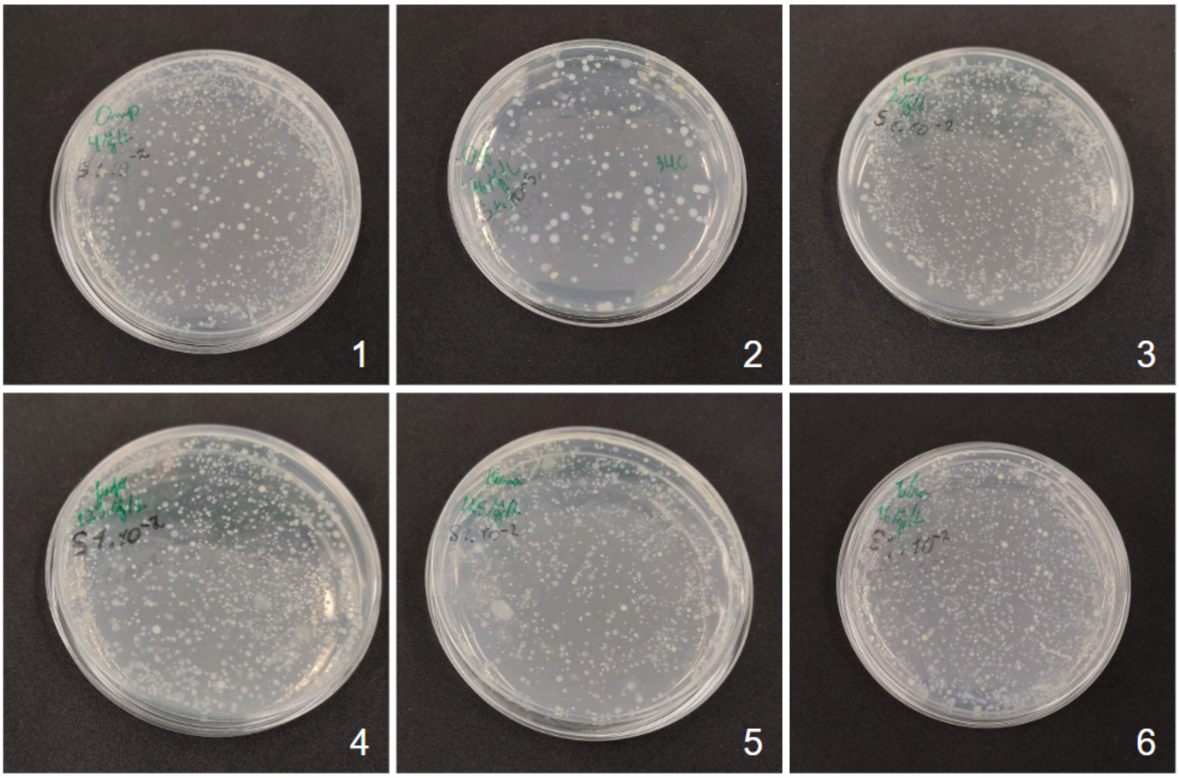


Fig. **7**: Sampling CFUs from plates with IC, illustrating the slight decrease in CFUs from the samples. 1- Ampicillin, 2- Azithromycin, 3- Ciprofloxacin, 4- Phosphomycin, 5- Kanamycin, 6- Tetracycline with a soil dilution of 10^{-2}.

As shown in table 1 and fig 7, the IC of the bacteria isolated in the six Arctic locations exceeds the values reported by Bengtsson-Palme and Larsson (2016). These results can be attributed to ARGs, whose regional dispersion can occur through several mechanisms, as Agudo and Reche (2024) described.

As exemplified in fig 8, without antibiotics, the samples presented several colonies with similar morphologies. Dilutions 1 and 2 presented many CFUs; however, the quantity made it difficult to identify and separate morphologically distinct colonies in dilution 1. In dilutions 2 and 3, it was possible to identify colonies with characteristics that were separate from each other and in an amount that was more representative of the biodiversity of the samples. On the other hand, dilutions 4, 5, and 6 presented a quantity of colonies that was too small to represent the biodiversity in the soil samples collected. Once the standard microbiological profile of the samples was identified, it was possible to compare the microbial growth between the control and growth in the presence of antibiotics.

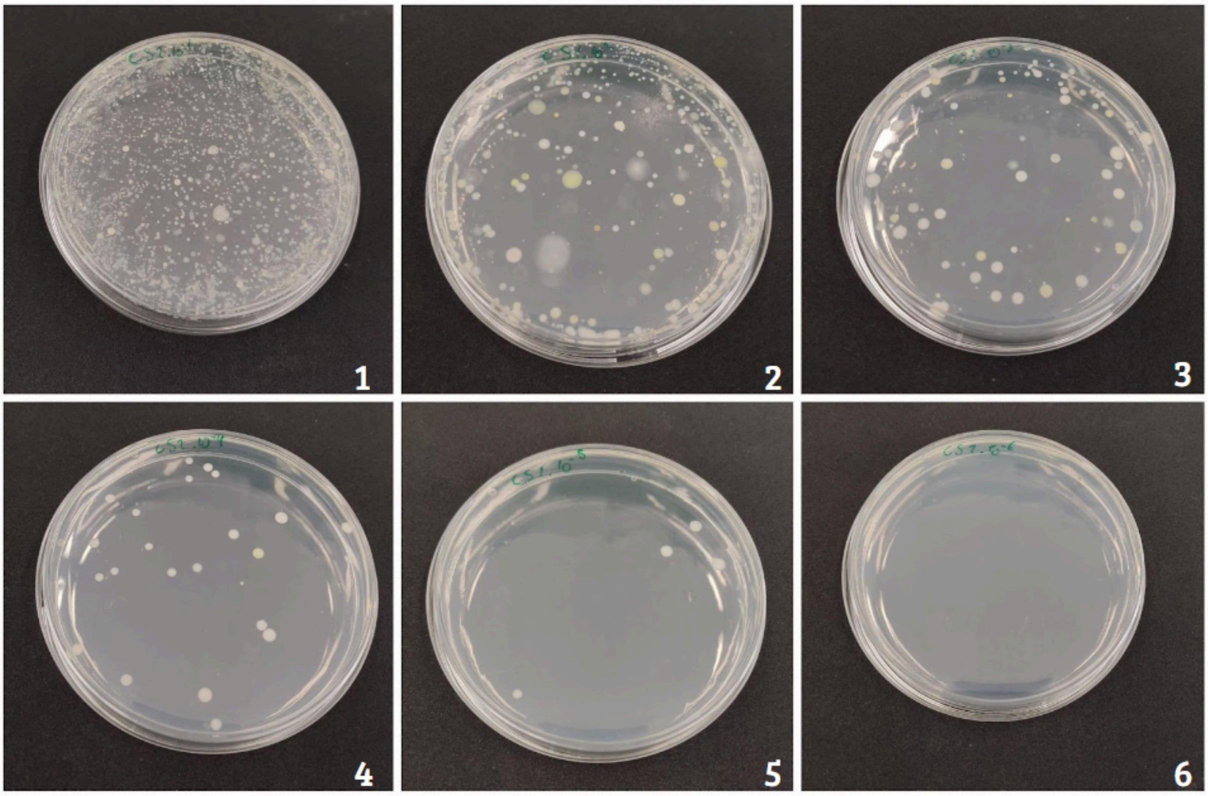
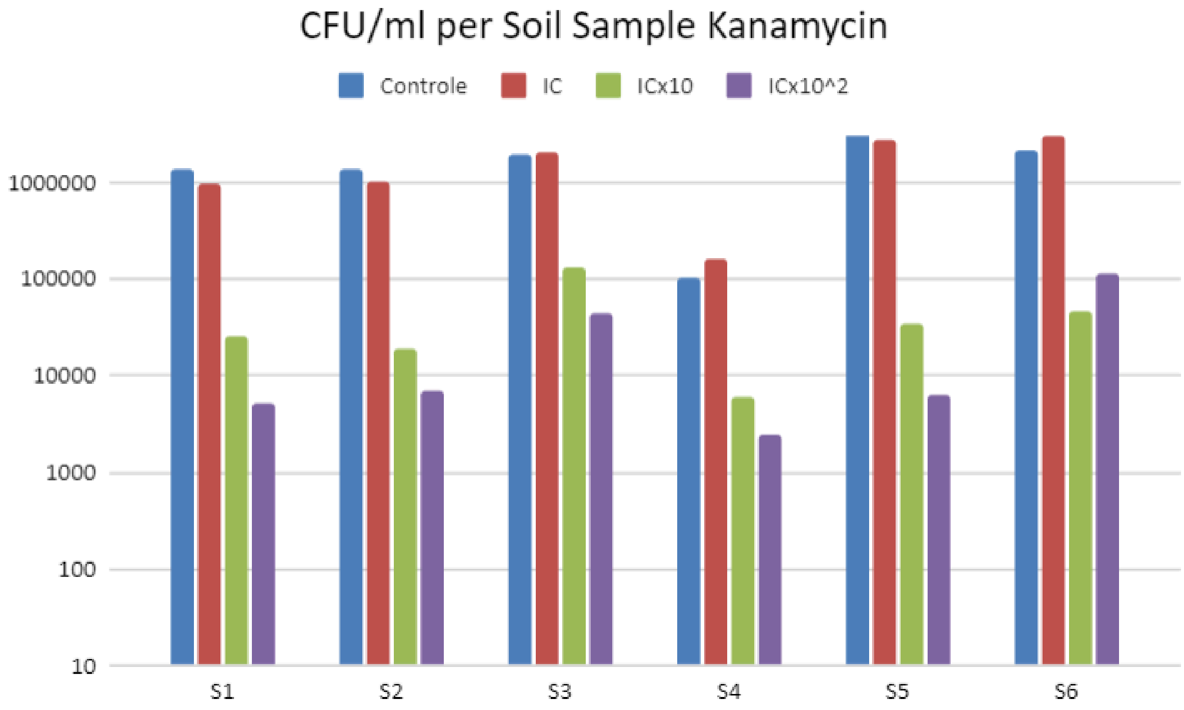


Fig. 8: Control group, numbered from 1 to 6, containing inoculations of soil 1 (Polish Polar Station) in dilutions 1: 10^{-1} to 10^{-6}, respectively.

After selecting bacteria with different morphologies, resistant to the established concentration of antibiotics, shown in table 1, PCR was performed for the 16S rRNA gene, thus confirming the presence of this gene in the selected organisms, which, according to Lao, H. Y. et al. (2024) is a bacterial identification gene. Growth in liquid R2A medium was observed within 72 hours, ensuring that O2 levels were not compromised. Approximately 26 morphologies were preserved at -80ºC on plates supplemented with ampicillin, 34 with azithromycin, 25 with ciprofloxacin, 24 with phosphomycin, 29 with kanamycin, and 30 with tetracycline, totaling approximately 176 samples. These samples were subjected to sequencing by the Illumina method for identification at the species level and genomic comparison with the metagenome.

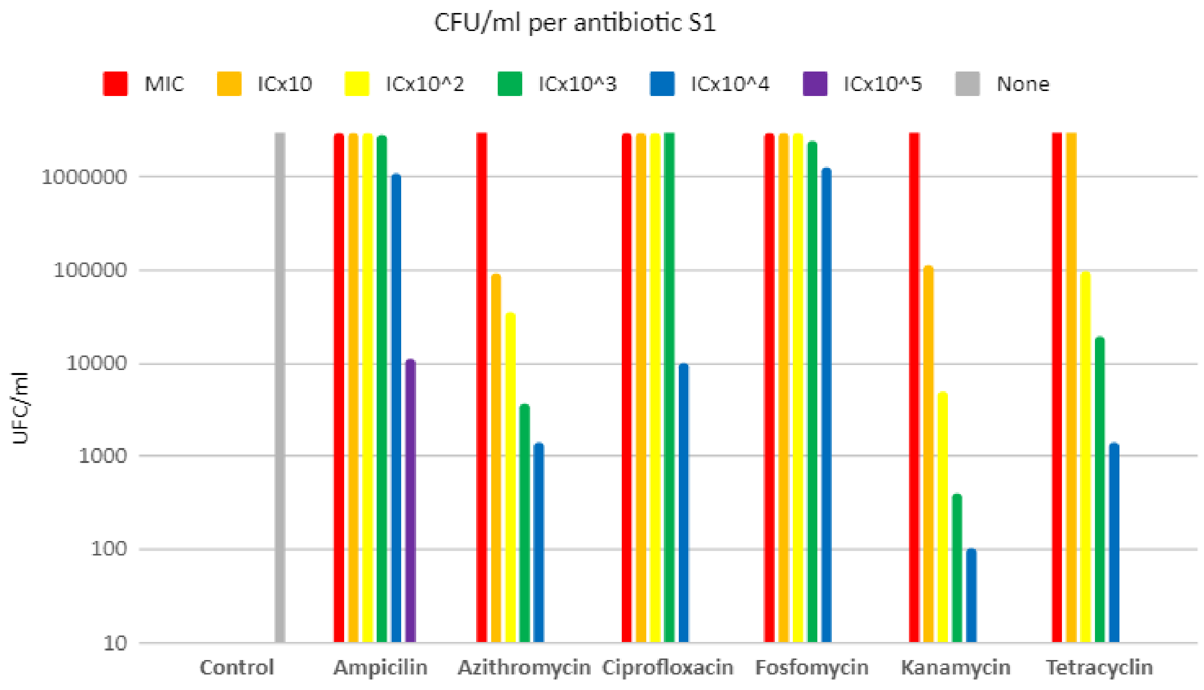
4.1.4. IMPACT OF ANTIBIOTICS ON CELL CULTURES AND SELECTION

The addition of antibiotics to plates containing R2A medium allowed a comparison of bacterial growth levels between control plates without antibiotics and those supplemented with different concentrations of antibiotics. After a 7-day incubation period, the plates were analyzed to ensure that CFUs were visually countable at various dilutions, i.e., within the range of 30 - 300 CFUs per plate (Tortora et al., 2012, p. 174). The results were compared between the different concentrations of each antibiotic, allowing the qualitative identification of the concentration that significantly reduced the number of CFUs. This process was essential to select antibiotic-resistant strains and to continue the construction of a metagenome composed only of these strains.



Graph 1: Log of CFU per milliliter, on all soil samples, with control plates and kanamycin antibiotic concentrations of IC to ICx10^2

The amount of CFUs in the plates containing the IC did not show an apparent reduction, having a count similar to that of the control plate in all soil samples. In graph 1, it's possible to see an example of this difference between the control samples and those containing different concentrations of kanamycin. Because of this, an investigation was carried out for each antibiotic to determine the concentration that would visually show a reduction in CFUs.



graph 2: Log of CFU per milliliter, on all antibiotics with antibiotic concentrations of IC to ICx10^5 and control.

As shown in graph 2, the antibiotics concentrations of ampicillin (4 x 10^5 ug/L), azithromycin (16 x 10^2 ug/L), ciprofloxacin (2 x 10^4 ug/L), phosphomycin (125 x 10^4 ug/L), kanamycin (125 x 10 ug/L), and tetracycline (16 x 10^3 ug/L) exhibited significant reductions. The same inhibitory concentration was obtained in all samples (see appendices). Ampicillin was the antibiotic that showed the closest proximity to the stock concentration being the ICx10^5, and the Kanamycin was the antibiotic with the concentration most similar with the IC, as being only ICx10.

4.2. MICROBIAL IN ARCTIC SOIL: MOLECULAR MICROBIOLOGY APPROACH

4.2.1. DNA COLLECTION AND PCR RESULTS

With the concentrations of antibiotics that demonstrated inhibition of bacterial growth, the different morphologies were collected within each set of dilutions; that is, among the three serial dilutions made for each soil, of these concentrations of antibiotics, all morphologies that were not identified with the naked eye as fungi were selected, to isolate them in single colonies and then perform a PCR with 16S rRNA to confirm the bacterial character.

Therefore, only the positive results (fig. 9) were taken to culture in liquid R2A medium for enrichment and verification of the storage possibility.

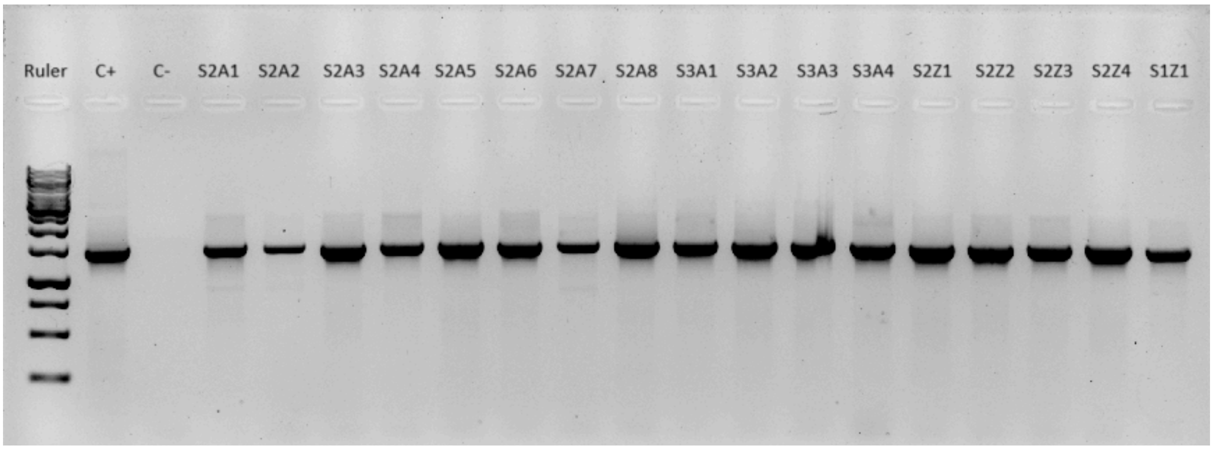


Fig. 9: PCR electrophoresis results after bacterial isolation and DNA extraction. The generuler can be seen in the first column, and the positive control used a known strain, and the negative control contained only the PCR master mix and nuclease-free water.

With this selection method using essential microbiology, only the bacteria that demonstrated resistance to antibiotics were isolated. The samples were enriched in liquid medium so that only those that grew in liquid medium underwent the DNA extraction process to have a higher concentration of DNA after extraction to meet the minimum requirements to be sent for Illumina sequencing.

To verify the quality of the extracted DNA, 0.8% (w/v) agarose gel electrophoresis was performed. This procedure sought to evaluate the integrity of the DNA and identify possible degradation resulting from the removal processes, both for total soil DNA (fig. 10) and isolated bacterial DNA (fig. 9).

The results found in some samples were of low abundance or evident signs of degradation (fig. 10: Fos S3). Given this, the DNA extraction protocol was repeated, and it was observed that some samples contained high amounts of extracellular polymeric substances (EPS), which stood out as the main difference between the samples with good abundance and lower distribution, that the EPS may have contributed to the damage during the removal process (Corcoll, N. et al. 2017).

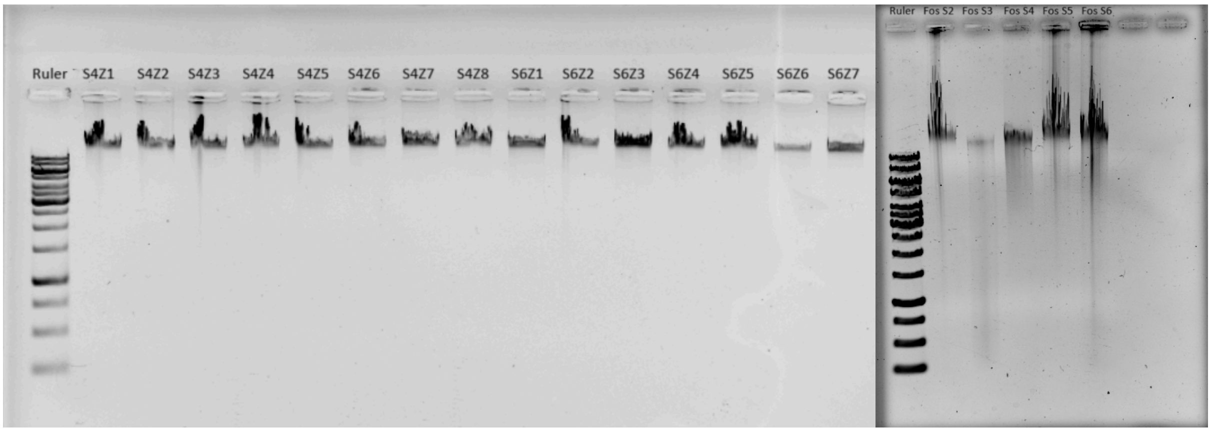
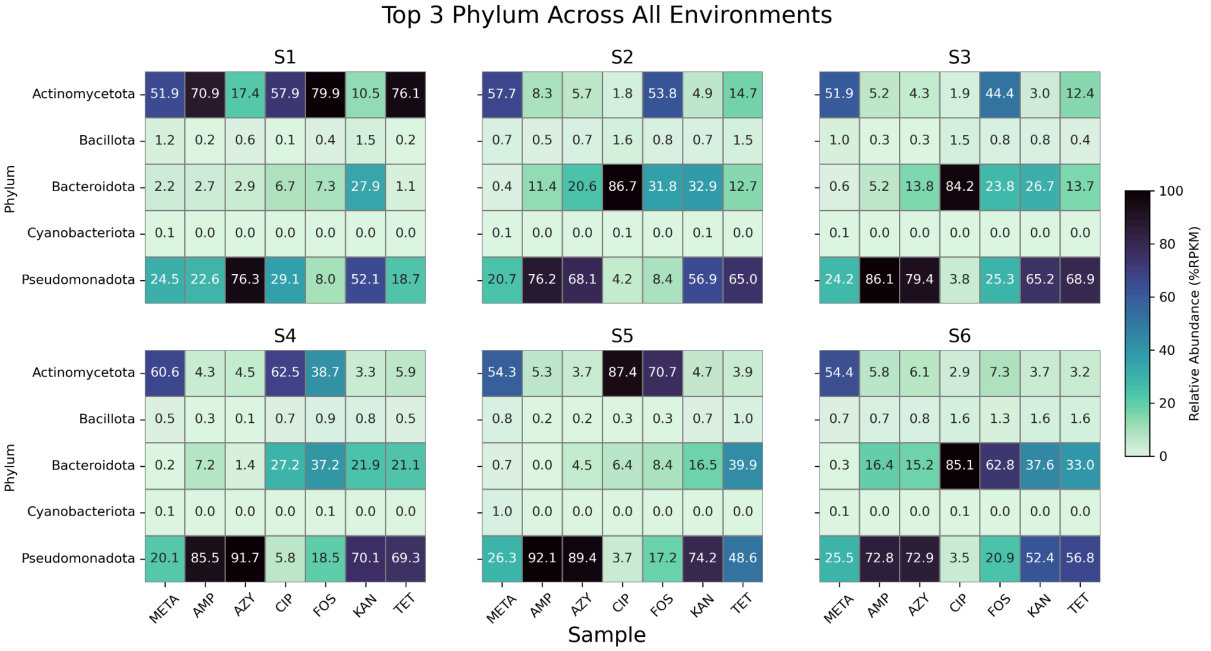


Fig. 10: on the left agarose gel containing ruler gene and DNA extracted from purified and enriched bacterial samples, which were isolated from azithromycin plates of soil samples 4 and 6 and on the right agarose gel containing gene ruler and metagenomic DNA from phosphomycin-enriched plates from soil samples 2, 3, 4, 5 and 6.

4.3. BIOINFORMATIC ANALYSIS

4.3.1. PHYLUM



S1

Phylum	META	AMP	AZY	CIP	FOS	KAN	TET
Actinomycetota	51.9	70.9	17.4	57.9	79.9	10.5	76.1
Bacillota	1.2	0.2	0.6	0.1	0.4	1.5	0.2
Bacteroidota	2.2	2.7	2.9	6.7	7.3	27.9	1.1
Cyanobacteriota	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Pseudomonadota	24.5	22.6	76.3	29.1	8.0	52.1	18.7

S2

Phylum	META	AMP	AZY	CIP	FOS	KAN	TET
Actinomycetota	57.7	8.3	5.7	1.8	53.8	4.9	14.7
Bacillota	0.7	0.5	0.7	1.6	0.8	0.7	1.5
Bacteroidota	0.4	11.4	20.6	86.7	31.8	32.9	12.7
Cyanobacteriota	0.1	0.0	0.0	0.1	0.0	0.1	0.0
Pseudomonadota	20.7	76.2	68.1	4.2	8.4	56.9	65.0

S3

Phylum	META	AMP	AZY	CIP	FOS	KAN	TET
Actinomycetota	51.9	5.2	4.3	1.9	44.4	3.0	12.4
Bacillota	1.0	0.3	0.3	1.5	0.8	0.8	0.4
Bacteroidota	0.6	5.2	13.8	84.2	23.8	26.7	13.7
Cyanobacteriota	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Pseudomonadota	24.2	86.1	79.4	3.8	25.3	65.2	68.9

S4

Phylum	META	AMP	AZY	CIP	FOS	KAN	TET
Actinomycetota	60.6	4.3	4.5	62.5	38.7	3.3	5.9
Bacillota	0.5	0.3	0.1	0.7	0.9	0.8	0.5
Bacteroidota	0.2	7.2	1.4	27.2	37.2	21.9	21.1
Cyanobacteriota	0.1	0.0	0.0	0.0	0.1	0.0	0.0
Pseudomonadota	20.1	85.5	91.7	5.8	18.5	70.1	69.3

S5

Phylum	META	AMP	AZY	CIP	FOS	KAN	TET
Actinomycetota	54.3	5.3	3.7	87.4	70.7	4.7	3.9
Bacillota	0.8	0.2	0.2	0.3	0.3	0.7	1.0
Bacteroidota	0.7	0.0	4.5	6.4	8.4	16.5	39.9
Cyanobacteriota	1.0	0.0	0.0	0.0	0.0	0.0	0.0
Pseudomonadota	26.3	92.1	89.4	3.7	17.2	74.2	48.6

S6

Phylum	META	AMP	AZY	CIP	FOS	KAN	TET
Actinomycetota	54.4	5.8	6.1	2.9	7.3	3.7	3.2
Bacillota	0.7	0.7	0.8	1.6	1.3	1.6	1.6
Bacteroidota	0.3	16.4	15.2	85.1	62.8	37.6	33.0
Cyanobacteriota	0.1	0.0	0.0	0.1	0.0	0.0	0.0
Pseudomonadota	25.5	72.8	72.9	3.5	20.9	52.4	56.8

Fig. 11: relative phylum abundance of bacteria non-sensitive to particular antibiotics across all environments.

The analysis of the control metagenome in all environments revealed that the predominant phylum is Actinomycetota, with an abundance greater than 50%, followed by

Pseudomonadota, which presents more than 20% of minimum representation. However, the presence of antibiotics significantly altered this distribution.

In environments S2 to S6, Pseudomonadota became the predominant phylum in most antibiotics, while in environment S1, Actinomycetota remained as the most abundant group. The exceptions in S1 were the antibiotics azithromycin and kanamycin, in which Pseudomonadota presented greater abundance (76.3% and 52.1%, respectively). For the antibiotic azithromycin, Actinomycetota was the second most abundant phylum (17.4%), while in kanamycin, the second predominant group was Bacteroidota (27.9%).

In the other environments, in the presence of the antibiotics ampicillin, azithromycin, kanamycin and tetracycline, Pseudomonadota predominated (<48%), followed by Bacteroidota. In S3, the abundance of Bacteroidota and Actinomycetota was equivalent (5.2%). In S4 (azithromycin) and S5 (AMP), Actinomycetota appeared as the second most abundant phylum.

In the presence of the antibiotic ciprofloxacin, environments S2, S3 and S6 showed a predominance of Bacteroidota, followed by Pseudomonadota. In contrast, in environments S4 and S5, Actinomycetota was the predominant phylum, followed by Bacteroidota.

In the presence of the phosphomycin, environments S1, S2, S3 and S5 showed a predominance of Actinomycetota, followed by Pseudomonadota. In environment S2, however, the second most abundant phylum was Bacteroidota. In environment S4, the difference in abundance between Actinomycetota (38.7%) and Pseudomonadota (37.2%) was minimal (<2%). In environment S6, Bacteroidota was the predominant phylum (62.8%), followed by Pseudomonadota (20.9%).

These results indicate that the presence of antibiotics significantly influences the composition of the microbial community, favoring specific taxa depending on the environment and the type of antibiotic present.

4.3.2. PHYLUM & ARGS ABUNDANCE

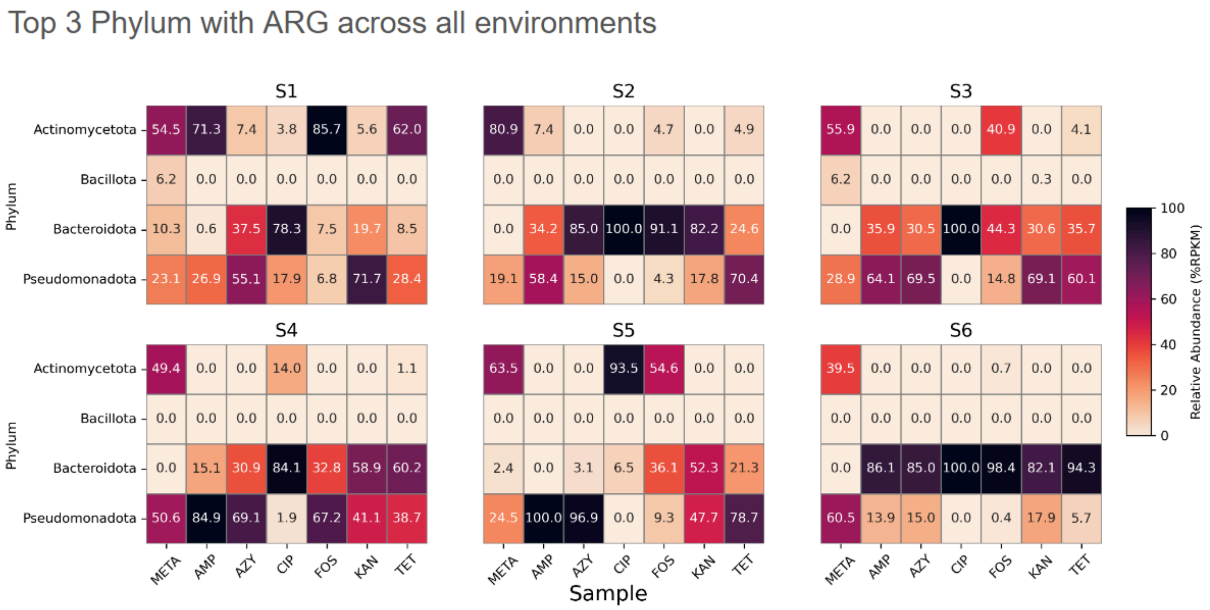


Fig. 12: relative phylum abundance with ARG of all antibiotics across all environments.

The analysis of the distribution of ARGs in the control metagenome revealed a predominance of the phylum Actinomycetota in S1 (54.5%), S2 (80.9%), S3 (55.28%) and S5 (63.5%), followed by Pseudomonodota. In environment S4, the relative abundance of Pseudomonodota (50.6%) and Actinomycetota (49.4%) showed a difference of less than 1.5%. In contrast, in environment S6, an inversion of this trend was observed, with Pseudomonodota predominating (>60%) and Actinomycetota representing 39.5%. In the presence of the other antibiotics, the predominant phylum in environment S6 was Bacteroidota, with abundances greater than 82%, followed by Pseudomonodota. In the presence of ampicillin, environments S2 to S5 showed a predominance of ARGs associated with Pseudomonodota (>58%), followed by Bacteroidota. However, S1 differed from the others, with a predominance of Actinomycetota (71.3%), followed by Pseudomonodota (26.9%). For the azithromycin, most environments (S1 to S5) presented ARGs predominantly associated with Pseudomonodota, followed by Bacteroidota, except S2, where the order was reversed.

In the presence of ciprofloxacin, most environments presented Bacteroidota as the predominant phylum in the abundance of ARGs, followed by Pseudomonodota in S1, Actinomycetota in S4 and Bacteroidota in S5. In environments S2, S3 and S6, the relative abundance of ARGs was 100% in Bacteroidota.

For phosphomycin, the distribution of ARGs among the Phyla varied even more in their respective environments. Environment S1 was the only one with a predominance of Actinomycetota (85.7%), followed by Bacteroidota (7.5%) and Pseudomonodota (6.8%), with a difference of less than 1% between the last two. In environments S2 and S6, Bacteroidota was predominant, with no significant values for the other phyla. In S3, Bacteroidota (44.3%) showed a difference of approximately 4.5% in relation to Actinomycetota (40.9%), followed by Pseudomonodota (14.8%). In environment S4, the predominance was of Pseudomonodota (67.2%), followed by Bacteroidota (32.8%). In S5, Actinomycetota (54.6%) was the most abundant phylum, followed by Bacteroidota (36.1%).

In the presence of kanamycin, environments S1 and S3 showed a predominance of ARGs associated with Pseudomonodota (>69%), followed by Bacteroidota. In contrast, in environments S2, S4, S5 and S6, Bacteroidota was the predominant phylum, followed by Pseudomonodota, although in S5 the difference between the two was less than 5%. Finally, in tetracycline, a predominance of Actinomycetota was observed in S1 (62%), followed by Pseudomonodota (28.4%). In environments S4 and S6, Bacteroidota was the most abundant phylum (>60%), followed by Pseudomonodota. In the other environments, Pseudomonodota predominated (>60%), followed by Bacteroidota.

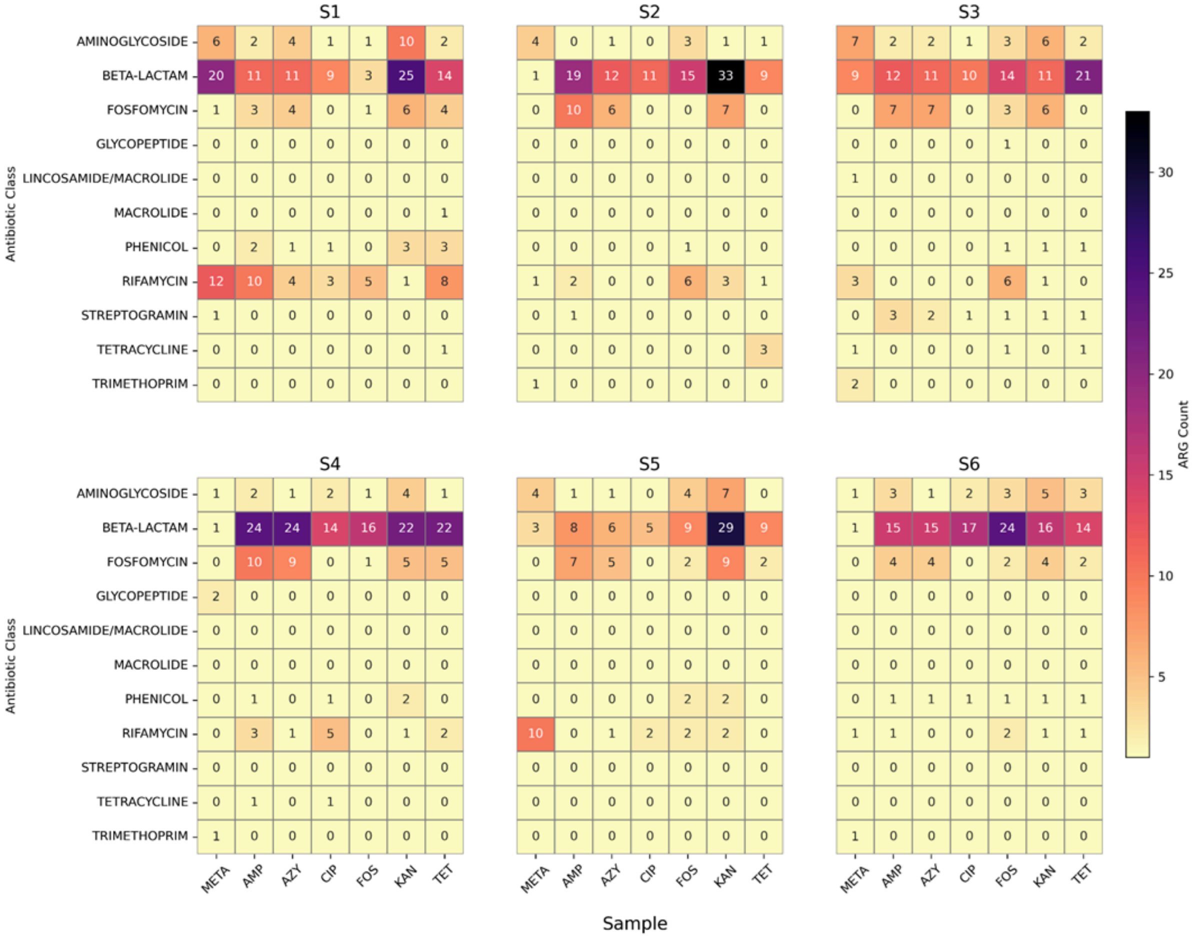
The analysis of microbial composition and the distribution of antibiotic resistance genes revealed distinct patterns among the predominant phyla, suggesting that the presence of antibiotics influences not only the community structure but also the resistance profile present in the microbiome. One of the most relevant aspects observed is the frequent association of Pseudomonodota with ARGs, which can be explained by their known ability to acquire exogenous genes through horizontal gene transfer (HGT) (Martínez, 2018). This phylum includes several highly versatile microorganisms, such as those of the genus Pseudomonas, which are recognized for their natural ability to capture genetic material from the environment (Davies & Davies, 2010).

The variation in ARG abundance even when phylum composition differed between environments suggests that resistance may be both autochthonous, i.e., intrinsic to certain microbial groups, and exogenous, acquired from the environment (Von Wintersdorff et al., 2016). This may explain why Pseudomonadota often exhibits a greater accumulation of ARGs, even in situations where it is not the predominant phylum in terms of relative abundance. This discrepancy may be attributed to the genomic plasticity of the group, which allows the rapid incorporation of resistance genes, increasing its adaptability in environments contaminated by antibiotics (Zhu et al., 2017).

In addition, the predominance of Bacteroidota in the presence of antibiotics such as ciprofloxacin and phosphomycin suggests that this phylum may play an important role in the maintenance and dissemination of ARGs in specific environments. This behavior has already been described in previous studies, where Bacteroidota showed a great capacity for adaptation to antibiotics due to the presence of mobile genetic elements associated with resistance (Salyers et al., 2004). On the other hand, Actinomycetota, which predominated in some control environments and in the presence of phosphomycin, may represent a natural reservoir of resistance, since many of the antibiotics currently used are derived from actinobacteria, which may favor the presence of intrinsic resistance genes in this group (D'Costa et al., 2006).

These findings reinforce the importance of understanding the mechanisms of resistance dissemination in environmental microbiomes, especially in extreme ecosystems such as Arctic soils. Combining classical microbiological techniques with metagenomic analyses allows not only to identify the prevalence and diversity of bacteria in these environments, but also to characterize the genetic mechanisms underlying resistance. Furthermore, by examining the taxonomic composition of bacterial hosts harboring resistance genes, this study contributes to the understanding of the evolution and dissemination of bacterial resistance in pristine environments, highlighting ecological and public health implications.

4.3.3 RESISTANCE GENES PER ANTIBIOTIC CLASS



S1

Antibiotic Class	META	AMP	AZY	CIP	FOS	KAN	TET
AMINOGLYCOSIDE	6	2	4	1	1	10	2
BETA-LACTAM	20	11	11	9	3	25	14
FOSFOMYCIN	1	3	4	0	1	6	4
GLYCOPEPTIDE	0	0	0	0	0	0	0
LINCOSAMIDE/MACROLIDE	0	0	0	0	0	0	0
MACROLIDE	0	0	0	0	0	0	1
PHENICOL	0	2	1	1	0	3	3
RIFAMYCIN	12	10	4	3	5	1	8
STREPTOGRAMIN	1	0	0	0	0	0	0
TETRACYCLINE	0	0	0	0	0	0	1
TRIMETHOPRIM	0	0	0	0	0	0	0

S2

Antibiotic Class	META	AMP	AZY	CIP	FOS	KAN	TET
AMINOGLYCOSIDE	4	0	1	0	3	1	1
BETA-LACTAM	1	19	12	11	15	33	9
FOSFOMYCIN	0	10	6	0	0	7	0
GLYCOPEPTIDE	0	0	0	0	0	0	0
LINCOSAMIDE/MACROLIDE	0	0	0	0	0	0	0
MACROLIDE	0	0	0	0	0	0	0
PHENICOL	0	0	0	0	1	0	0
RIFAMYCIN	1	2	0	0	6	3	1
STREPTOGRAMIN	0	1	0	0	0	0	0
TETRACYCLINE	0	0	0	0	0	0	3
TRIMETHOPRIM	1	0	0	0	0	0	0

S3

Antibiotic Class	META	AMP	AZY	CIP	FOS	KAN	TET
AMINOGLYCOSIDE	7	2	2	1	3	6	2
BETA-LACTAM	9	12	11	10	14	11	21
FOSFOMYCIN	0	7	7	0	3	6	0
GLYCOPEPTIDE	0	0	0	0	1	0	0
LINCOSAMIDE/MACROLIDE	1	0	0	0	0	0	0
MACROLIDE	0	0	0	0	0	0	0
PHENICOL	0	0	0	0	1	1	1
RIFAMYCIN	3	0	0	0	6	1	0
STREPTOGRAMIN	0	3	2	1	1	1	1
TETRACYCLINE	1	0	0	0	1	0	1
TRIMETHOPRIM	2	0	0	0	0	0	0

S4

Antibiotic Class	META	AMP	AZY	CIP	FOS	KAN	TET
AMINOGLYCOSIDE	1	2	1	2	1	4	1
BETA-LACTAM	1	24	24	14	16	22	22
FOSFOMYCIN	0	10	9	0	1	5	5
GLYCOPEPTIDE	2	0	0	0	0	0	0
LINCOSAMIDE/MACROLIDE	0	0	0	0	0	0	0
MACROLIDE	0	0	0	0	0	0	0
PHENICOL	0	1	0	1	0	2	0
RIFAMYCIN	0	3	1	5	0	1	2
STREPTOGRAMIN	0	0	0	0	0	0	0
TETRACYCLINE	0	1	0	1	0	0	0
TRIMETHOPRIM	1	0	0	0	0	0	0

S5

Antibiotic Class	META	AMP	AZY	CIP	FOS	KAN	TET
AMINOGLYCOSIDE	4	1	1	0	4	7	0
BETA-LACTAM	3	8	6	5	9	29	9
FOSFOMYCIN	0	7	5	0	2	9	2
GLYCOPEPTIDE	0	0	0	0	0	0	0
LINCOSAMIDE/MACROLIDE	0	0	0	0	0	0	0
MACROLIDE	0	0	0	0	0	0	0
PHENICOL	0	0	0	0	2	2	0
RIFAMYCIN	10	0	1	2	2	2	0
STREPTOGRAMIN	0	0	0	0	0	0	0
TETRACYCLINE	0	0	0	0	0	0	0
TRIMETHOPRIM	0	0	0	0	0	0	0

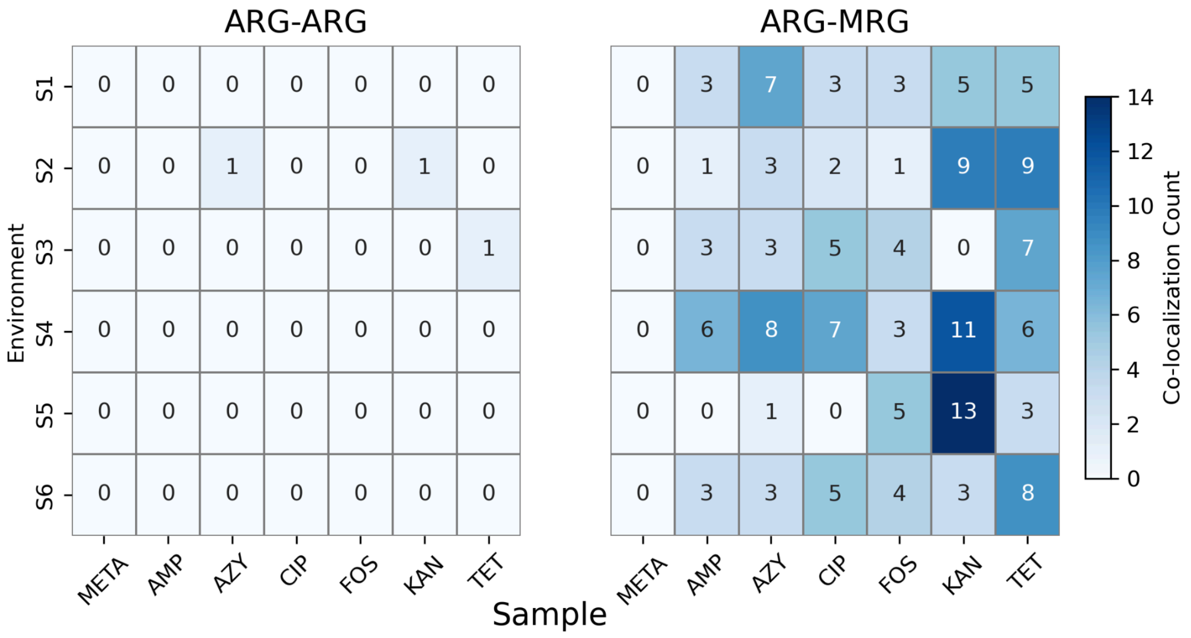
S6

Antibiotic Class	META	AMP	AZY	CIP	FOS	KAN	TET
AMINOGLYCOSIDE	1	3	1	2	3	5	3
BETA-LACTAM	1	15	15	17	24	16	14
FOSFOMYCIN	0	4	4	0	2	4	2
GLYCOPEPTIDE	0	0	0	0	0	0	0
LINCOSAMIDE/MACROLIDE	0	0	0	0	0	0	0
MACROLIDE	0	0	0	0	0	0	0
PHENICOL	0	1	1	1	1	1	1
RIFAMYCIN	1	1	0	0	2	1	1
STREPTOGRAMIN	0	0	0	0	0	0	0
TETRACYCLINE	0	0	0	0	0	0	0
TRIMETHOPRIM	1	0	0	0	0	0	0

Fig. 13: ARGs count per antibiotic class, across all environments.

The high abundance of antibiotic resistance genes (AGRs) classified as β-lactams can be observed regardless of the antibiotic class used in the culture medium of the bacteria analyzed in the metagenome. This phenomenon probably results from the low accuracy of older protein identification methods used to construct the databases employed in the analysis. Such methods were based on predictors for the classification and identification of β-lactamases, which presented lower accuracy compared to current approaches (ASHRAF et al., 2021). In addition, it is possible that genes responsible for resistance to other classes of antibiotics, selected in different culture media, are underrepresented due to the high relative abundance of β-lactam resistance genes.

4.3.4 ARGS CO-LOCALIZATION WITH MRG



	META	AMP	AZY	CIP	FOS	KAN	TET
S1	0	0	0	0	0	0	0
S2	0	0	1	0	0	1	0
S3	0	0	0	0	0	0	1
S4	0	0	0	0	0	0	0
S5	0	0	0	0	0	0	0
S6	0	0	0	0	0	0	0

	META	AMP	AZY	CIP	FOS	KAN	TET
S1	0	3	7	3	3	5	5
S2	0	1	3	2	1	9	9
S3	0	3	3	5	4	0	7
S4	0	6	8	7	3	11	6
S5	0	0	1	0	5	13	3
S6	0	3	3	5	4	3	8

Fig. 14: ARG-ARG and ARG-MRG Co-localization of all environments in each antibiotic.

Few co-localizations are observed between ARG-ARG, while in ARG-MRG co-localization is much more frequent, with several cells showing high values, especially in kanamycin in sample S5. This suggests that there is a stronger relationship ARG-MRG than between ARG-ARG.

The co-occurrence of antibiotic resistance genes and metal resistance genes has been widely studied due to its relevance in the spread of antimicrobial resistance. Studies indicate that this association is more frequent in human pathogens than in environmental bacteria, suggesting a possible selective advantage in clinical settings. For example, Pal et al. (2017) analyzed a large collection of complete genomes and identified a significantly higher co-occurrence of ARGs and MRGs in human-associated bacteria than in microorganisms from soil and aquatic environments.

Furthermore, investigations of microbial communities on marine plastics suggest that the plastisphere may act as a reservoir for ARGs and MRGs. Studies have shown that network analysis revealed non-random co-occurrence patterns between ARG and MRG subtypes, indicating complex interactions between these genes in the marine environment (Zettler, Mincer, & Amaral-Zettler, 2013).

These findings highlight the importance of understanding the co-occurrence of ARGs and MRGs in different ecosystems, as they may influence the spread of antimicrobial resistance and impact mitigation strategies.

5. CONCLUSION

This study investigated the diversity and antibiotic resistance of microbial communities in Arctic soils using classical microbiology and metagenomic approaches. The results revealed that antibiotic concentrations did not significantly reduce bacterial growth at initial concentrations, leading to further investigations to determine the MIC of six antibiotics: ampicillin, azithromycin, ciprofloxacin, phosphomycin, kanamycin, and tetracycline. The MICs obtained were higher than those previously reported in the literature, suggesting that Arctic microbial communities possess intrinsic or acquired resistance mechanisms.

The culture-based approach identified a diverse microbiota across all sampling sites, with antibiotic supplementation selecting for resistant bacterial populations. The presence of resistant bacteria was confirmed by PCR targeting the 16S rRNA gene, followed by DNA extraction and sequencing. Bioinformatic analyses demonstrated significant shifts in microbial community composition due to antibiotic exposure. In control conditions, Actinomycetota was the predominant phylum, but antibiotic-treated samples exhibited increased relative abundances of Pseudomonadota and Bacteroidota, suggesting selective pressure favoring these groups.

The metagenomic analysis of ARGs indicated a high prevalence of resistance determinants across all samples, with notable enrichment in Pseudomonadota and Bacteroidota. The frequent association of Pseudomonadota with ARGs highlights its potential role as a reservoir of resistance genes, likely facilitated by horizontal gene transfer. Additionally, the variation in ARG abundance across different environments, despite shifts in phylum composition, underscores the complex interplay between microbial community structure and resistance gene distribution.

Overall, this study provides critical insights into the resilience and adaptability of Arctic soil microbiomes in the presence of antibiotics. The findings highlight the widespread presence of antibiotic resistance in remote environments, emphasizing the need for continued monitoring of ARG dissemination in natural ecosystems. Further investigations into the mechanisms driving resistance gene transfer and persistence in these microbial communities are essential to better understand the ecological and evolutionary implications of antibiotic resistance in extreme environments.

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