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Dispersal of antibiotic resistant microbes in alpine snow and its consequences

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I also want to extend a big THANK YOU to Tobias for bringing in so much expertise where I would have otherwise struggled, for almost overnight reviewing my papers, and for always being just one call away to support. You definitely helped me become a better scientist.

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Last, but definitely not least, I want to thank Lilli. The most stunning, heartfelt person who decided to spend the rest of her life by my side. You've heard all of my talks 20 times, calmed my mind whenever necessary, proofread all the (German) blog articles I wrote, and still became my wife despite me still being a student. I could not have done this PhD without you. Thank you for being my better half!

I feel like I could go on forever and fill many more pages simply thanking all the wonderful people around me. But as this should be a scientific piece of work in the end, I will conclude here with a final THANK YOU TO ALL OF YOU. Know that you've been part of this.

Thesis Structure

The thesis is structured as follows: In **Chapter 1**, necessary background information within the scope of this thesis is presented including an overview of antibiotic resistance, its environmental aspects, and the challenges in communicating the matter to the broader public. This is followed by a Chapter on the existing knowledge gaps and the main research questions of this work to help close them (**Chapter 2**). In **Chapter 3**, the key results of **Paper I**, **Paper II**, and **Paper III** are discussed together. Major challenges in study design and methods are presented in **Chapter 4** which highlights potential methodical limitations as well as workaround solutions used while conducting these studies. Emerging future research needs and applications as well as an overall conclusion are presented in **Chapter 5**. Finally, the individual publications are presented individually in full length including supplementary material.

Paper I: Gattinger D, Pichler K, Weil T, Sattler B. **A comparative approach to confirm antibiotic-resistant microbes in the cryosphere**. Front Microbiol. 2023 Aug 3;14:1212378. doi: 10.3389/fmicb.2023.1212378. PMID: 37601352 PMCID: PMC10435281.

Paper II: Gattinger D, Schlenz V, Weil T, Sattler B. **From remote to urbanized: Dispersal of antibiotic-resistant bacteria under the aspect of anthropogenic influence.** Sci Total Environ. 2024 May 10;924:171532. doi: 10.1016/j.scitotenv.2024.171532. Epub 2024 Mar 6. PMID: 38458439.

Paper III: Gattinger D, Schlenz V, Weil T, Sattler B. **Shaping Antibiotic Resistance: Human Influence in Earth's Coldest Realms** (Submitted to Frontiers for Young Minds)

Summary

Antibiotic resistance is considered one of the greatest global challenges of the 21st century. However, relatively little is known about its distribution outside urban areas, and especially the cryosphere. These regions, often regarded as one of the most pristine ones on Earth, provide an interesting environment for studying both intrinsic resistances and estimating the extent of anthropogenic pollution by antibiotic resistance. The impact of humans on the spread of resistant microorganisms in the cryosphere has been largely unexplored. Glacier regions are not isolated areas, and the downstream effects on the circulation of resistant microorganisms and their resistance genes remain unclear. The lack of standardized and comparable methods for such investigations poses a particular challenge for determining the resistome of the cryosphere.

This work addresses these existing knowledge gaps and explores the potential of microorganisms in the cryosphere to survive antibiotics in toxic concentrations, as well as the role of anthropogenic influence.

Paper I and **Paper II** focus on the distribution of phenotypic resistances of cold-loving bacteria in the cryosphere. We demonstrated the limited effectiveness of up to eight different antibiotics on cold-loving microorganisms from these environmental samples. Resistance was not limited to natural antimicrobials but also included semi-synthetic and synthetic ones. Bacterial isolates with reduced susceptibility to antibiotics were found in ice, snow, high mountain lakes, glacial meltwaters, and downstream rivers. Overall, we found that more than 50% of all isolates were multi-resistant, which highlights the underestimated potential of cryospheric resistome.

In **Paper I**, we also investigated the effects of various parameters on traditional cultivation-based antibiotic susceptibility tests for evaluating phenotypic resistance in psychrophilic and psychrotrophic organisms. Standardized tests for studying antibiotic resistance in the cryosphere are essential to increase comparability between studies. To lay groundwork for that, the agar disk diffusion method, the gold standard in clinical settings,

was adapted and optimized for the growth conditions of cold-loving microorganisms while adhering to EUCAST guidelines. We showed that the choice of nutrient medium and agar concentration significantly influenced the interpretation of results while adjustments to the growth conditions of cold-loving bacteria had a much smaller effect. Thus, we demonstrated that an optimized agar diffusion test, following certain guidelines such as the choice of the correct nutrient medium, is a suitable method for studying antibiotic resistance in the cryosphere comparably and reliably.

Paper II addresses the effect of the anthropogenic influence on antibiotic resistance in the cryosphere. Using a novel approach and leveraging publicly available heatmaps from sports tracking providers, we compared the resistome of glacier regions in relation to their direct human influence as a first. Within this study, a clear correlation between direct human presence and an increase in antimicrobial resistance on glaciers was observed. This trend was also confirmed for further samples of the study, consisting of snow, flowing waters, and lakes, where "CORINE Land Cover" data was used in the respective catchment areas of the sampling sites to evaluate the human influence. Across all our samples, the average number of resistant bacteria was 18.5 % to 29.8 % higher in heavily influenced samples. Additionally, a tendency towards increased multi-resistance (+40%) was observed in isolates from regions with more direct human activity. Resistance to synthetic antibiotics was also more prevalent in these sampling sites.

Paper III deals with effective knowledge transfer to individuals outside the scientific discourse. Antibiotic resistance is a global issue that also faces a communication problem. As a "One-Health" problem, it requires the participation of everyone, and bridging the gap between scientists and non-scientists is essential to achieve this. The "Frontiers for Young Minds" platform provides a suitable opportunity to successfully convey knowledge to younger individuals without losing scientific rigor. This endeavor from Paper III was further supported by an actively curated blog and a website with data from **Paper II**.

Zusammenfassung

Antibiotikaresistenzen gelten als eine der größten weltweiten Herausforderungen des 21. Jahrhunderts. Dennoch ist vergleichsweise wenig über sie bekannt, insbesondere wenn es um ihre Verbreitung außerhalb von städtischen Gebieten geht, einschließlich der Kryosphäre. Gerade diese Region, die oft als vergleichsweise "unberührt" betrachtet wird, stellt eine interessante Umgebung für die Untersuchung sowohl intrinsischer Resistenzen als auch für die Abschätzung des Ausmaßes anthropogener Verschmutzung durch Antibiotikaresistenzen dar. Der Einfluss des Menschen auf die Verbreitung resistenter Mikroorganismen in der Kryosphäre ist bisher kaum erforscht worden. Zudem sind Gletscherregionen keine isolierten Gebiete, und die Auswirkungen flussabwärts auf die Zirkulation resistenter Mikroorganismen und ihrer Resistenzgene sind noch unklar. Das Fehlen standardisierter und vergleichbarer Methoden für entsprechende Untersuchungen stellt eine besondere Herausforderung für die Ermittlung des Resistoms der Kryosphäre dar.
Diese Arbeit adressiert vorhandene Wissenslücken und erforscht mit innovativen Ansätzen das Potenzial der Mikroorganismen in der Kryosphäre, Antibiotika in toxischen Konzentrationen zu überstehen, und welche Rolle dabei der anthropogene Einfluss spielt.

Paper I und **Paper II** befassen sich mit der Verbreitung von phänotypischen Resistenzen von Bakterien der Kryosphäre. Dabei konnten wir die limitierte Effektivität von bis zu acht verschiedenen Antibiotika auf Mikroorganismen aus Umweltproben zeigen. Die Resistenz war dabei in fast allen Untersuchungsstellen nicht nur auf natürliche antimikrobielle Substanzen beschränkt, sondern umfasste auch Semisynthetische und Synthetische. Resistente Mikroorganismen konnten in Eis, Schnee, Hochgebirgsseen und Gletscherabflüssen nachgewiesen werden. Mehr als 50 % aller Isolate waren dabei multiresistent und zeigten eindrucksvoll das unterschätzte Potential der Mikroorganismen aus Kryosphärenhabitaten, trotz der Präsenz von Antibiotika zu überleben.

In **Paper I** untersuchen wir außerdem die Auswirkungen verschiedener Parameter auf die klassischen kulturbasierenden Antibiotika-Tests für die Testung von phänotypischer Resistenz bei Organismen Kryosphärenhabitaten. Standardisierte Tests für die Erforschung

von Antibiotikaresistenzen in der Kryosphäre sind essentiell, um künftig die Vergleichbarkeit zwischen Studien zu erhöhen. Der Agardiffusionstest - der Goldstandard in klinischen Umgebungen - wurde dazu auf die Wachstumsbedingungen kälteliebender Mikroorganismen bei gleichzeitig möglichst genauer Einhaltung der Richtlinien der EUCAST adaptiert und optimiert. Dabei zeigte sich vor allem, dass die Wahl des Nährmediums und insbesondere der Agar-Konzentration einen signifikanten Einfluss auf die Interpretation der Ergebnisse hat. Anpassungen an die Wachstumsbedingungen von psychrophilen und psychrotrophen Bakterien hingegen haben einen deutlich kleineren Effekt. Damit konnten wir zeigen, dass ein optimierter Agardiffusionstest unter Einhaltung bestimmter Richtlinien, wie der Wahl des korrekten Nährmediums, ein geeignetes Verfahren ist, um Antibiotikaresistenzen in der Kryosphäre vergleichbar und verlässlich zu untersuchen.

Paper II behandelt den Effekt des anthropogenen Einflusses auf Antibiotika-Resistenzen in der Kryosphäre. Mithilfe eines neuartigen Ansatzes und der Nutzung öffentlich verfügbarer “Heatmaps” von “Sports-Tracking-Anbietern” konnte dabei unseres Wissens nach erstmals eine relative Quantifizierung von Gletscherregionen untereinander hinsichtlich ihres direkten humanen Einflusses erfolgen. Dabei zeigte sich eine klare Korrelation zwischen der menschlichen Präsenz und steigender Zahl an antibiotikaresistenten Bakterien. Dieser Trend konnte auch für weitere Proben der Studie bestehend aus Schnee, Fließgewässern, und Seen beobachtet werden, wobei “CORINE Land Cover” Daten in den jeweiligen Einzugsgebieten der Probenstellen herangezogen wurden, um den menschlichen Einfluss zu bestimmen. Im Durchschnitt war die Zahl der resistenten Bakterien um 18.5 % bis 29.8 % höher in stark beeinflussten Proben.
Zusätzlich zeigte sich auch eine Tendenz erhöhter Multiresistenz (+ 40 %) in Isolaten aus Regionen mit mehr direktem Kontakt zu menschlichen Aktivitäten. Auch die Resistenz gegen synthetische Antibiotika war in diesen Probenstellen erhöht.

Paper III befasst sich mit der effektiven Wissensvermittlung an Menschen außerhalb des wissenschaftlichen Diskurses. Antibiotikaresistenz ist ein globales Thema, welches außerdem einem Kommunikationsproblem unterliegt. Als "One-Health"-Problem erfordert es das Mitwirken eines jeden Einzelnen und eine Brücke zwischen Wissenschaftlern und Nicht-Wissenschaftlern ist daher unerlässlich, um dies zu erreichen. Die Plattform “Frontiers

for Young Minds" bietet dabei eine entsprechende Möglichkeit, Wissen erfolgreich an die nächste Generation zu übermitteln, ohne dabei die Wissenschaftlichkeit zu verlieren. Unterstützt wurde dieses Vorhaben aus **Paper III** außerdem durch einen aktiv betreuten Blog und eine Website mit Daten aus **Paper II**.

Author contributions

All of the publications presented were collaborative work and the author's contributions are the following:

Paper I: Gattinger D, Pichler K, Weil T, Sattler B. **A comparative approach to confirm antibiotic-resistant microbes in the cryosphere**.
DG, BS, and TW designed the study. DG and BS collected the samples. DG and KP conducted lab work and evaluation of data (DG: main contributor). DG and BS wrote the manuscript (DG: main contributor) that was reviewed by all DG, BS, KP, and TW. All authors contributed to the article and approved the submitted version.

Paper II: Gattinger D, Schlenz V, Weil T, Sattler B. **From remote to urbanized: Dispersal of antibiotic-resistant bacteria under the aspect of anthropogenic influence.**
DG, BS, and TW designed the study. DG and BS collected the samples. DG and VS conducted lab work and evaluation of data (DG: main contributor). DG and BS wrote the manuscript (DG: main contributor) that was reviewed by all DG, BS, TW, and VS. All authors contributed to the article and approved the submitted version. BS was responsible for the project administration. BS, DG, and TW did funding acquisition.

Paper III: Gattinger D, Schlenz V, Weil T, Sattler B. **Shaping Antibiotic Resistance: Human Influence in Earth's Coldest Realms**
DG, BS, and TW designed the study. DG and BS collected the samples. DG and VS conducted lab work and evaluation of data (DG: main contributor). DG and BS wrote the manuscript (DG: main contributor) that was reviewed by all DG, BS, TW, and VS. DG designed the graphical supporting figures. All authors contributed to the article and approved the submitted version.

Abbreviations & Glossary

Acquired resistance: Specific antibiotic resistance caused by mutations, transformation, conjugation, or transduction

Antibiotic Susceptibility: The degree to which bacteria are vulnerable to the effects of specific antibiotics

AR: Antibiotic Resistance

AMR: Antimicrobial Resistance

ARG: Antibiotic Resistance Gene

AWaRe: Access, Watch, Reserve (classification of antibiotics)

CDC: Centers for Disease Control and Prevention

CLC: CORINE Land Cover

EUCAST: European Committee on Antimicrobial Susceptibility Testing

Intrinsic resistance: Nonspecific defense mechanisms to protect bacteria from toxic substances like antibiotics

MDR: Multi-drug resistance

MP: Microplastic

Multi-drug resistance: Antibiotic resistance against three or more antibiotics

Natural antibiotics: Naturally occurring antibiotics that are often derived from environmental microorganisms (e.g. gentamicin, vancomycin)

Resistome: Collection of all antibiotic resistance genes within a microbial community

Semisynthetic antibiotics: Antibiotics derived from natural sources but modified chemically for improved efficacy or other properties (e.g. ampicillin)

Synthetic antibiotics: Antibiotics created entirely through chemical synthesis, rather than being derived from natural sources (e.g. linezolid, nitrofurantoin)

Chapter 1 - Introduction

The evolution of antibiotic resistance

Almost 100 years ago, Alexander Fleming started to make a discovery that would reshape the landscape of medicine. His discovery of the first antibiotic, Penicillin, proved to be a pivotal moment for medicine and marked the start of what we nowadays call the golden age of antimicrobials. However, the successful usage of the newly found antibacterial substance quickly started to vanish as the phenomenon of antibiotic resistance (AR) arose within pathogenic bacteria. The first resistance to Penicillin was reported shortly after its first prescription in 1940 (Abraham and Chain 1940, Barber and Rozwadowska-Dowenzko 1948, Rammelkamp and Maxon 1942), and infections with staphylococcal strains became untreatable with the world's first antibiotic in more than 80% of the cases by the late 1960s (Lowy 2003). With the development and discovery of new natural, semisynthetic, and synthetic antimicrobials over the following years, the problem became neglectable. Today, the human arsenal of antibacterial substances comprises a diverse range that target various essential structures and processes within bacterial cells, including but not limited to inhibition of DNA, RNA, protein, and cell wall synthesis, as well as disruption of metabolic pathways, membrane integrity, and essential enzyme activities.

Table 1 - Information and overview of the antibiotics used for antibiotic susceptibility testing throughout this thesis including their WHO AWaRe classification (WHO 2021).

Antibiotic	**Class**	**Synthesis**	**Mechanism of Action**	**AWaRe classification**
Ampicillin	Penicillins	Semisynthetic	Cell Wall Synthesis Inhibitor	Access
Chloramphenicol	Amphenicols	Semisynthetic	Protein Synthesis Inhibitor	Access
Gentamicin	Aminoglycosides	Natural	Protein Synthesis Inhibitor	Access
Linezolid	Oazolidinones	Synthetic	Protein Synthesis Inhibitor	Reserve
Nitrofurantoin	Nitrofuran-derivatives	Synthetic	Metabolic Inhibitor	Access
Novobiocin	Aminocoumarins	Natural	DNA Synthesis Inhibitor	Access
Trimethoprim	Trimethoprim-derivatives	Synthetic	DNA Synthesis Inhibitor	Access
Vancomycin	Glycopeptides	Natural	Cell Wall Synthesis Inhibitor	Watch

However, diverse factors like human overuse (Read and Woods 2014) and misuse (Luyt et al. 2014), agricultural applications (Bartlett et al. 2013, The antibiotic alarm 2013), and the lack of newly discovered antibiotics (Allen et al. 2010, Piddock 2012) accelerated the rise of AR bacteria quickly to the point where AR has risen to a global threat. The release of sub-inhibitory concentrations of antibiotics into the environment can facilitate the selection for antimicrobial resistance (AMR) (Sanchez-Cid et al. 2023). The extensive array of defensive strategies against antibiotic resistance, including mutations (Davies 1997, Woodford and Ellington 2007), horizontal gene transfer (Lerminiaux and Cameron 2019), and intrinsic resistance mechanisms (D'Costa et al. 2011), underscores the complexity of combatting this global threat. With 458 antibiotic resistance gene (ARG) families and 5010 references now listed in the Comprehensive Antibiotic Resistance Database (Alcock et al. 2023), each

corresponding to one of the four primary mechanisms of resistance - membrane impermeability, alteration of the antibiotic target site, modification of the antibiotic itself, and efflux of the antibiotic (Blair et al., 2015) - targeting antibiotic resistance becomes increasingly challenging. These complexities are further compounded by alarming rates of resistance observed in clinically relevant bacterial strains across 72 countries, signaling a pressing public health concern (GLASS report 2022). Moreover, projections suggest that mortality due to infections with antibiotic-resistant bacteria could reach 10 million annually by 2050 (Review on Antimicrobial Resistance 2016).

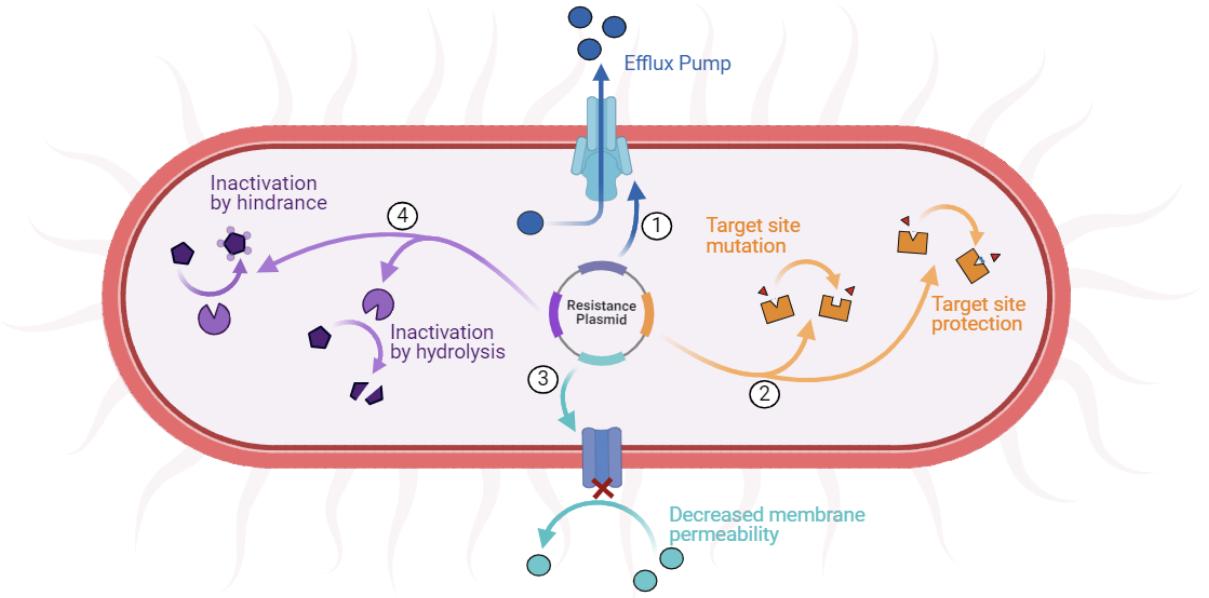

Figure 1 - Schematic of the main defensive strategies (acquired and intrinsic) adopted by antibiotic-resistant bacteria (Blair et al. 2015). This includes (1) the removal of toxic substances from the inside of the cell, (2) alteration of the target site, (3) reduced membrane permeability for the antibiotic, and (4) degradation of the antimicrobial through enzymes. This figure was created with BioRender.com.

However, it's crucial to recognize that the scope of this global challenge extends beyond medical settings. Considering that many antibiotics originate from soil (Nesme and Simonet, 2015), the evolution of defensive strategies against antimicrobials has also been shaped by co-evolutionary processes in natural environments. Additional duality in the function of some antibiotics as signal molecules that are being produced in sub-lethal doses (Fajardo and Martínez 2008) is likely also contributing to the evolution and persistence of antibiotic resistance in non-urbanized areas. Intrinsic resistance, a natural phenomenon known to be present in many bacterial species (Cox and Wright 2013), is often caused by nonspecific

efflux pumps, reduced membrane permeability (Fajardo et al. 2008), or strategies like extreme slow growth (Gray et al. 2019). Especially gram-negative bacteria are renowned for their capability of being intrinsically resistant to antibiotics (Alvarez-Ortega et al. 2011, Poole 2011, Impey et al. 2020) with up to a 100-fold reduced outer membrane permeability offering protection to some species (Hancock and Brinkman 2002). Additional selective pressure through anthropogenic usage of antimicrobials can increase the transformation of ARGs in naturally competent cells (Winter et al. 2021).

Antibiotic resistance (AR) in the cryosphere

In addition to soil ecosystems, antibiotic resistance has been detected globally, spanning beyond clinical and agricultural settings. This includes urban air (Li et al., 2018), clouds (Rossi et al., 2023), isolated cave ecosystems (Pawlowski et al., 2016), and various cryospheric habitats (e.g. Hernández and González-Acuña 2016, McCann et al., 2019; Segawa et al., 2013).

The cryosphere, previously often perceived as a sterile environment, consists of seasonal snow cover, sea ice, glaciers, and permafrost and is full of majorly microbial life that is ideally adapted to low temperatures and nutrient scarcity (Anesio and Laybourn-Perry 2012, Jungblut et al. 2022, Margesin and Miteva 2011). Despite these harsh conditions, the prevalence of AR bacteria in these environments has been vastly underestimated.

The mechanisms facilitating the transmission of AMR into high altitude, and latitude areas remain poorly understood. However, three primary factors are likely contributing to its spread: dissemination through physical and biological vectors (Allen et al. 2010, Laborda et al. 2022), direct human activity (Depta and Niedźwiedzka-Rystwej 2023), and intrinsic bacterial resistance mechanisms (Cox and Wright 2013). Given that prior exposure to natural antibiotics can increase the probability of acquiring resistance mechanisms against clinically relevant antimicrobials (Raaijmakers and Mazzola 2012), the additional selective pressure from human activities is expected to intensify the development and spread of antibiotic resistance in microbial populations. In addition, genes known for resistance against β-lactam, tetracycline, and glycopeptide antibiotics have existed since before the first antibiotic was even discovered (D'Costa et al. 2011) and indicate the potential of environmental bacteria to withstand certain antibiotics. These intrinsic resistance

mechanisms often rely on nonspecific efflux pumps, reduced membrane permeability, and other factors such as antibiotic degradation and target modification (Fajardo et al. 2008, Delcour 2009).

It is generally agreed that all of these factors play a crucial role in the ubiquitous presence of AR bacteria. However, the full extent of the spread of AMR in cryospheric habitats still lacks a lot of information.

Antibiotic resistance as human pollution

Extensive human activities and globalization have reshaped our perspective of “pristine” environments, with remote cryospheric habitats now being the ideal sampling sites to study the anthropogenic impact on the environment (McConnel et al. 2018). Antibiotic resistance emerges as one of these persistent pollutants (Kim and Aga 2007, Laborda et al. 2022, Zhang et al. 2009), where human activities potentially play a central role. However, data on the spread of this kind of human pollution into remote habitats is relatively sparse. This is especially true for high altitude and high latitude areas which are generally considered one of the Earth’s most pristine regions.

The additional selective pressure of human antimicrobial usage is a known driver for the development of AMR (Tello et al. 2012). Therefore, main hotspots for AR bacteria are usually closely associated with strong anthropogenic impact and include healthcare settings, wastewater treatment plants, and agricultural areas. Even for natural environments, the direct human presence appears to be a factor in the spread of antimicrobial resistance (Depta and Niedźwiedzka-Rystwej 2023). However, the effect of the anthropogenic influence on the cryospheric resistome remains poorly understood and needs further investigation.

Understanding environmental habitats like the cryosphere is crucial as it is increasingly acknowledged to contribute to the spread of AR (European Union 2017).

Furthermore, cryospheric areas are not isolated but rather interconnected to other environments. Incorporating AR bacteria and their corresponding ARGs through melting processes can potentially affect the resistome of downstream ecosystems. Respective resistance genes can then spread through conjugation (Peterson and Kaur 2018, Wintersdorff et al. 2016) or transformation (Winter et al. 2021) even if bacterial isolates cannot survive in their new environment which could potentially also affect vulnerable

pathogenic bacteria (Larsson and Flach 2022, Maeusli et al. 2020, Wright 2010). Therefore, it is crucial to close the existing knowledge gaps of antibiotic resistance in the cryosphere and consider these regions as part of a cycle for ARGs and resistant bacterial strains.

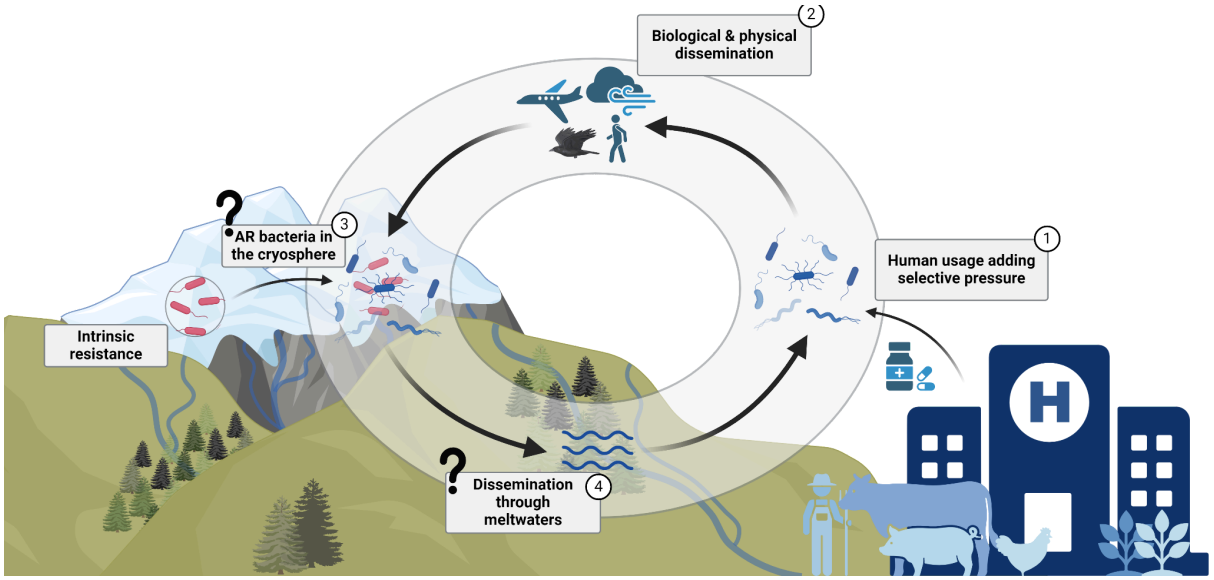

Figure 2 - Scheme of how antibiotic resistance can potentially cycle between urbanized areas and the cryosphere. (1) Additional selective pressure from human activities (e.g. agricultural or medical usage) shapes the resistome in urbanized areas (2) Resistant bacteria and respective ARGs get disseminated into cryospheric areas like high alpine glaciers through biological or physical long-range transport or human activities (3) Antibiotic-resistant microbes from anthropogenically influenced areas and intrinsic resistance mechanisms shape the antibiotic resistome of the cryosphere (4) Melting process translocate potentially resistant bacteria and respective genes downstream and back into human-associated areas. (3) and (4) are still big unknowns and the main research questions of this thesis. This figure was created with BioRender.com.

Antibiotic resistance as a 'One Health' problem and the central role of science communication

The COVID-19 pandemic impressively showed that clear and effective science communication is of critical importance. Studies showed that especially during times of uncertainty, people's information uptake from media increases (Lyu 2012, Maal and Wilson North 2019). However, contradictory information or even fake news, especially due to easy access to social media (Sharma et al. 2019, Paine 2015, Mitchell et al. 2020), can cause uncertainty among the general public. Fake news can travel faster and wider than the truth for all kinds of information through social networks (Vosoughi et al. 2018) and impact all topics of interest (Aïmeur et al. 2023). This transmission of misinformation also affects

health matters during a global pandemic by reducing vaccination willingness and best practices to avoid infections (Giotakos 2022).

Fake news is a complicated matter and a variety of factors like prior exposure (Pennycook et al. 2018) and ideological beliefs (Shu et al. 2017) can shape someone's perception of respective information. Therefore, fighting fake news is challenging (Sharma et al. 2019), and things like fact-checking will most likely not be able to keep up with dynamic changes in incorrect information (Potthast et al. 2018).

The success of fake news is also a potential trust issue. Increasing trust in science can only be achieved by effectively communicating scientific knowledge. However, conveying scientific information to the non-scientific community is generally a complex matter in itself, as it requires reaching a diverse audience of various ages and levels of pre-existing knowledge (Patel and Prokop 2015). Additional challenges arise from data often being interpreted in more than one way, and the way of presenting scientific knowledge as communication between scientists and non-scientists usually happens indirectly (National Academies of Sciences, Engineering, and Medicine, 2017). The COVID-19 pandemic-associated 'infodemic' impressively demonstrated how society can be affected by all sorts of information through a variety of different media channels and communicators (Balakrishnan et al. 2022). Therefore, it underscores the importance of a better bridge between the scientific and non-scientific worlds.

Similar to a viral pandemic, antibiotic resistance can be considered a silent pandemic and is acknowledged as a 'One Health' problem (Review on Antimicrobial Resistance 2016, WHO 2019, Taylor et al. 2014) that needs the combined global efforts of scientists, healthcare workers, stakeholders, and every individual to be effectively combated. Yet, there is still a broad unfamiliarity with the concepts of antibiotic resistance (WHO 2015, Wellcome Trust 2015) which underscores the importance of clear communication and simplification of the respective terminology (Mendelson et al. 2017) and scientific theories. Different target groups with differing obligations can further complicate how information about AMR is effectively transported (King et al. 2022). However, imminent drastic consequences of a post-antibiotic era (Burnham 2021, Review on Antimicrobial Resistance 2016) urge for a quick change and a clear strategy for a general understanding of the benefits and risks of antibiotic usage is needed for appropriate agent use and minimizing second-hand harms (Langford et al. 2019).

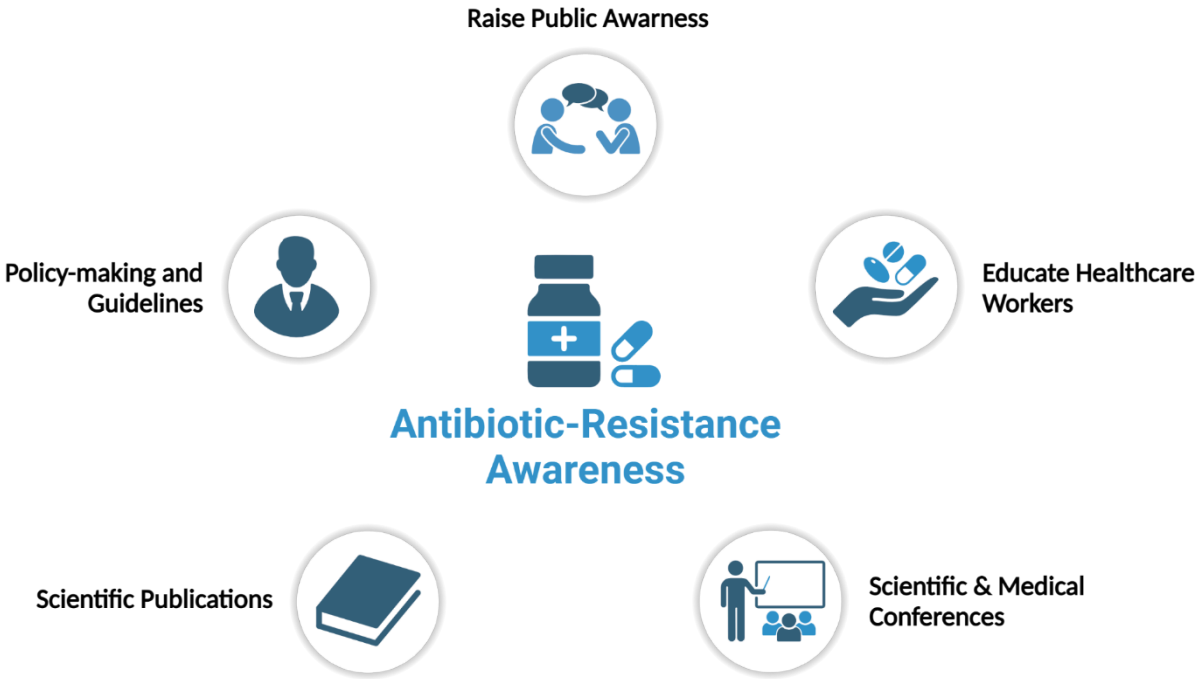

Figure 3 - Scheme of key points on how to raise awareness for antibiotic resistance according to the WHO. As a One-Health problem it requires the involvement of science, policy-makers, health care and the general public to be successfully tackled. This figure was created with BioRender.com.

The cryosphere as a study site

The key findings of this thesis are based on the sampling of the Tyrolean Alpine space that took place during 2019 and 2020. The selected area depicted the ideal study site as it offers the possibility to sample remote high-latitude areas and seasonal snow from urbanized regions. Furthermore, Tyrol's touristic character allowed a direct comparison of industrialized and relatively pristine glacier regions. Finally, to analyze freshwater environments, transects from remote glacial melting waters to rivers of higher order and in densely populated areas as well as high-alpine lakes compared to bathing lakes were sampled within the region.

Samples were processed and analyzed at the Institute of Ecology at the University of Innsbruck except for sequencing which was performed at the Fondazione Edmund Mach in Italy.

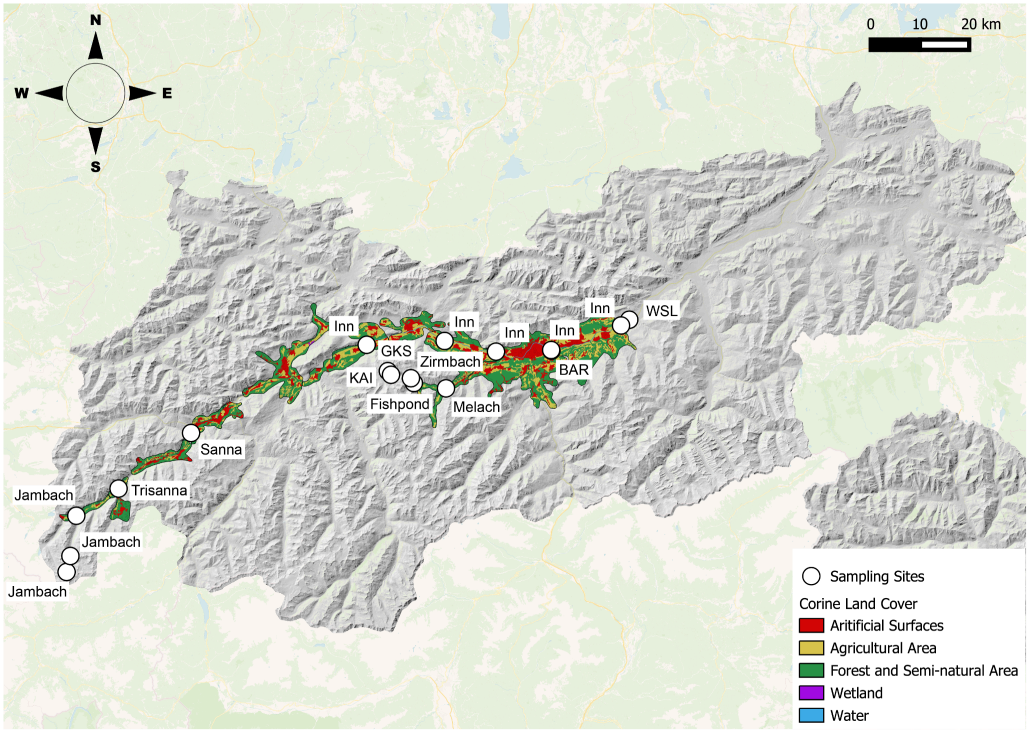

Figure 4 - Freshwater samples and CLC data for relevant catchments of the respective lakes and streams. The land cover in the catchments was used to compare these sampling sites relative to their anthropogenic impact.

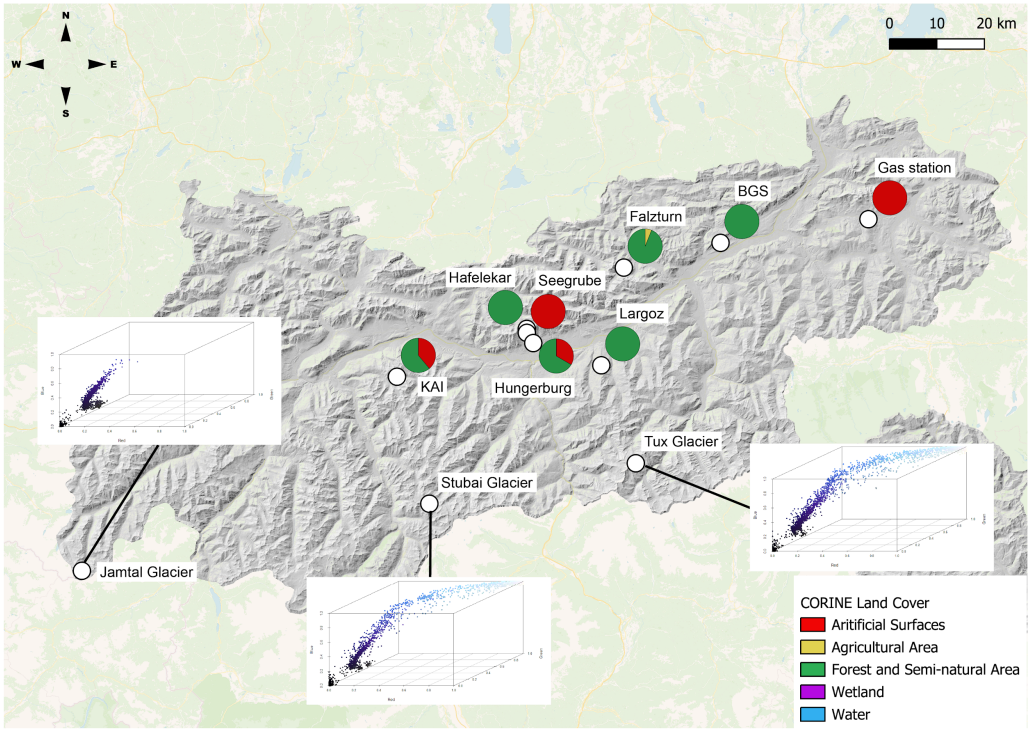

Figure 5 - Snow and ice samples taken in the Tyrolean Alpine space. CLC data was used for the evaluation of the relative anthropogenic influence on sites where seasonal snow was collected. For glacier regions publicly available heatmaps from Strava were used to compare these samples regarding their respective human impact.

Chapter 2 - Research question and objectives

When talking about AMR, the research focus so far has mostly been on clinical and agricultural settings. However, there is a lack of knowledge on how widespread this phenomenon is in Earth's most pristine environments. The influx of substantial microbial populations into melting waters driven by global warming underscores the significance of understanding antibiotic resistance in cryospheric habitats. Through horizontal gene transfer, freshly distributed ARGs could potentially spread into the existing urban resistome (Maeusli et al. 2020, Wright 2010). While some research on AR in the cryosphere exists, they all have one thing in common: the lack of standardized methods. Popular approaches for most of these studies are either metagenomics which solely focuses on detecting existing ARGs or some cultivation-based (non-standardized) procedures. This decreases comparability between data and therefore reduces our ability to estimate the cryospheric resistome.

Additionally, we lack knowledge on how humans contribute to the spread of AMR in cold habitats.

Apart from the urgent need to learn more about the spread of antibiotic-resistant bacteria and the effect of the anthropogenic influence on it, AR is a global problem that needs to be tackled as such. Information and education of the broader public is still lacking and combined efforts are needed to increase the knowledge in all age groups to effectively combat AMR.

As a result, the goal of this PhD thesis is to contribute to closing knowledge gaps on antibiotic resistance in the cryosphere as well as creating new and exciting distribution pathways for information regarding this sensible topic. The key goals and research questions answered in the individual papers that are part of this thesis are:

- **Paper I & II:** Investigate the extent and distribution patterns of antibiotic-resistant microbes within cryospheric and relatively remote freshwater environments, aiming to elucidate the prevalence and spatial distribution of antimicrobial resistance in these ecologically significant habitats.
- **Paper I:** Propose an adapted and optimized version of the antimicrobial susceptibility test by Kirby-Bauer (Bauer et al. 1959) for the detection of AMR in psychrophilic and

psychrotrophic bacteria as a standardized method for reliable and comparable results.

- **Paper II:** Evaluate the impact of human presence and activities on the antibiotic resistome in cryospheric samples along a latitudinal gradient from remote glaciers to urban samples.
- **Paper III:** Increase effective science communication for all age groups by breaking down complex structures into easy terminology and concepts to tackle the problem of AMR as a global phenomenon that needs the active participation of every individual.

The thesis is embedded within the project 'ANTHROPO.SNOW: Snow and Ice as Testimonial for Pristine Environments Suffer Severely from Human Imprint,' funded by the TAWANI Foundation USA, which aims to detect the anthropogenic influence on cryospheric habitats in high altitude and high latitude areas. Additionally, this work was supported by a 12-month doctoral scholarship of the University of Innsbruck.

Chapter 3 - Key results and discussion

Prevalence of culturable antibiotic-resistant bacteria in cryospheric and freshwater environments

Antibiotic resistance in human-associated environments is extensively studied and recognized as a pressing global concern. It has emerged as a significant challenge in healthcare (WHO 2019) and poses substantial economic burdens (Review on Antimicrobial Resistance 2016), with its prevalence accelerating rapidly. However, existing research predominantly focuses on anthropogenic settings and pathogenic bacteria, often overlooking the natural environment. Despite AR's status as a global threat, there remains a critical knowledge gap regarding the extent to which AMR has disseminated into more remote regions, such as high-altitude or -longitude areas. Not only could this give valuable insights into the distribution patterns but also increases our understanding of how antibiotic resistances circulate. After all, the interconnectedness of all worldwide habitats could potentially lead to the transfer of ARGs back to clinically relevant bacterial strains and further accelerate the problem (Maeusli et al. 2020, Wright 2010).

Using a cultivation-based approach optimized for the needs of psychrophilic habitats we could provide valuable insight into the culturable resistome along an altitudinal gradient (**Paper I**) and a variety of remote and urbanized cryospheric and freshwater habitats (**Paper II**). By testing the phenotypic resistance of the bacterial isolates against eight different antibiotics (see Table 1), we gained valuable insights into the diversity of existing antimicrobial resistance in cold habitats. These antimicrobials contained natural, semisynthetic, and synthetic substances and covered different mechanisms of action as well as all groups of the WHO AWaRe classification (WHO 2021).

With our findings, we support the idea that antibiotic resistance is a widespread phenomenon present in remote areas like high alpine lakes (**Paper I + II**), seasonal snow, and glacier ice (**Paper II**). Notably, the prevalence of multidrug-resistant (MDR) bacteria exceeded 50 % (**Paper II**), indicating the limited effectiveness of the tested antibiotics. Additionally, our results reveal a low susceptibility of the isolates with only 17.8 % being

susceptible to all antimicrobials tested. Among these, ampicillin and trimethoprim were the least effective, while linezolid and gentamicin exhibited the highest success rate in inhibiting bacterial growth (**Paper II**).

Overall, the number of chloramphenicol resistance from our study corresponded well with what has been found in other natural habitats like the Arctic (Mogrovejo et al. 2020) and Antarctica (Tam et al. 2015).

Concerns about the rise of resistance against oxazolidinones (Bender et al. 2018, Fioriti et al. 2020) like linezolid are supported by our study despite it showing comparably lower numbers when looking at other antibiotics tested (**Paper II**).

Overall *Pseudomonadacea*, known for their intrinsic resistance mechanisms like reduced outer membrane permeability (Hancock and Brinkman 2002), efflux pumps, and antibiotic-inactivating enzymes (Pang et al. 2019), were the most (**Paper II**) and second most (**Paper I**) bacterial family within our studies. This highlights the possible importance of intrinsic resistance in cryospheric environments, especially when looking at remote regions.

Standardizing antibiotic susceptibility tests for culturable psychrophilic and psychrotrophic bacteria

Existing studies on AMR in cryospheric habitats are often based on different approaches that decrease comparability. Molecular biological approaches have become the preferred choice today due to their clear advantages, such as the ability to detect a variety of present ARGs. However, they provide limited information on gene expression levels and, consequently, the actual utilization of these resistance genes (Kumar et al. 2020). The so-called phenotypic resistance on the other hand could be easily detected with cultivation-based approaches like the well-established agar-disk-diffusion test albeit needing adaptation and optimization to fit the needs of psychrophilic microbes. However, there is a clear lack of standardized procedures to evaluate the culturable resistome in cryospheric habitats. Simple cultivation tests in the presence of antibiotics (D'Costa et al. 2006, Walsh and Duffy 2013) can give estimates on how well bacterial strains tolerate antimicrobials but lack reading guides and adjusted minimum inhibition concentrations for each species. The results from this method can therefore differ in up to 24 % of the cases when compared to the results of the agar-disk-diffusion method (**Paper I**). This demonstrates the importance of a standardized testing procedure to increase future comparability for antibiotic susceptibility testing of cryospheric samples.

Procedures like the agar-disk-diffusion test have clear guidelines, produce reliable results since their inception in the mid-20th century (Bauer et al. 1959), and are still the a widely used method for many cases. However, one of the main challenges of cultivation-based approaches is their design for mesophilic microorganisms.

As they offer a lot of the advantages described above, we focused on optimizing the agar-disk-diffusion test for psychrophilic and psychrotrophic bacteria (**Paper I**). To fit the needs of cold-loving microorganisms, a decrease in incubation temperature and subsequent prolonged incubation period is necessary. This is known to have a significant impact when testing mesophilic bacteria for antibiotic susceptibility (Smith and Kronvall 2015, Smith et al. 2018). However, for psychrophilic and psychrotolerant isolates, the effect seems to be comparably lower albeit still visible for certain antibiotics (**Paper I**). Lower temperatures could specifically have an effect on the uptake of small molecules with intracellular targets

as cells up-regulate membrane transport proteins (Maayer et al. 2014). For vancomycin, an antibiotic with a much bigger molecular weight and that is targeting the bacterial cell wall, no differences between tests at 20° and 4° C could be determined (**Paper I**).

Additionally, the choice of nutrition media to perform the agar-disk-diffusion test is of major importance. While Reasoner's 2 agar (R2A) is a suitable choice for growing microorganisms from oligotrophic habitats (Rüthi et al. 2023), it can cause significantly different results for the agar-disk-diffusion test if used instead of the recommended Mueller-Hinton agar (MH) (**Paper I**). Especially the differences in the agar concentration (1.7 % for MH, 1.5 % for R2A) lead to increased inhibition zone sizes that potentially impact the classification of the resistance profile of the tested isolates. If MH agar is replaced with R2A agar for the Kirby-Bauer test, inhibition zone sizes can differ by 44-74 % of the threshold values given by the breakpoint table to classify bacterial strains into susceptible, intermediate, or resistant (**Paper I**). A portion of this can be explained by the decreased viscosity of the nutrition media and the inversely proportional diffusion rate (Fick 1855). However, even adjusting agar concentration to 1.7 % causes differences in inhibition zone size (**Paper I**).

Therefore, our data suggests that sticking as close as possible to the original protocols produces the most reliable results. With small adjustments in terms of incubation temperature and period, the agar-disk-diffusion method is applicable to gain insights into antibiotic susceptibility profiles of culturable isolates from the cryosphere and could increase comparability between studies.

Human impact on antibiotic resistance in natural environments

The increased selective pressure that derives from human antibiotic use and misuse is known to be the main driving factor for the rise of AR bacteria (Rodríguez-Rojas et al. 2013). Resistant microorganisms or potential pollutants like the antibiotics themselves can reach aquatic environments through sewage or superficial water runoffs (Marti et al. 2014). Distribution into more remote regions is a little more complex but most likely includes the impact of a variety of biological and physical forces (Allen et al. 2010). The perception of high altitude and high latitude areas to be pristine and unaffected has long changed and antibiotic-resistant bacteria are a known phenomenon in these habitats (e.g. McCann et al. 2019, Ushida et al. 2010, Segawa et al. 2013, Lagana et al. 2019, Laborda et al. 2022). However, there is only limited knowledge about the effect of human activities on the presence of AR bacteria in cryospheric samples

To close this knowledge gap and view antibiotic resistance in snow, ice, and water samples under the aspect of anthropogenic influence, we designed a study to contain samples from areas with different levels of human activities (**Paper II**). These were evaluated with the help of publicly available heatmaps from tracking devices (Strava 2023) and CORINE Land Cover (CLC) data (European Union 2018) in the respective catchment areas of the sampling sites (**Paper II**). Therefore, a classification of the anthropogenic influence into the three categories low, medium, and high, and a relative comparison between sites was possible.

As a result, we could find the trend of human activities to be the driving force for antibiotic resistance to be true in cryospheric samples and freshwater environments (**Paper II**). This aligns well with results from studies on different environmental samples like sediment (Bhattacharyya et al. 2019), rivers (Guan et al. 2022), subalpine lakes (Eckert et al. 2018), and tropical regions (Pontes et al. 2009).

For our study, antibiotic resistances among bacterial isolates from sampling sites with high anthropogenic impact were on average 18.5 % and 29.8 % higher than in regions with medium and low influence respectively (**Paper II**). This included significantly increased numbers in AR from three glaciers with different levels of direct human presence. Here, the site facing the highest anthropogenic influence (Nature Ice Palace - Tux Glacier) showed numbers that are comparable to clinical settings (Nuñez et al. 2016) and hospital effluents (Varela et al. 2013, Rodriguez-Mozaz et al. 2015) for some antibiotics. Out of the eight

antibiotics tested, only two could effectively combat the majority of the isolates, while the total number of MDR exceeded 95 %. Especially resistances against synthetic antibiotics, ranging from 10 to 90 % respectively, support the assumption that resistances in this highly influenced habitat are to a certain extent acquired (**Paper II**).

In comparison, samples from the Jamtal Glacier, a relatively remote glacier that lacks skiing infrastructure and consequently experiences significantly lower anthropogenic pressure, showed a different picture. With almost 40 % fewer MDR isolates and reserve antibiotics like linezolid and vancomycin being effective against every bacterium, it is a clear indicator that human impact is a significant factor in the dissemination of AR into cryospheric samples (**Paper II**). Similar to glaciers, the trend remained the same for seasonal snow cover as well as lake samples. A higher anthropogenic influence corresponded with more MDR bacteria and detected AR against every antibiotic. Once again, the highest numbers of antibiotic-resistant isolates, MDR bacteria, and resistances against synthetic antibiotics were found in snow and lake samples in urbanized areas (**Paper II**). Especially, resistances against synthetic and semisynthetic antibiotics as found in all samples (**Paper I, Paper II**), are potential indicators for acquired resistance.

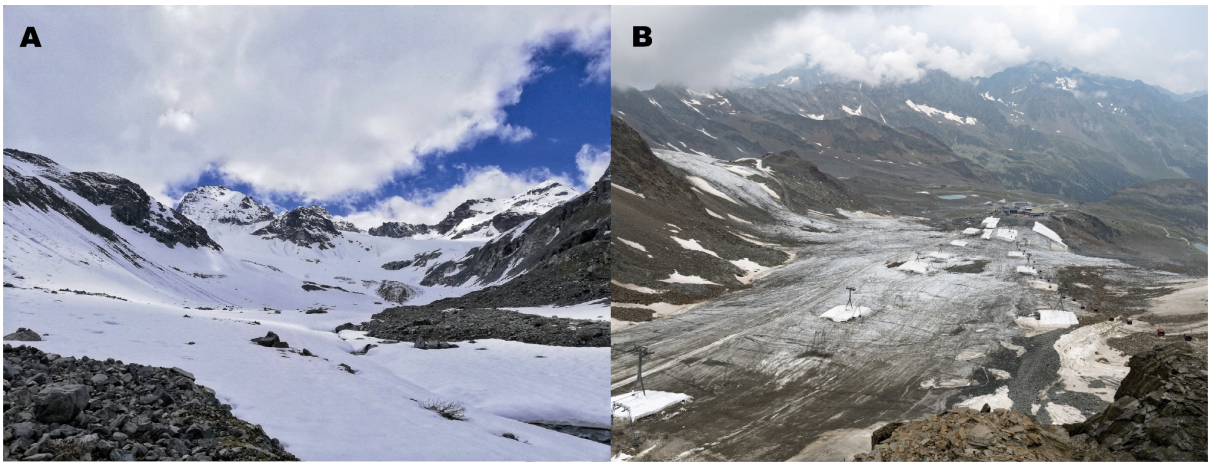

Figure 6 - Two of the glacier sampling sites that were used in Paper II. (A) The relatively pristine Jamtal Glacier. (B) The industrialized Stubai Glacier with its respective skiing area (Photo taken by Klemens Weisleitner).

Furthermore, human-associated isolates (*Escherichia coli* and *Enterobacter cloacae*) derived from two lakes experiencing relatively high levels of direct and indirect anthropogenic influence exhibited a multi-drug resistant phenotype. This is another indicator for a strong

correlation between human activities and the emergence of antibiotic resistance in the environment (**Paper II**).

However, the observed reduced antibiotic susceptibility in relatively remote environments suggests that AMR in these habitats is a complex topic dependent on additional variables. These likely include long-range transport (Allen et al., 2010, Ly et al. 2024) and intrinsic resistance mechanisms (Tamae et al. 2008, D'Costa et al. 2011), which are frequently attributed to various ecological interactions of antibiotics in natural settings (Raaijmakers and Mazzola 2012). Additionally, intrinsic resistance mechanisms often prevalent in environmental bacteria (Cox and Wright 2013), likely contribute to the number of AR found in sampling sites with a comparably low anthropogenic impact (**Paper II**).

Public outreach

Common science communication performance, such as peer-reviewed articles and conferences, have limited accessibility to the general public. While they ensure high-quality standards, independent proof by other experts, and facilitate discussions and collaborations between scientists of the respective field, these channels lack knowledge transfer outside of the scientific bubble.
This limitation persists mainly due to two factors:

- Many publications are still gated and can only be accessed with subscription models.
- Science is often a complex matter and peer-reviewed articles consist of technical jargon and an overall complex language.

While open access to scientific literature has increased in recent years, a lot of articles are still inaccessible to the public without a subscription. The trend to public availability is increasing, yet there are still only approximately 50% of new publications that utilize some form of open access. One of the approaches seemingly getting more interest from the science community is the “Frontiers for Young Minds” issue where kids of different age groups are the target audience and the “reviewers” simultaneously. While this platform is only part of the solution, it helps to bridge the gap and enhance the communication between the scientific and non-scientific communities, while ensuring high quality standards of the scientific work.

The goal of **Paper III** was to enhance knowledge transfer regarding global topics such as antibiotic resistance and raise awareness of its dispersal into the cryosphere. Through the effective breakdown of scientific jargon and the use of accessible language tailored to a target group of 8-11-year-olds, we tried to overcome communication barriers and impart valuable knowledge to future generations (**Paper III**).

This approach was supported by developing a website and blog with similar goals although designed for a wider target group. While the website (alpineresistancemap.com) was primarily intended to describe the overall goal of our research, it also contains an interactive

map with the most recent results from **Paper II**. This interactive feature allows visitors to freely navigate the data, enabling playful exploration of scientific findings. Research has shown that interactivity improves information processing and memory retention (Xu and Sundar 2016), making the interactive map a valuable tool for enhancing our scientific output. The map was created with R (version 4.2.2) and the leaflet (version 2.1.1) package (Cheng et al. 2024). It displays the sampling sites including the number of environmental isolates, the percentage of MDR bacteria and the number of isolates with resistances against the eight antibiotics tested.

The main focus of the blog (blog.alpineresistancemap.com) is to explain scientific concepts of antibiotic resistance, current developments, and some other environmental challenges like microplastic pollution. The daily visitor numbers, peaking at 372 on April 1st, 2022 (Google Analytics), are a clear proof of concept that show demand from the non-scientific community for easily comprehensible science content. Additionally, both the website and the blog are available in English and German to maximize the reach. A version of **Paper III** will be embedded on the website to increase visibility as soon as the original article is published.

Figure 7 – QR-Code to access the website and blog targeted at a non-scientific audience.

Chapter 4 - Challenges in study design and methods

Low Biomass environments and isolation of psychrophilic bacteria

The cryosphere is a low biomass environment and can be challenging for the cultivation of existing isolates. While the cryosphere harbors a diverse and viable microbiome with many cryospheric genera (Anesio and Laybourn-Perry 2012, Bourquin et al. 2022) the bacterial count of culturable strains from snow and ice is usually low (Shivaji et al. 2013, Zhang et al. 2008). In addition, the psychrophilic character of the bacteria requires lower incubation temperatures, longer incubation periods, and an oligotrophic nutrition media like the R2A agar (Rüthi et al. 2023)(**Paper I, Paper II**).

In addition, sampling and processing low biomass environments are especially vulnerable to contamination (Eisenhofer et al. 2019). To address this concern, we implemented rigorous protocols, including the use of sterile equipment and working in controlled environments. Additionally, we adhered to the recommendations outlined by Eisenhofer et al. (2019), which involved randomizing samples, selecting wind-protected or wind-averse sampling sites, and wearing protective clothing. Negative controls were incorporated at various stages including sampling, cultivation, and DNA processing to monitor and mitigate potential contamination (**Paper I, Paper II**). Genomic contaminants are especially prevalent in various scientific equipment (e.g., Glassing et al. 2016, Nogami et al. 1998, Salter et al. 2014, Shen et al. 2006), and the steps taken help ensure the reliability of our scientific findings.

Agar-disk-diffusion test and standardization

One of the main challenges when studying antibiotic resistance in the cryosphere is the lack of standardized procedures. As a result, most research done commonly uses either cultivation-based (e.g. D'Costa et al. 2006, Walsh and Duffy 2013, Pawlowski et al. 2016) or molecular biological approaches (e.g. McCann et al. 2019, Segawa et al., 2013, Wang et al. 2024) to address respective research questions. In **Paper I** we tried to tackle this problem by comparing different antibiotic susceptibility tests. However, common testing strategies like the agar-disk-diffusion test are optimized for the needs of mesophilic, fast-growing, and pathogenic bacteria (eucast.org 2021) while isolates from cryospheric environments are

usually slow growers, oligotrophic, and prefer low temperatures (Anesio and Laybourn-Perry 2012, Jungblut et al. 2022, Margesin and Miteva 2011). Therefore, necessary adaptations included the reduction of incubation temperature and period (**Paper I**). However, using this method in cryospheric environments presents its challenges, as the prolonged incubation time can affect results, particularly for mesophilic organisms. (Smith and Kronvall 2015, Smith et al. 2018). However, to the best of my knowledge, no data is available for the respective impact on the results when this antibiotic susceptibility test is performed on psychrophiles. Our tests revealed that changes in the zone of inhibition size were only noticeable for certain antibiotics (**Paper I**).

In addition, the results of the agar-disk diffusion test rely on correctly reading the results which include measuring the inhibition zone size and comparing it to respective values given in the EUCAST breakpoint table (eucast.org 2023). Since the threshold values in this document are given individually for pathogenic bacteria of interest, we needed to find a workaround for environmental isolates. To achieve this, we took the mean of the respective breakpoints for each antibiotic and bacterial isolate given and used these to categorize our isolated strains into susceptible, resistant, or (if available) intermediate (**Paper I, Paper II**)

Finally, cultivation-based approaches, albeit a reliable strategy to evaluate bacterial antibiotic susceptibility, only paint part of the picture. While it is helpful to determine the existing phenotypic resistance in samples of interest (**Paper I, Paper II**), it is limited to testing culturable strains and selected antibiotics.

Evaluating the anthropogenic impact

Assessing the anthropogenic impact on an absolute level is impossible due to the lack of a single reliable parameter that accounts for the diverse factors that contribute to it. As a result, many studies resort to visually interpreting and categorizing sampling sites into various levels of human influence (e.g. Bhattacharyya et al. 2019, Guan et al. 2022, Sanchez-Cid et al. 2022) (**Paper I**). This leaves room for subjectivity and potentially carries the trap of falling into a bias, which is why novel ideas are needed to enhance the objective evaluation of the anthropogenic impact. The CLC data offers a reliable, up-to-date dataset for the European region with a resolution of 100 m (European Union 2018) and wide usage across different disciplines. By implementing this into our study and assessing the amount of

human-associated land cover (urbanized and agricultural areas) within the respective catchment areas of our sampling sites, we could show that the information gathered can help estimate the relative anthropogenic influence objectively (**Paper II**). However, the CLC data can only serve as a proxy for direct human impact and neglect any form of long-range transport. By including additional parameters like main wind direction and calculating back trajectories, this model could be further enhanced to provide an even better picture of the relative anthropogenic influence.

Although land cover data adequately serves low-altitude regions, distinguishing between sampling sites regarding their human impact on glaciers and high-latitude areas remains challenging. Therefore, assessments of antibiotic resistance under the perspective of anthropogenic influence are often limited to human-inhabited regions (Miller et al. 2009). In **Paper II**, we opted for the innovative approach of using data from tracking devices (Strava) to estimate the direct human presence in different glacier areas. Tracking devices such as Strava (120 million users as of 2023, Business of Apps 2023) and Garmin gather movement data of millions of individuals. With the help of the publicly available heatmap from Strava which represents the relative intensity of human activities worldwide, we got valuable insights into how frequently the glaciers in our study faced anthropogenic presence (**Paper II**). Once again, this lacks data on long-range transport which is responsible for the dispersal of antibiotic resistance into remote areas to a certain extent (Allen et al. 2010, Rossi et al. 2023).

Scientific vs. science communication

In addition to scientific and methodological challenges, bridging the gap between science and effective science communication presents a distinct set of obstacles. While scientific work demands rigor and precision, translating complex matters into accessible language for the wider public requires a completely different skill set. It generally needs consideration of the target group and a clear communication goal (Sharma et al. 2019) which can be challenging due to the heterogeneity of the recipient of the information (Patel and Prokop 2015). Furthermore, it is important to cut down scientific insights into smaller pieces and cherry-pick one key insight to transport rather than several. Considering the avoidance of

technical jargon and complex sentence structure can further enhance the chances of keeping non-scientific readers engaged and interested. In addition, the use of clear visuals is considered one of the most important factors for effectively transporting knowledge to readers (Barry 2002). All of this has been implemented in our online presence and publications to educate the general public on the topic of antibiotic resistance and to contribute to tackling this worldwide challenge also on the non-scientific front (**Paper III**, alpineresistancemap.com, blog.alpineresistancemap.com).

Chapter 5 - Conclusion and future direction

With this work, we contributed to closing the existing knowledge gap on the culturable antibiotic resistome of the cryosphere especially under the aspect of anthropogenic influence. While also laying the groundwork for better standardization in testing antibiotic susceptibility in crysopheric environments, we also used a novel approach to quantify the direct human impact on a relative basis for high alpine regions. This together allowed us to successfully answer the following research questions:

Paper I & Paper II: What is the extent and distribution of antibiotic-resistant microbes in cryospheric and relatively remote freshwater environments?

AMR is widespread in the cryosphere as well as in freshwater environments. Independent of the proximity of sampling sites to urbanized areas, antibiotic-resistant bacteria can be found in snow, ice, cryoconite, melting waters, lakes, and river systems along the Tyrolean Alpine space. In many cases, bacterial isolates show resistance against several different kinds of antibiotics including natural, semisynthetic, and synthetic ones.

Paper I: How can the agar-disk-diffusion test (Bauer et al. 1959) be adapted and optimized to effectively detect AMR in psychrophilic and psychrotrophic bacteria, thereby establishing a standardized method to ensure reliable and comparable results?

The Kirby-Bauer test is a reliable method to look into the culturable cryospheric resistome. However, the bacterial needs should be respected while sticking as close as possible to the original protocol (eucast.org 2021) to achieve the most reliable results. The most important factors influencing the results of this susceptibility test are the choice of nutrition media and agar concentration followed by incubation temperature and period. To deal with unknowns in terms of breakpoint thresholds to classify isolates as either resistant, susceptible, or intermediate, we recommend calculating the given means (eucast.org 2023) for all values for the antibiotic of interest.

Paper II: What is the impact of human presence and activities on the antibiotic resistome within cryospheric samples across a latitudinal gradient, spanning from remote glacier environments to urban settings?

Human activities shape the antibiotic resistome within the cryosphere. Sampling sites within the Tyrolean Alpine space showed a significant correlation of increased AR with increased anthropogenic influence. This trend was consistent throughout glacier ice, seasonal snow, running waters, and lake water samples. A higher number of MDR isolates as well as resistances against synthetic and semisynthetic antibiotics further support this assumption. However, AMR is prevalent throughout all sampling sites, and even in remote glacier regions, some isolates showed decreased susceptibility to many antibiotics. Several factors like long-range transport from urbanized areas and intrinsic resistance are potential explanations for this phenomenon. However, it is unclear to what extent AR is acquired in these relatively remote samples.

In addition, we could increase effective science communication for age groups usually not targeted by scientific knowledge by breaking down complex structures of antibiotic resistance into easy terminology and concepts in **Paper III**. With the help of the journal "Frontiers for Young Minds" results from our studies on how far AR has spread into cryospheric environments can be easily shared with future generations which could help to tackle the global phenomenon as it needs active participation of every individual. Our contribution aims to help clear up communication problems while raising awareness among a wider audience about AMR, as effective communication has the potential to enhance the situation and reduce antibiotic-resistance associated risks (Langford et al. 2019).

In **Paper I** and **Paper II** we show how far antibiotic resistance has spread into environments that were so far overlooked in AR research. The culturable resistome of snow, ice, and freshwater samples is diverse, and bacterial isolates seem to have adopted defensive mechanisms to antibiotics of natural, semisynthetic, and synthetic origin. Multi-drug resistance is common (> 50%) in all cryospheric samples and again underscores the underestimated presence of AMR in cold regions. Similar or even higher numbers of resistant microbes in melting waters and along an altitudinal gradient indicate the potential of the spread of these resistances back into urbanized areas.

In **Paper I** we successfully showed that the agar-disk-diffusion test, which is the gold standard for antibiotic susceptibility testing in clinical settings, can be an effective method to investigate the culturable resistome of cryospheric samples. Adaptations regarding incubation temperature, and incubation period need to be considered to fit the needs of psychrophilic and psychrotolerant bacteria. Calculating the mean of all breakpoints to get thresholds for environmental isolates instead of known pathogens appears to be a sufficient approach to identify AR in respective isolates. Finally, our study showed that the best and most reliable results could be achieved when using the highest possible incubation temperature (below 37 °C), the suggested MH agar, and the shortest incubation period which is advisable to be tested before the actual antibiotic susceptibility test. Using a standardized approach would help improve comparability between studies. Furthermore, evaluating the AR of culturable bacteria gives valuable information on phenotypic resistance and active ARG utilization.

In **Paper II** we show that the human impact has a significant effect on the presence of AR microbes in the cryosphere. Our study revealed this phenomenon for glacier ice, seasonal snow, high alpine- and bathing lakes, and flowing waters. Resistance against WHO's reserve antibiotics linezolid and vancomycin was the highest in areas with high human activity. Furthermore, with up to 80 % of all isolates being MDR, habitats under strong anthropogenic influence showed a significantly bigger diversity in defensive mechanisms. Overall, our results suggest that the effectiveness of many antibiotics is diminishing even in environmental bacterial strains and a strong correlation between human presence and antibiotic resistance exists even in cold areas.

With **Paper III** we could contribute to the lack of knowledge transfer for a global topic generally considered a 'One-Health' problem. This is especially important since tackling antibiotic resistance requires the involvement of science, politics, healthcare, as well as the general public. Here we broke down complex scientific matters into easy-to-understand terms to reach the youngest audience and educate them on a pressing topic. This form of outreach through 'Frontiers of Young Minds' gives a platform to connect with interested

children and was further supported by our website (alpineresistancemap.com) and blog (blog.alpineresistancemap.com).

Overall, our extensive research on the culturable resistome in the cryosphere of the Tyrolean Alpine space suggests that AR has spread further into more remote regions than previously expected. Direct human presence appears to be a significant driver for the emergence of AMR in cold habitats. However, it is essential to consider other potential factors, such as natural microbial community dynamics and respective intrinsic resistance mechanisms as well as the long-range transport of resistant bacteria. Despite these complexities that shape the antibiotic resistome in cryospheric habitats, glaciers facing strong anthropogenic pressure can harbor more antibiotic-resistant microbes than some clinical settings (Nuñez et al. 2016)(**Paper II**). Additionally, even relatively remote areas with minimum direct human presence seem to contain a diverse and complex array of AR with defensive strategies against a variety of natural, semisynthetic, and synthetic antibiotics of all groups from the WHO AWaRe list.

To get a better understanding of how these resistances spread and if they are mostly intrinsic or acquired defensive mechanisms, the scope of future studies could be a combined approach of a cultivation-based susceptibility test like the one presented in **Paper I** and metagenomic analysis. Through a combination of phenotypic resistance testing and the identification of respective ARGs we could gain more insights into the resistome of cold habitats. Comparisons with popular databases like CARD which contains information on over 300.000 alleles corresponding to antibiotic resistance (Alcock et al., 2023) and including mobile genetic elements in the analysis, could provide valuable knowledge on the spread of acquired resistance genes in these environments. Performing qPCR analysis with either selected resistance genes or an extensive primer set (e.g. Primer Set 2.0, Stedtfeld et al. 2018) could provide an additional layer of information in a combined approach with cultivation based antibiotic susceptibility testing. In addition, anthropogenic influence should be considered when picking sampling sites. Improvement of our respective models that rely on CLC and human activity data could be achieved by including data from weather models to include the effects of long-range transports which are known to translocate microbial communities (Weil et al. 2017) and ARGs (Rossi et al. 2023). Animal feces in

high-altitude areas could further address and improve our understanding of the role of animal migration in the distribution of AR bacteria into relatively remote areas. Long-distance transport through animals could translocate bacterial strains across continents (Allen et al. 2010) but the effect on the antibiotic resistome in high-altitude regions has not yet been investigated.

Additionally, other factors such as microplastics (MP) might play a key role in the distribution of antibiotic resistance in cryospheric habitats. MP, which are widespread anthropogenic pollutants (Parolini et al. 2021, González-Pleiter et al. 2021), possess biofilm-enhancing properties that allow complex and diverse microbial communities to form on their surfaces (Oberbeckmann et al., 2015). These plastic pieces can act as vectors and sinks for pollutants (Wang et al. 2021, He et al., 2022) and harbor significantly higher numbers of antibiotic-resistant bacteria than surrounding environments (Sucato et al. 2021, Liu et al., 2021). Furthermore, plastic debris can act like a taxi for the long-range transport of antibiotic-resistant microbes into remote regions like the Arctic (Laganà et al., 2019). However, the connection between plastic pollution, their associated biofilms, and potential as hotspots for antibiotic resistance in cryospheric environments and melting waters remains poorly understood and requires further investigation.

Future research should also address the transport of resistant bacteria and their respective ARGs from remote glacial areas back into urbanized areas. Increased melting processes through climate change lead to the integration of comparably high bacterial biomass. This would also translocate antibiotic-resistant bacteria and their respective ARGs and influence the existing resistome in environments closer to urbanization. A transfer of resistance genes to susceptible pathogenic bacteria in urbanized regions from there on is a real possibility (Larsson and Flach 2022, Maeusli et al. 2020, Wright 2010) and has not been assessed so far. Monitoring melting waters and the respective effluent of AR bacteria as well as their effect on local communities downstream could help to understand this potential. Additionally, intrinsic resistance and evolutionary processes suggest that the cryosphere also has the potential to be a source of antibiotics as even antarctic soils contain natural antibiotics (Van Goethem et al. 2018). This capacity is untapped and could pose a new area of antimicrobial research, particularly considering that the global microbiome could be an underestimated source for new antibiotics (Santos-Júnior et al. 2024).

Finally, a socioecological study on how the general public perceives the topic of AMR could help tailor future science communication for increased effectiveness. Understanding the knowledge gaps within the non-scientific community is crucial for enhancing communication strategies and addressing this global One-Health issue more effectively.

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Chapter 6 - Publications

Paper I - A comparative approach to confirm antibiotic-resistant microbes in the cryosphere

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 03 August 2023
DOI 10.3389/fmicb.2023.1212378

Check for updates

A comparative approach to

MH agar is set as a standard for the agar disk diffusion method (The European Committee on Antimicrobial Susceptibility Testing, 2021a).

2.4.3. EUCAST agar disk diffusion test

For the agar disk diffusion test, adjustments of the standardized procedure by EUCAST (The European Committee on Antimicrobial Susceptibility Testing, 2021a,b) were needed for psychrophilic and slow-growing organisms. Those adaptations included the previously described reduction of the incubation temperature to 20 and 4°C, respectively, and an increased incubation time for slow-growing cultures of up to 2 weeks. To address one of the major problems when applying the agar disk diffusion test to bacteria from the cryosphere – the lack of breakpoints for non-pathogens – the mean threshold for vancomycin (14.83 mm) was calculated based on all given diameter breakpoints (The European Committee on Antimicrobial Susceptibility Testing, 2021b, see Supplementary Table S3). For novobiocin, which is not included in the EUCAST breakpoint table, thresholds (< 16 mm = resistant) were applied according to the existing literature (Harrington and Gaydos, 1984). Following the EUCAST reading guide, bacteria showing a zone of inhibition that is smaller than the defined breakpoints were considered resistant. The isolates with an inhibition zone diameter bigger or equal to the calculated sensitive breakpoint were considered sensitive to the antibiotic. Apart from these adaptations, we followed the official protocol given by the EUCAST.

To evaluate the effect of nutrition media and incubation temperature on the size of the zone of inhibition, we tested all cultures capable of growing at 4 and 20°C at both temperatures and on R2A and MH.

To examine the reason for potential differences in the size of the zone of inhibition, another experimental design focused on the adjustment of the agar concentration for each growth media. The agar concentration, which is usually 15 g/L in the R2A agar, was adapted to the level of the original MH agar recipe (17 g/L) and the other way around. This resulted in a total of four different agar compositions (hitherto named R2A15, R2A17, MH15, MH17).

2.5. Identification of gram (–) bacteria: KOH-test

As vancomycin's effect is limited on gram-positive bacteria, a gram test was performed beforehand to the actual antibiotic susceptibility test. To determine the cell wall structure, we followed the protocol of the Ryu non staining KOH technique (Ryu, 1938). Only gram-positive isolates were tested for vancomycin resistanceLife Science Identifiers.

2.6. 16S sanger sequencing

DNA of all pure cultures was extracted using Qiagen Blood and Tissue DNA Extraction Kit following the recommended procedure of the manufacturer. For the PCR, the universal primers 27F cycling conditions were as follows: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 1 min, and final elongation at 72°C for 7 min. Gel electrophoresis was performed to check the correct length of the PCR fragment with a 1 kb + DNA ladder as a reference. Finally, the amplified products were purified using ExoSAP-IT reagent (Thermo Fisher). Sanger sequencing was then performed at the Fondazione Edmund Mach.

Bioinformatics was done in R (4.0.3) and RStudio (1.1.463.0) using the sangeranalyseR package (Chao et al., 2021). The threshold for the quality score was set at 30. Trimmed sequences were blasted against the NCBI database and submitted to the GenBank®.

2.7. Statistics

All statistics were performed in R (4.0.3) and RStudio (1.1.463.0) including the following packages: tidyverse, ggpubr, rstatix, networkD3, dplyr, and dabestr. To evaluate differences in the diameter of the zone of inhibition, the dataset was filtered so that cultures that showed zero inhibition in their growth by the antibiotic at any condition (zone of inhibition = 0 mm) were not part of the analysis. If agar plates showed a zone of inhibition on at least one testing condition, the data was included in the statistical calculations. Estimation plots were performed to estimate the paired mean difference between the conditions MH15, MH17, R2A15, and R2A17. The influence of single parameters on the inhibition zone was tested with a Wilcoxon signed-rank test. Differences between the two cultivation-based antibiotic susceptibility tests were evaluated using descriptive statistics.

3. Results

3.1. Cultivation of isolates from cryospheric samples

In total, we were able to isolate 35 bacterial colonies from all water samples, 21 from ice, and 6 from snow samples. Morphological differences were heeded within but not between sampling sites. Four cultures initially isolated on R2A agar did not grow after transferring them to liquid media, and one culture did not show any sign of growth after the first antibiotic susceptibility test, which is why in total five isolates were excluded from further analysis. Furthermore, no isolate was able to grow at 37°C while all remaining 57 cultures did successfully form colonies on agar plates at 20°C. Seventy percent thereof were able to grow at 4°C. This confirmed the prior assumption that 37°C is not suitable for the growth of cryospheric bacteria. Hence, all further tests were carried out at 20 and 4°C, respectively. All isolates formed colonies on R2A and MH agar. Of the 57 isolates used for further testing, 37 strains were identified as gram-positive bacteria with the KOH test.

(Table 1) as in general, more resistances were observed on both nutrition media when the incubation temperature was decreased.

According to the cultivation method performed at 20°C, most resistances in the water samples were found in the river Melach (75% novobiocin; 80% vancomycin), followed by the fishpond (60%; 88%), Zirmbach (45%; 59%), KAI (17%; 67%) and GKS (17%; 33%). Samples from the 'Nature Ice Palace' located at the Hintertux Glacier contained the highest level of resistant isolates (100%; 81%) within all samples. While snow samples collected next to Lake Kaiser had moderate numbers of antibiotic-resistant bacteria (50%; 50%). On average, these numbers were higher in all habitats when the incubation temperature was decreased to 4°C.

3.3. EUCAST agar disk diffusion method

On average, 61% of the isolates were resistant to novobiocin and 64% of the gram-positive bacteria were resistant against vancomycin when the agar disk diffusion method was performed at 20°C. These numbers increased to 75% for novobiocin and 78% for vancomycin when the incubation temperature was reduced to 4°C. However, results differed between different incubation conditions such as temperature, nutrition media, and agar concentration. When performed at 20°C, the agar disk diffusion method detected the majority of resistant bacteria within samples from the touristic ice cave at the Hintertux Glacier (98% novobiocin; 81% vancomycin). Among the other sampling sites, Melach contained the highest number of resistant isolates (50%; 50%) followed by the fishpond (50%; 50%), Zirmbach (32%; 64%), and Gossenkoellesee (33%; 33%). The least amount of resistance was observed within the isolates from Kaiser-See (25, 42%) while isolates from snow samples collected next to the Kaiser-See were resistant in 50 and 60% of the cases. The relative number of resistances increased with a decrease in the incubation temperature from 20°C to 4°C in all sampling sites except for the river Zirmbach.

3.4. Comparison of two different cultivation-based antibiotic susceptibility testing strategies

The two cultivation-based antibiotic susceptibility tests used in this study delivered different results regarding the classification of the bacterial isolates (resistant or sensitive) in approximately 23.8% of the cases. In total, 147 antibiotic susceptibility tests were performed across different nutrition media and testing strategies, including 57 isolates for novobiocin susceptibility tests at 20°C and 40 at 4°C, as well as 31 and 19 gram-positive bacteria being tested for vancomycin resistance at each temperature, respectively. Among these tests, 36.1% of the test results differed when the antibiotic susceptibility tests were performed on R2A agar, and 32% of the test results differed when testing isolates for antibiotic resistance with both testing procedures on MH agar (see Figure 2).

TABLE 1 Antibiotic resistance within all sampling sites with the two different cultivation based antibiotic susceptibility testing strategies performed at different testing conditions with two antibiotics.

Method	Agar	Antibiotic	4°C	20°C
Cultivation method	MH	Novobiocin	80%	63%
		Vancomycin	85%	67%
	R2A	Novobiocin	83%	68%
		Vancomycin	79%	73%
Agar disk diffusion method	MH	Novobiocin	75%	67%
		Vancomycin	77%	64%
	R2A	Novobiocin	75%	54%
		Vancomycin	79%	64%

3.5. Influence of the agar concentration, incubation temperature, and nutrition media on the size of the inhibition zone

Compared with the officially recommended MH agar (The European Committee on Antimicrobial Susceptibility Testing, 2021a) with an agar concentration of 17 g/L, all other nutrition media compositions used in this study resulted in increased inhibition zone sizes (Figure 3). The biggest mean difference could be observed between MH17 and R2A15 with 9.87 mm, while MH17 and R2A17 only resulted in a mean difference of 5 mm. On average, the zone of inhibition increased by 6.66 mm on MH15 compared to MH17. The difference in the inhibition zone size caused by the varying agar concentration was significant ($p < 0.001$). Furthermore, different nutrition media ($p < 0.001$) and agar concentrations show significance when using R2A ($p < 0.001$) and MH ($p < 0.001$).

A reduction of the agar concentration from 1.7 to 1.5% resulted in a significant increase of the inhibition zone by 5.17 mm for novobiocin ($p < 0.001$) and 4.36 mm for vancomycin ($p < 0.01$) on R2A agar. Similarly, on MH agar, the inhibition zone increased by 8.74 mm for novobiocin ($p < 0.001$) and 3.18 mm for vancomycin ($p < 0.05$). The largest absolute mean difference was observed between MH17 and R2A15 for novobiocin (+11.8 mm) and vancomycin (+6.6 mm). A significant effect of the incubation temperature could only be seen for novobiocin ($p < 0.001$) but not for vancomycin ($p = 0.3$).

3.6. 16S sequencing

Sequences trimmed and controlled for quality had an average length of ~675 bp. No chimeric sequences were found. A total of 15 cultures did not yield satisfactory BLAST results either due to low percentage identity (< 97%) or insufficiently long reads. The Gram status from the KOH test was confirmed in all but six cases. The *Bacillaceae* family was found to be the most prevalent (14) among the isolated colonies with *Pseudomonadacea* following close behind (8) (see Supplementary Table S4).

4. Discussion

The emergence of antibiotic resistance has become a major public health concern. With this study, we sought to address the lack of standardized methods for cryospheric communities by comparing different cultivation-based approaches to detect antibiotic resistance in cold habitats.

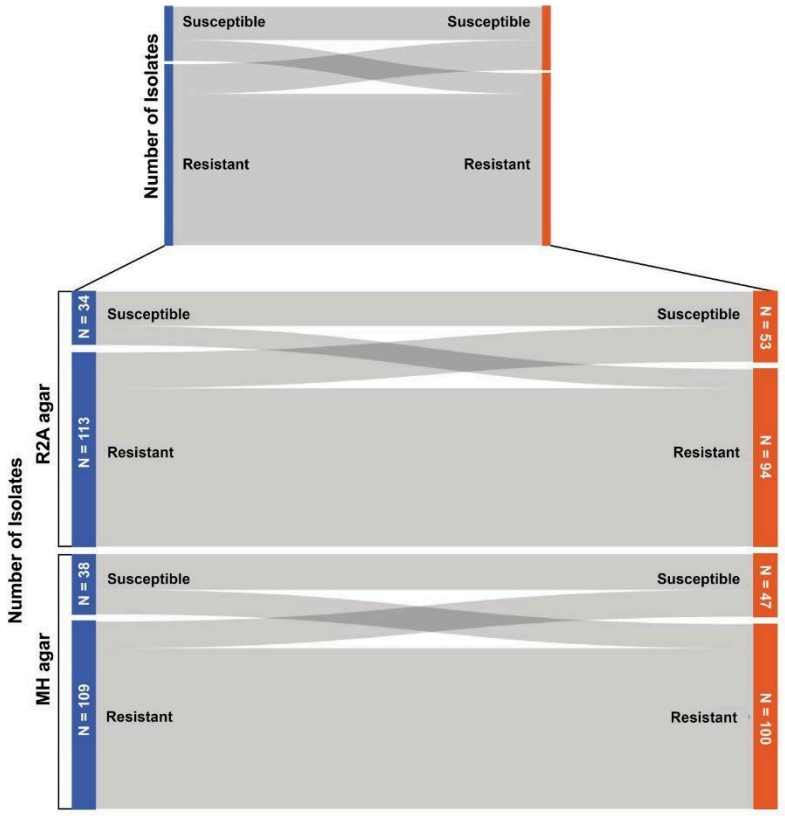

FIGURE 2
Sankey diagram to display the number of bacteria that changed their resistance status (susceptible/resistant) when tested with the two different antibiotic susceptibility tests.

4.1. Cultivation of isolates from cryospheric samples

Out of the initial isolate yield (62), 57 bacterial colonies were successfully grown in liquid media (Luria-Broth) and tested for antibiotic susceptibility.

All cultures used in this study did grow at 20°C whereof 43 cultures had the ability to grow at 4°C, which highlights the psychrophilic character of the isolates usually growing best between 0 and 20°C (Morita, 1975). However, due to limitations in the cultivation of microorganisms from environmental and cold habitats, isolates in this may not be strictly limited to low growing temperatures.

Even though 56% of all bacterial colonies isolated during this study originated from water samples, none were able to grow at 37°C (the recommended temperature for common antibiotic susceptibility tests). This emphasizes the necessity of adaptations for the cultivation-based antibiotic susceptibility tests for bacteria from environmental and especially cryospheric habitats.

4.2. Antibiotic resistance and anthropogenic influence

Antibiotic-resistant microorganisms were found in every sampling site of this study. However, the percentage of resistant bacteria increased along the altitudinal transect from the high alpine lakes (GKS and KAI) to the river Melach, located in a highly populated area. Even though it is difficult to actually measure human impact, an increase in the anthropogenic influence downstream of the water-sample transect can be assumed. On a relative scale, the river Melach, which is showing higher proximity to urbanized areas than any other water sample in this study, is likely to face the highest level of anthropogenic influence. On the other hand, GKS and KAI are only minimally impacted directly by humans, whereas the relative abundance of antibiotic-resistant isolates in these sampling sites is high compared to other little human-impacted areas (Scott et al., 2020). The rural Zirmbach is flowing through some sparsely populated areas and, the observed increase in antibiotic-resistant microbes downstream of the sampled water transect (Figure 4) goes hand in hand with an increased anthropogenic influence due to proximity to

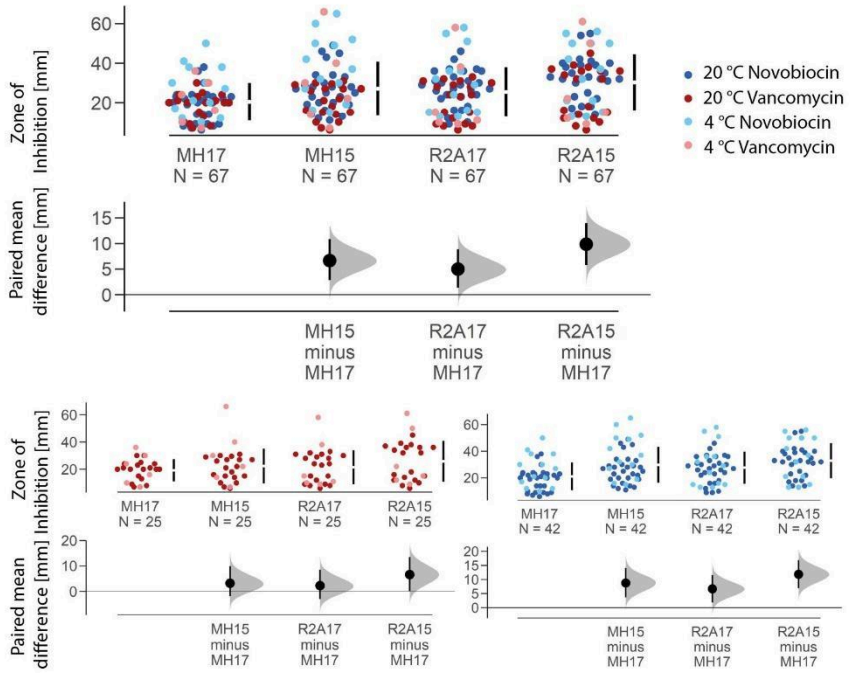

FIGURE 3
Estimation plot showing the paired mean difference of the zone of inhibition size for the two different nutrition media and agar concentrations used. The mean size of the inhibition zone of MH15, R2A17, and R2A15 is compared to the officially recommended MH agar with an agar concentration of 17 g/L. The biggest mean difference (in total and for each antibiotic) was observed between MH17 and R2A15, resulting in a 9.8758 mm larger zone of inhibition on the Reasoner's 2A agar.

humans. These results agree with the general assumption that antibiotic resistance is to a large extent caused by anthropogenic pressure and thus especially present in environments shaped by humans (Kim and Aga, 2007; Berendonk et al., 2015). Furthermore, the same increase in antimicrobial resistance with increased proximity to areas with bigger anthropogenic influence has already been reported before in river networks (McConnell et al., 2018). The effect has been revealed by the selection and the character of the transect but was not the prior scope of this study. The fishpond along the course of the Zirmbach embodies a special case, as it is used for commercial aquaculture. Generally, antibiotics are still widely applied in the fish farming industry across the world and include antibiotics from different classes like penicillins or quinolones (Tuševljak et al., 2012). The use of antimicrobial substances would explain the high numbers of resistance found within the fishpond (Figure 4), even though it is situated outside urbanized areas. However, vancomycin and novobiocin are not approved for antibacterial therapies in aquacultures within the European Union (VO 37/2010 EU), which indicates that these resistances are likely not caused by the use of antibiotic therapies in treating potential fish pathogens.

The river Melach revealed the highest resistances along this transect (Figure 4) which might also be in context with populated areas. In order to cover a high variety of cryospheric habitats for our comparative study, we included an englacial cave open for touristic usage: "Nature Ice Palace" located at the Hintertux Glacier and which is not connected to the transect. The cave is exceptional due to heavy pressure by *ca.* 50.000 yearly visitors and the lack of ventilation. Large numbers of antibiotic-resistant bacteria were found originating from englacial ice. In comparison to other glacial environments around the world, the results indicate that antibiotic resistance does occur in very high numbers within the ice cave (Segawa et al., 2013) (see Figure 4).

4.3. Comparison of two different cultivation-based antibiotic susceptibility testing strategies

The comparison of the two different antibiotic susceptibility tests clearly showed that they potentially provide different results regarding the resistance status of bacterial isolates. While it cannot be stated for sure which method is more accurate, the agar disk diffusion method has some clear methodical advantages. It has been tested for years and was already described as an applicable method to evaluate antibiotic resistance in the mid-20th century (Bauer et al., 1959). Apart from the disk-diffusion test being a standardized procedure with an official protocol to follow, it also considers the antibiotic concentration to match its minimum inhibition concentration for each bacterial species – an aspect the cultivation method lacks completely. This absence may be the main reason for the differences between the two methods. The

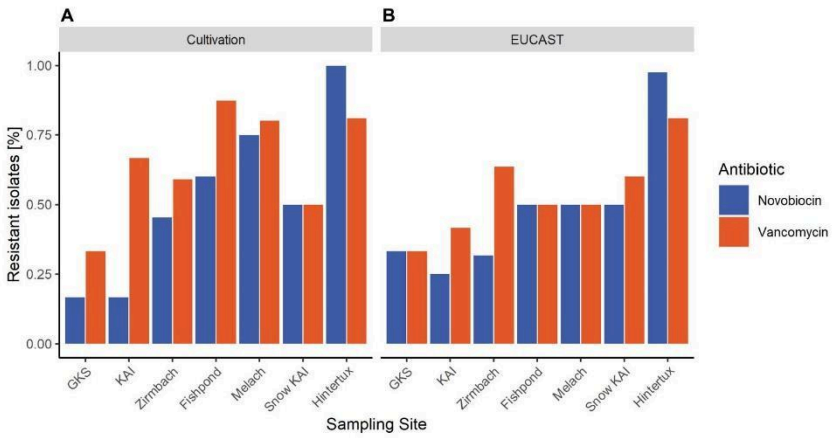

FIGURE 4
Average percentage of resistant isolates within the sampling sites based on the results of the antibiotic susceptibility tests performed on different nutrition media and incubation temperatures. **(A)** displays the results achieved with the cultivation method at 20°C, while in **(B)** the relative abundance of antibiotic-resistant bacteria detected with the agar-disk-diffusion method at 20°C is mapped.

more precise reading guide connected to breakpoints of the agar disk diffusion method – which identifies an isolate as either resistant, susceptible, or (in some cases) intermediate – poses another major cause for differences in the resistance profile of an isolate based on the testing procedure. In total, the two methods delivered different results in ~24% of the treatments (all incubation temperatures and nutrition media). This again highlights the importance of a standardized testing procedure for antibiotic susceptibility testing of environmental and especially cryospheric samples.

4.4. Influence of nutrition media, agar concentration, and incubation temperature on the size of the zone of inhibition

In general, an effect on the size of the zone of inhibition has been observed by nutrition media, agar concentration, and incubation temperature.

The effect of the nutrition media on the size of the zone of inhibition is mainly caused by the differences in the agar concentration. A decrease of the agar concentration from 1.7% (original recipe of MH) to 1.5% (original recipe of R2A) ultimately leads to an increased zone of inhibition size at both nutrition media tested. This is likely caused by the enhanced diffusion rate of the antibiotics at a higher diffusion coefficient. According to Fick's (1855) law the diffusion rate is inversely proportional to the viscosity of the media the molecule has to pass through. The reduction of the agar concentration does decrease the viscosity of the nutrition media, which causes an increased diffusion coefficient and thus an increased diffusion rate for the antibiotics. When comparing R2A15 with R2A17 and MH15 with MH17, respectively, highlights the strong significant effect the agar concentration has on the size of the zone of inhibition (Figure 3).

While other factors like starch may also influence the diffusion rate of antimicrobial substances, these seem to have a much lower impact. However, the differences in the nutrition media compositions might explain the discrepancy between MH15 and R2A15 or MH17 and R2A17 (Figure 3). The significance of the differences indicates that the agar concentration itself is not the only factor that influences the diffusion rate of the antibiotics. Especially, the increased starch concentration of MH agar potentially impacts how easily molecules can diffuse through the agar. Nonetheless, the size of the inhibition zone differed the least when MH17 was compared with R2A17 for both novobiocin (+6.64 mm) and vancomycin (+2.24 mm). Overall, these results suggest that the use of different nutrition media and especially of a different agar concentration can lead to a change in inhibition zone size and, therefore, to a potential misinterpretation of the antibiotic susceptibility of an isolate. Using R2A agar (with 1.5% agar concentration) instead of the officially recommended MH agar (with 1.7% agar concentration) resulted in an average increase of the inhibition zone by 11.8 mm for novobiocin and 6.6 mm for vancomycin, respectively. These differences caused by different nutrition media and agar concentrations can have a drastic impact on the final result of the antibiotic susceptibility test. This is especially relevant because these increases equal roughly 74% (novobiocin) and 44% (vancomycin) of the thresholds given (according to the EUCAST breakpoint table, The European Committee on Antimicrobial Susceptibility Testing, 2021b) to identify a bacterium as either resistant or susceptible to the antibiotic and could therefore lead to a misinterpretation of the actual susceptibility.

The different increase in the zone of inhibition size between the two antibiotics can potentially be explained by the differences in their molecular weight (novobiocin 612.6 g/mol, vancomycin 1449.3 g/mol) as it is again inversely proportional to the diffusion coefficient. According to Fick's law, diffusion decreases with an increasing radius of the diffusing molecule. Therefore, the diffusion rate is expectedly higher for novobiocin than for vancomycin, which is also represented in the results (Figure 3).

Previous studies described an effect of a decreased incubation temperature and the consequent prolonged incubation period on the results of the agar disk diffusion (e.g., Smith and Kronvall, 2015; Smith et al., 2018) which has also been observed in this study. However, these experiments are usually performed on mesophilic

bacteria, while, to the best of our knowledge, nothing is yet reported for psychrophilic and psychrotolerant microorganisms. Our results show that a decrease in the incubation temperature from 20°C to 4°C can cause an increase in the zone of inhibition diameter size when the agar disk diffusion method is applied to psychrophilic and psychrotolerant bacteria. This result is contrary to the expected decreased diffusion rate of molecules at lower temperatures. However, this effect seems to be irrelevant due to the prolonged incubation period of up to 2 weeks for cultures to visibly appear. Interestingly, the significant difference in the size of the zone of inhibition at the two temperatures of interest could only be observed when the antibiotic susceptibility test was performed with novobiocin – a molecule that needs to enter the bacterial cell to inhibit its growth by inhibiting the DNA and/or RNA synthesis (Smith and Davis, 1967). In general, the transport of molecules into bacteria can be mediated by either passive diffusion, facilitated diffusion, or active transport through energy-dependent systems (Chopra, 1989). However, passive diffusion of hydrophilic antibiotics through the cytoplasmic membrane is limited to a size of a maximum of 100 g/mol (Franklin and Leive, 1973). Nevertheless, it is known that even though passive diffusion rates across membranes are lower at cold temperatures, bacterial cells up-regulate membrane transport proteins (de Maayer et al., 2014). This could impact the effective novobiocin uptake at lower temperature levels and explain the average increase in the inhibition zone size. However, even though the zone of inhibition increased significantly when isolates were tested for novobiocin susceptibility at 4°C, the relative number of bacteria determined as resistant did not change. No significant change in the size of the zone of inhibition was observed when the agar disk diffusion method was performed with vancomycin at 20°C and 4°C.

5. Conclusion

Overall, our results suggest that the standardized agar disk diffusion test with the recommended nutrition media (MH with an agar concentration of 17 g/L) is well-suited for the analysis of the culturable cryospheric antibiotic susceptibility. The advantage of the established test is not only the standardized protocol and guidelines provided by the EUCAST but also years of research and experience that enhanced the robustness thereof. In addition, the agar disk diffusion test considers the minimum inhibitory concentration and, therefore, relies on the usage of specific antibiotic concentrations and respective breakpoints. However, the latter is species-dependent and only given for common human pathogens, which complicates the application of this method for non-pathogenic environmental bacteria. As the breakpoints within the provided reading table (The European Committee on Antimicrobial Susceptibility Testing, 2021b) do only differ slightly between different bacterial strains, we suggest that using the mean of these thresholds is an appropriate procedure to evaluate antibiotic susceptibility of bacterial strains that are not listed.

The EUCAST protocol for the agar disk diffusion method is optimized for mesophilic pathogenic bacteria and can therefore not be followed regarding incubation temperature and time. This presents another challenge that needs to be tackled to evaluate the antibiotic susceptibility of culturable isolates from environmental and especially cryospheric samples. Sticking as close as possible to the original protocol ensures high-quality standards and is, therefore, strongly recommended. Adaptations regarding the incubation temperature to meet the optimal needs of the bacteria potentially influence the outcome of the results, which is why we suggest using temperatures as close as possible to the official EUCAST guidelines. Apart from that, the nutrition media seem to be an important factor when evaluating the zone of inhibition. Hence, growth on MH agar is strongly advised based on our results. If any other nutrition media is chosen for the testing procedure (e.g., to match the bacterial growing conditions), the adaptation of the agar concentration to 17 g/L might be able to reduce variance in inhibition zone size to a minimum. However, this would need further testing and the most reliable results are to be expected by the use of the suggested nutrition media.

All in all, we suggest that antibiotic susceptibility testing with an adapted version (in terms of incubation temperature and time) of the standardized agar disk diffusion method is applicable to evaluate the culturable resistome of cryospheric habitats and could further enhance the comparability between studies. This method seems especially interesting when complemented with antibiotic resistance gene screening to also assess phenotypic and unknown resistance mechanisms.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

DG, BS, and TW designed the study. DG and BS collected the samples. DG and KP conducted lab work and evaluation of data (DG: main contributor). DG and BS wrote the manuscript (DG: main contributor) that was reviewed by all DG, BS, KP, and TW. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1212378/full#supplementary-material

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Supplementary Material

A comparative approach to confirm antibiotic-resistant microbes in the cryosphere

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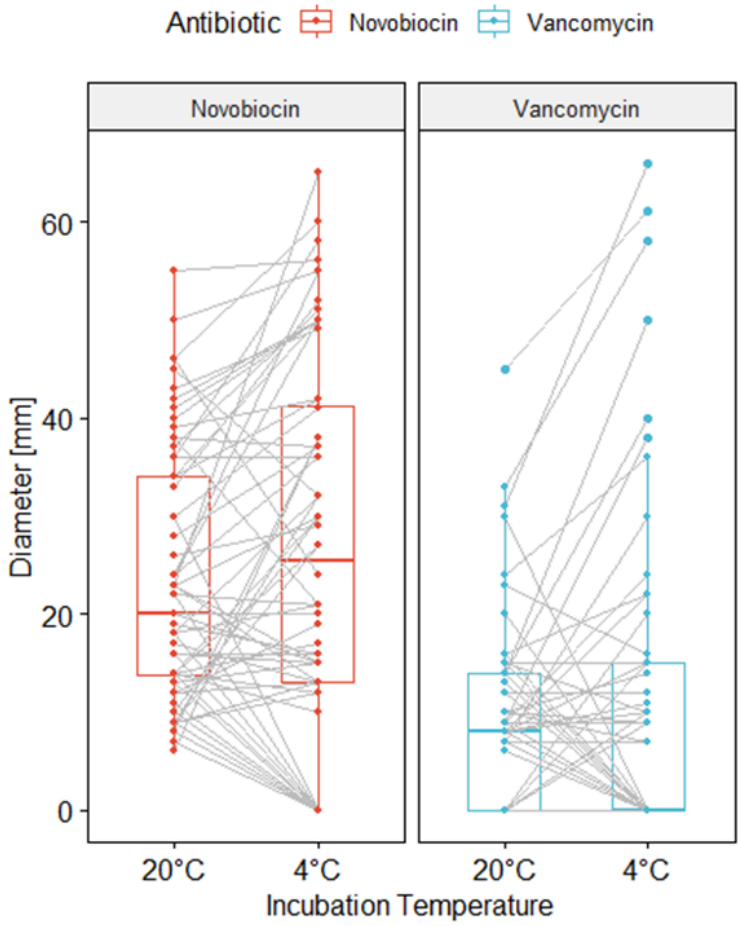

Supplementary Figure 1. Effect of the incubation temperature on the size of the zone of inhibition for novobiocin and vancomycin.

Supplementary Table 1. List of all sampling sites including exact geolocations and sample type. GKS = Gossenkoellesee, KS = Kaiser See, NIP = Nature Ice Palace.

Site	Sample type	GPS coordinates	Number of bacterial isolates
GKS	Lake/Water	47°13'45.4"N 11°00'51.4"E	5
KS	Lake/Water	47°13'22.7"N 11°01'24.4"E	7
Snow KS	Snow	47°13'22.9"N 11°01'26.5"E	7
Zirmbach	Stream/Water	47°13'00.5"N 11°04'34.1"E	11
Fishpond	Fishpond/Water	47°12'29.3"N 11°05'01.5"E	5
Melach	Stream/Water	47°11'54.9"N 11°10'08.5"E	6
NIP	Glacier/Ice	47°03'42.4"N 11°40'47.2"E	21

Supplementary Table 2. List of the bacterial isolates from all sampling sites including their morphology, gram status and ability to grow at 20 and 4 °C. The gram status has been assessed with the KOH test after Ryu et al. (1938)

				Growth	
Culture Name	**Sampling Site**	**Morphology**	**Gram status**	**4 °C**	**20 °C**
RK 1	Melach	round, entire, flat, white	positive	-	+
RK 2	Melach	round, entire, flat white	positive	+	+
RK 4	Melach	irregular, filiform, flat, white	negative	-	+
RK 5	GKS	round, filiform, raised, white	positive	-	+
RK 6	GKS	irregular filiform, flat, white	positive	-	+
RK 7	Zirmbach	round, entire, flat, red	negative	-	+
RK 8	Zirmbach	round, entire, flat, yellow	positive	-	+
RK 9	Zirmbach	round, entire, flat, white	positive	-	+
RK 10	Zirmbach	irregular, undulate, flat, yellow	positive	+	+
RK 11	Zirmbach	irregular, filiform, flat, white	positive	-	+
RK 12	KS	round, entire, flat, orange	positive	-	+
RK 13	KS	round, entire, raised, brown	positive	-	+

RK 14	KS	round, entire, flat yellow	positive	+	+
RK 15	KS	round, entire, flat, white	positive	-	+
RK 17	Snow KS	round, entire, flat, yellow	negative	+	+
RK 18	Snow KS	irregular, undulate, flat, white	positive	-	+
RK 19	Snow KS	round, entire, flat, white	negative	-	+
RK 20	Snow KS	irregular, undulate, flat, yellow	negative	-	+
RK 21	Fishpond	round, entire, flat, yellow	positive	-	+
RK 22	Fishpond	round, entire, flat, yellow	positive	-	+
RK 23	Fishpond	round, entire, raised, white	positive	-	+
RK 24	Snow KS	round, entire, fat, yellow	positive	+	+
RK 25	Snow KS	irregular, filiform, flat, yellow	positive	-	-
RK26	Snow KS	round, entire, raised, translucent	positive	+	+
RK 27	Zirmbach	round, entire, raised, white	negative	+	+
RK 28	Zirmbach	round, entire, flat, purple	positive	+	+
RK 29	Zirmbach	irregular, filiform, flat, yellow	negative	+	+
RK 30	Zirmbach	round, entire, raised, yellow	negative	+	+
RK 31	Zirmbach	round, entire, raised, red	positive	+	+
RK 32	Zirmbach	irregular, filiform, flat, white	negative	+	+
RK 33	Fishpond	round, entire, flat, white	negative	+	+
RK 34	Fishpond	round, entire, flat yellow	positive	+	+
RK 35	Melach	round, entire, raised, yellow	positive	+	+
RK 36	Melach	round, entire, raised, white	negative	+	+
RK 37	Melach	irregular, entire, flat, yellow	positive	+	+
RK 38	KS	round, filiform, flat, white	negative	+	+
RK 39	KS	round, entire, convex, yellow	positive	-	-
RK 40	GKS	round, entire, raised, white	positive	-	-
RK 41	KS	round, entire, raised, white	positive	-	-
RK 42	GKS	round, entire, convex, translucent	negative	+	+

RK 43	GKS	round, entire, raised, white	positive	-	-
HT 1	NIP	round, entire, flat, pink	negative	+	+
HT 2	NIP	round, entire, flat, yellow	negative	+	+
HT 3	NIP	round, entire, raised, white	positive	+	+
HT 4	NIP	irregular, undulate, flat, yellow	positive	+	+
HT 5	NIP	round, entire, flat, white	positive	+	+
HT 6	NIP	irregular, entire, flat, yellow	positive	+	+
HT 7	NIP	round, entire, raised, red	positive	+	+
HT 8	NIP	round, undulate, flat, white	negative	+	+
HT 9	NIP	round, entire, flat, yellow	positive	+	+
HT 10	NIP	round, undulate, flat, white	negative	+	+
HT 11	NIP	round, entire, flat, white	positive	+	+
HT 12	NIP	round, entire, flat translucent	positive	+	+
HT 13	NIP	round, undulate, flat, yellow	positive	+	+
HT 14	NIP	round, entire, flat, white	positive	+	+
HT 15	NIP	round, entire, flat, yellow	positive	+	+
HT 16	NIP	round, undulate, flat, translucent	positive	+	+
HT 17	NIP	round, entire, flat, white	negative	+	+
HT 18	NIP	round, entire, raised, white	negative	+	+
HT 19	NIP	round, entire, raised, translucent	negative	+	+
HT 20	NIP	round, translucent, flat, white	negative	+	+
HT 21	NIP	round, entire, raised, white	negative	+	+

Supplementary Table 3. Breakpoint/Size of the zone of inhibition for the agar disk diffusion test based on recommended susceptibility thresholds by the EUCAST (eucast.org 2021b). *Harrington and Gaydos 1984

Phyla	**Antibiotic Family**	**Antibiotic**	**Disk content [µg]**	**S ≥ [mm]**	**R < [mm]**
Enterococcus spp.	Glycopeptides	Vancomycin	5	12	12

Streptococcus groups A, B, C, D	Glycopeptides	Vancomycin	5	13	13
Streptococcus pneumoniae	Glycopeptides	Vancomycin	5	16	16
Viridans group streptococci	Glycopeptides	Vancomycin	5	15	15
Corynebacterium spp.	Glycopeptides	Vancomycin	5	17	17
Aerococcus sanguinicola/urniae	Glycopeptides	Vancomycin	5	16	16
Calculated mean value				**14,83**	**14,83**
-	Aminocumarines	Novobiocin	5	16*	16*

Supplementary Table 4. Results of the 16S rRNA Sanger sequencing. Sequences were submitted to GenBank®.

Culture Name	**Family**	**Genus**	**Accession number**
RK 1	*Bacillaceae*	*Bacillus*	OR225929
RK 2	*Bacillaceae*	*Bacillus*	OR225930
RK 4	*Pseudomonadaceae*	*Pseudomonas*	OR225931
RK 5	*Bacillaceae*	*Bacillus*	OR225932
RK 6	*Bacillaceae*	*Bacillus*	OR225933
RK 7	*Pseudomonadaceae*	*Pseudomonas*	OR225934
RK 8	*Micrococcaceae*	*Paenarthrobacter*	OR225935
RK 9	*Bacillaceae*	*Bacillus*	OR225936
RK 10	*Micrococcaceae*	*Agreia*	OR225937
RK 11	-	-	
RK 12	*Bacillaceae*	*Bacillus*	OR225938
RK 13	-	-	
RK 14	*Microbacteriaceae*	*Plantibacter*	OR225939
RK 15	*Bacillaceae*	*Bacillus*	OR225940

RK 17	*Oxalobacteraceae*	*Janthinobacterium*	OR225941
RK 18	*Bacillaceae*	*Bacillus*	OR225942
RK 19	*Comamonadaceae*	*Acidovorax*	OR225943
RK 20	*Rhodobacteraceae*	*Paracoccus*	OR225944
RK 21	*Flavobacteriaceae*	*Flavobacterium*	OR225945
RK 22	*Bacillaceae*	*Bacillus*	OR225946
RK 23	*Bacillaceae*	*Bacillus*	OR225947
RK 24	*Bacillaceae*	*Lysinibacillus*	OR225948
RK26	-	-	
RK 27	-	-	
RK 28	*Flavobacteriaceae*	*Flavobacterium*	OR225949
RK 29	-	-	
RK 30	*Flavobacteriaceae*	*Flavobacterium*	OR225950
RK 31	-	-	
RK 32	*Oxalobacteraceae*	*Massilia*	OR225951
RK 33	*Flavobacteriaceae*	*Flavobacterium*	OR225952
RK 34	-	-	
RK 35	*Pseudomonadaceae*	*Pseudomonas*	OR225953
RK 36	*Flavobacteriaceae*	*Flavobacterium*	OR225954
RK 37	-	-	
RK 38	*Oxalobacteraceae*	*Janthinobacterium*	OR225955
RK 42	-	-	
HT 1	*Pseudomonadaceae*	*Pseudomonas*	OR225914
HT 2	*Sphingomonadaceae*	*Sphingomonas*	OR225915
HT 3	-	-	
HT 4	*Pseudomonadaceae*	*Pseudomonas*	OR225916

HT 5	-	-	
HT 6	-	-	
HT 7	*Bacillaceae*	*Bacillus*	OR225917
HT 8	*Staphylococcaceae*	*Staphylococcus*	OR225918
HT 9	*Sphingomonadaceae*	*Sphingomonas*	OR225919
HT 10	*Yersiniaceae*	*Yersinia*	OR225920
HT 11	-	-	
HT 12	*Bacillaceae*	*Bacillus*	OR225921
HT 13	*Oxalobacteraceae*	*Massilia*	OR225922
HT 14	-	-	
HT 15	*Yersiniaceae*	*Yersinia*	OR225923
HT 16	*Bacillaceae*	*Bacillus*	OR225924
HT 17	*Oxalobacteraceae*	*Janthinobacterium*	OR225925
HT 18	*Pseudomonadaceae*	*Pseudomonas*	OR225926
HT 19	*Pseudomonadaceae*	*Pseudomonas*	OR225927
HT 20	*Pseudomonadaceae*	*Pseudomonas*	OR225928
HT 21	-	-	

Paper II - From remote to urbanized: Dispersal of antibiotic-resistant bacteria under the aspect of anthropogenic influence

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From remote to urbanized: Dispersal of antibiotic-resistant bacteria under the aspect of anthropogenic influence

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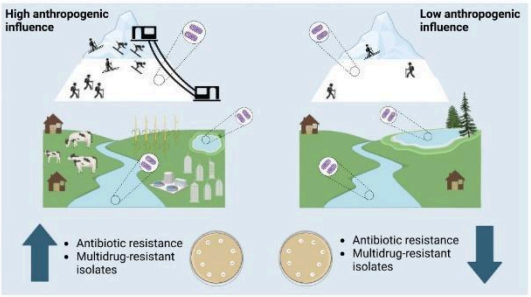
HIGHLIGHTS

- Decline in antibiotic efficacy is not limited to clinical settings but is also observed in environmental samples
- Over 50% of isolated microorganisms exhibit multidrug resistance (MDR), indicating limited antibiotic effectiveness
- The decreasing effectiveness of antibiotics, including that of reserve antibiotics, has raised concerns
- Innovative approach assesses anthropogenic impact using Corine Land Cover data and heatmaps from sports activity trackers
- Clear link between increased human influence and higher AR levels in glacier, snow, and lake samples

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotic resistance is a growing global concern, but our understanding of the spread of resistant bacteria in remote regions remains limited. While some level of intrinsic resistance likely contributes to reduced susceptibility to antimicrobials in the environment, it is evident that human actions, particularly the (mis)use of antibiotics, play a significant role in shaping the environmental resistome, even in seemingly distant habitats like glacier ice sheets.

Our research aims to bridge this knowledge gap by investigating the direct influence of human activities on the presence of antibiotic-resistant bacteria in various habitats. To achieve a comprehensive assessment of anthropogenic impact across diverse and seemingly isolated sampling sites, we developed an innovative approach utilizing Corine Land Cover data and heatmaps generated from sports activity trackers. This method allowed us to make meaningful comparisons across relatively pristine environments.

Our findings indicate a noteworthy increase in culturable antibiotic-resistant bacteria with heightened human influence, as evidenced by our analysis of glacier, snow, and lake water samples. Notably, the most significant concentrations of antibiotic-resistant and multidrug-resistant microorganisms were discovered in two highly impacted sampling locations, namely the Tux Glacier and Gas Station Ellmau.

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1. Introduction

The discovery of Penicillin in the mid-20th century and the following 'golden era' with the discovery of many new classes of antibiotics revolutionized modern medicine. However, the extensive human use of these drugs quickly raised a new challenge in the form of antimicrobial resistance (AMR). Almost 100 years later, bacteria seem to be outperforming human capabilities to find and produce new effective antibiotics. As of 2023, the Comprehensive Antibiotic Resistance Database (CARD) lists over 300.000 alleles that represent known unique variants of antibiotic resistance genes (ARGs) (Alcock et al., 2023) and while the number of AMR continues to rise, the discovery of new effective substances to inhibit bacterial growth has declined drastically (Ventola, 2015) thus posing a worldwide challenge that urgently needs to be tackled (WHO, 2019). Not only does AMR pose a major threat to public health, but it also has economic implications. According to The Review on Antimicrobial Resistance (2016), antibiotic resistance (AR) could cost the global economy up to $100 trillion until 2050 if no action is taken.

Beyond clinical settings, AMR has emerged as a serious environmental issue. There is increasing evidence that AR is not only limited to populated areas but also is of concern for natural environments including the cryosphere (Gattinger et al., 2023).

The cryosphere encompasses a variety of habitats such as glaciers, seasonal snow cover, permafrost, sea ice, and others that were once thought to be void of life. Nowadays, it is well-known that the cryosphere harbors a variety of life which includes bacteria, archaea, viruses, and fungi that have adapted to cold and nutrient-depleted environments (e.g. Jungblut et al., 2022) Moreover, these cold habitats can contain significant numbers of antibiotic-resistant bacteria (Hernández and González-Acuña, 2016; McCann et al., 2019; Ushida et al., 2010; Segawa et al., 2013). Yet, despite these sensible habitats being highly interconnected with other ecosystems, AMR research in the cryosphere is still in its early days and mainly focused on the detection of ARGs.

It is generally agreed that animal migration, global wind and water systems, and human travel activities are potentially contributing to the worldwide spread of AMR (Allen et al., 2010), however, transport mechanisms to remote areas are not yet fully understood.

Antibiotic resistance has mainly originated from the additional selective pressure provoked by extensive human use of antibiotics. Here, a variety of factors like overuse and misuse of antibiotics, inadequate sanitation, and use of antibiotics in agri- and aquaculture have contributed massively to the current state of AMR.

Therefore, it is essential to consider the relevance of anthropogenic influence in the context of AMR within cryospheric habitats. Despite the traditional perception of these habitats as pristine and unaffected by human activities, increasing evidence suggests otherwise. Anthropogenic activities play a crucial role in introducing antimicrobial resistance genes (ARGs) into these environments through various entry pathways that are closely connected to humans like plastic pollutants that act as carriers for ARGs, facilitating their transport into remote glacial environments (Laganà et al., 2019). Similarly, the migration of animals can introduce resistant bacteria into these habitats, contributing to the dissemination of AMR (Laborda et al., 2022). Human activities such as tourism and research expeditions in polar regions can also cause spreading of AMR (Depta and Niedźwiedzka-Rystwej, 2023). Finally, recent research suggests that AMR may be disseminated through atmospheric processes, particularly in mountainous regions, highlighting the complexity of the pathways involved (Rossi et al., 2023).

Despite these entry pathways, it is worth noting that AMR can also emerge within cryospheric habitats independent of direct human influence. Native bacterial populations inhabiting these environments can exhibit intrinsic resistance to antimicrobials, contributing to the overall resistome (Pawlowski et al., 2016). This intrinsic resistance underscores the need for comprehensive research to elucidate the dynamics of AMR in cryospheric habitats, considering both anthropogenic and natural factors.

With this study, we aimed to achieve a better understanding of the human impact on AMR in different environmental habitats including cryospheric and freshwater samples. To this end, we analyzed available data that are usually considered sources for AR such as direct human presence and land cover data associated with hotspots for AR.

Our samples included a) glacial snow and ice, b) seasonal snow in areas with different levels of human impact as well as c) river systems (from melting waters to larger rivers), and d) different types of lakes (high altitude to swimming lakes).

The spanning from remote to urbanized areas allowed us to compare the prevalence of AMR in culturable bacteria at different levels of anthropogenic influence (low, medium, and high).

By analyzing the data collected from various environmental and cryospheric sources, the work presented here paves the way for a better understanding of the influence of human activities on the spread of AR in our environment.

2. Material and methods

2.1. Sampling sites

Snow, ice, and water were sampled during several field campaigns between November 2018 and July 2020 (Fig. 1). Sampling sites were chosen based on three different levels of anthropogenic influence (low, medium, high), which was determined by several factors such as direct human activity (glaciers), disposal/catchment areas, and land use data (lakes, rivers, snow).

Glacier samples consisted of snow as well as ice samples and were taken from the surface of the glacial ice sheets. The Tux Glacier was a unique sampling site in this study due to sampling in a walkable crevasse (Nature Ice Palace [NIP]) resembling a cave being available for tourism. Samples were mostly taken from a variety of locations within the ice cave with 50,000 visitors/year, (pers. comm. Roman Erler).

2.2. Evaluating anthropogenic influence

It is impossible to determine the absolute human impact with precision because there is no single reliable parameter that comprehensively accounts for all the diverse factors that contribute to it. However, some indicators can be used to differentiate between different levels of anthropogenic influence. These include among others human presence, population density, land use, sewage, and disposal area.

To compare anthropogenic influence between sampling sites, catchment areas for snow and lakes were delineated using QGIS (QGIS Version 3.16) with the GRASS and PCRraster add-on using a digital elevation model of Tyrol with a resolution of 5 m (Land Tirol - data.tirol.gv.at, 2023). Disposal areas derived from Tiris Maps provided by the Amt der Tiroler Landesregierung (2023) were used as the relevant area to evaluate human impact on flowing waters.

Within catchment and disposal areas, the percentage of anthropogenically used land was calculated based on the freely available CORINE Land Cover (CLC) data provided by the European Union (2018). For that purpose, levels of the CLC were reclassified and aggregated to fit into 5 major categories: artificial surfaces, agricultural areas, forests and semi-natural areas, wetlands, and water. Snow, lake, and water sampling sites have then been categorized into low (< 33 % artificial/agricultural areas), medium (33–66 % artificial/agricultural areas), and high (> 66 % artificial/agricultural areas).

For snow and ice from glacial samples, the main indicator of human impact was direct human presence. To compare relevant areas, heat maps (Strava, 2023) were used. These color-coded heatmaps (see Supplementary 1) represent the relative abundance of human activity in specific regions with brighter colors usually indicating higher activity. Using this information we calculated the percentage of the area of interest (glacier surface) subjected to varying levels of direct human

impact. Color channels were therefore split into four categories depicting no, low, medium, or high human presence at a specific pixel.

2.3. Sampling

Snow and superficial ice were scratched off with a cleaned stainless-steel shovel whereas deeper layers of ice were sampled with a Kovacs Ice Corer (Mark III, ⌀ 7,25 cm). The respective original sample was used to clean all sampling devices. Snow and ice samples were sampled in Whirl-Packs(R) and stored at −20 °C until further use.

Water was collected into 250 ml sterile screw-cap bottles, transported to the home institute, and stored at 4 °C before proceeding with cultivation experiments.

2.4. Isolation of pure cultures

To isolate pure cultures 1 ml of each snow, ice, and water sample was plated on Reasoner's 2 A agar (R2A, Merck) and incubated at 4 °C and 20 °C respectively until visible growth was observed. The choice of incubation temperature was a compromise in terms of the tolerability of cold-loving bacteria and temperatures as close as possible to the official recommendation by the EUCAST. All samples were treated the same way except for an initial melting step at 4 °C for snow and ice samples kept at −20 °C after sampling. R2A agar was selected due to its oligotrophic properties which fit the preference of cryospheric organisms.

Distinct colonies were described for morphology, color, and optical growth before the transfer into 7 ml of liquid media (lysogeny broth, LB) with a sterile loop. Seven days of incubation in LB media at the respective temperatures allowed for sufficient biomass growth to perform further tests. The purity of all isolates was then confirmed by streaking. LB was the media of choice due to its suitability for subsequent agar disk diffusion tests.

2.5. Agar disk diffusion test

To perform the agar disk diffusion test standardized by EUCAST (eucast.org, 2021), some modifications were necessary for psychrophilic, slow-growing organisms (Gattinger et al., 2023). In brief, these changes involved the reduction of the incubation temperature from 37 °C to 20 and 4 °C respectively as well as the extension of the incubation time to up to two weeks for slow-growing bacteria. The lack of breakpoints for non-pathogenic bacteria was overcome by calculating all available diameter breakpoints from the official guidelines (eucast.org, 2023, see Supplementary 1). For novobiocin, no diameters are listed in the EUCAST breakpoint table. Therefore, thresholds were applied based on existing literature (Harrington and Gaydos, 1984).

Besides these adjustments, we adhered to the official guidelines for the agar disk diffusion method.

Following the EUCAST reading guide, bacteria with a zone of inhibition smaller than the defined breakpoint are classified as resistant. Isolates that showed an inhibition zone size equaled or exceeded the calculated thresholds were considered sensitive to the antibiotic.

2.6. Antibiotics

A total of 8 antibiotics were used to test the susceptibility of isolated bacteria. These covered a wide range in mechanisms of action (including but not limited to inhibition of translation, transcription, and cell wall biosynthesis), antibiotic synthesis (natural, semi-synthetic, synthetic), the latest WHO AWaRe classification (access, watch, reserve), and suitability for the agar disk diffusion test (Table 1). Strains were classified as multidrug-resistant (MDR) when they were resistant to at least three different antimicrobials.

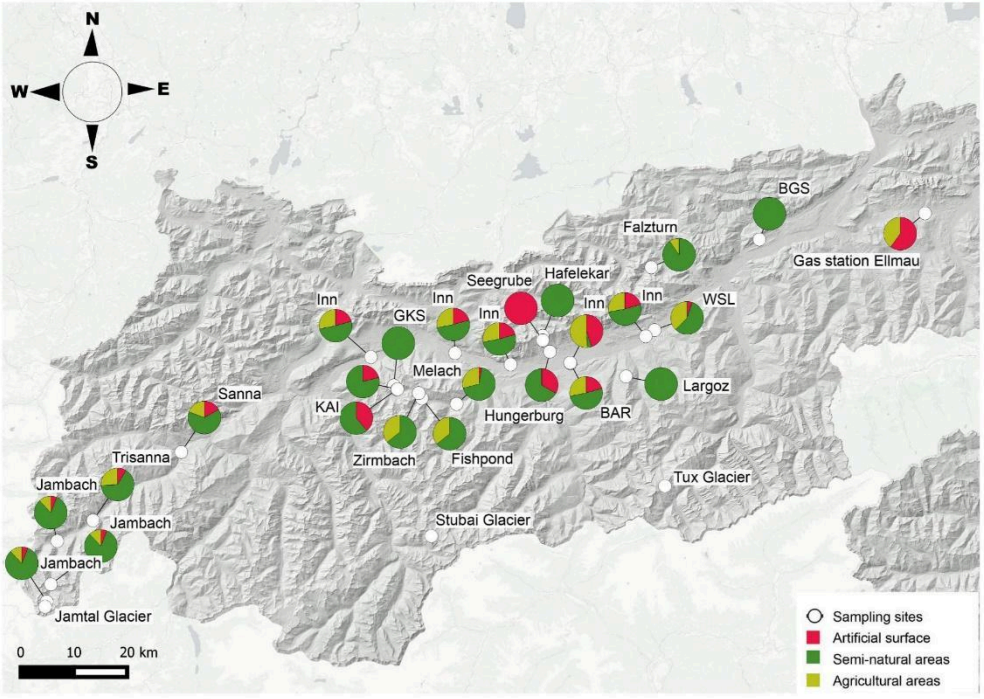

Fig. 1. Sampling sites throughout the Tyrolean alpine space including an overview of the properties of the surrounding land cover from CLC. Glacier samples were not investigated for land cover but rather heatmaps from tracking services.

2.7. DNA extraction

The DNA of all isolated and purified cultures was extracted using the DNeasy Blood & Tissue kit (Qiagen). As starting material for the extraction, bacterial isolates were inoculated in 5 ml of LB media for 7–14 days. After centrifugation, all further steps were performed following the protocol for bacterial isolates provided by the manufacturer. DNA quantity and quality were evaluated with a nanodrop spectrophotometer.

2.8. PCR and Sanger sequencing

PCR amplification was performed by targeting the hypervariable regions V1 to V9 of the 16S rRNA gene with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR reactions were carried out using 2× PCR Master Mix (Thermo Fisher) and 50 ng template DNA. The cycling conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 1 min, and a final elongation step at 72 °C for 7 min. PCR products were checked by gel electrophoresis and purified using the ExoSAP-IT™ reagent (Applied Biosystems) according to the manufacturer's instructions. The purified PCR products were sequenced at the Fondazione Edmund Mach on a 3730xl Genetic Analyzer (Applied Biosystems).

2.9. Bioinformatics and statistics

Data generated through sequencing underwent initial processing using the sangeranalyseR package (Chao et al., 2021) in R (version 4.2.2) for quality trimming (QS 30). This allowed the generation of Sanger reports and fasta files which were then used to perform a BLAST search against the NCBI database.

To calculate the glacier area with human activity from heatmaps, R together with the 'countcolors' package (Hooper et al., 2020) was used.

For the statistical analysis, R (version 4.2.2) was utilized with the packages 'dplyr', 'ggpubr', and 'ggplot2'. After testing for assumptions, ANOVA was employed to assess the impact of the anthropogenic influence on AR in environmental and cryospheric sampling sites. Subsequently, a Tukey post hoc test was conducted to identify significant pairwise differences between the groups.

In cases where one or more assumptions of the ANOVA were not met and data transformation did not suffice, a Kruskal-Wallis test followed by a Pairwise Wilcoxon test was performed.

3. Results

3.1. Human activity heatmaps

Tracking data from one of the main providers (Strava), was used to estimate the relative direct human presence on each glacier of interest. The highest anthropogenic activity was observed in the glacial skiing areas on the Tux and Stubai Glacier. Additionally, samples from Tux Glacier were taken within the NIP. Data collected revealed that the human impact on the Jamtal Glacier is low, on the Stubai Glacier medium, and high on the Tux Glacier (Table 2).

3.2. Corine land use data

The CLC data of the respective catchment areas of snow and lake samples as well as of the disposal area of flowing waters revealed that one lake (BAR) and two snow sampling sites (Seegrube, Ellmau) are under high anthropogenic pressure. On the contrary, no flowing water was found to be highly influenced by humans, and for three snow samples (BGS, Hafelakar, Largoz) and two lake samples (GKS, KAI) only a comparably slight anthropogenic influence could be detected (Table 3).

3.3. Isolation of pure cultures

In total, 264 isolates (see Supplementary 2) were successfully cultivated and grown in pure cultures with the methods described. Morphological distinction was observed within but not between sampling sites. 31.82 % of the isolates were identified as gram-positive.

180 bacterial colonies showed visible growth at 20 °C on R2A and MH agar. 84 out of the initial 264 bacterial colonies grew at 4 °C on both nutrition media.

3.4. Agar disk diffusion test

Out of 264 bacterial isolates tested for antibiotic susceptibility, 54.92 % were MDR and 17.80 % of the bacterial isolates could successfully be inhibited in their growth by all antibiotics tested (Fig. 2). The percentage of resistant isolates was the lowest against Linezolid and Gentamicin and the highest in Trimethoprim and Ampicillin (see Table 4).

Overall, AR bacteria were isolated from all habitats but one (Hafelekar) with Gentamicin being the most effective antibiotic throughout all sampling sites. On average, AR was higher in cryospheric samples (snow and ice) than in freshwater ecosystems.

The highest percentage of MDR microorganisms were isolated from

Table 2
The relative level of anthropogenic influence on glacier samples. Determination was based on colored heatmaps (Strava) highlighting human activities. This allowed the classification of the sampling site based on the intensity of direct human presence as well as additional information. Samples from Tux Glacier were mostly taken within the touristic ice cave NIP.

	Direct human presence				
Site	Low	Medium	High	Additional Info	Relative level of anthropogenic influence
Jamtal Glacier	40 %	2 %	0 %	No skiing area	Low
Stubai Glacier	21 %	21 %	28 %	Skiing area	Medium
Tux Glacier	20 %	22 %	36 %	50,000 visitors/year	High

Table 1
Overview of the antibiotics used including general information about them and concentration of the filter disks used for the agar disk diffusion test.

Antibiotic	Class	Concentration	AWaRe group	Synthesis
Ampicillin	Beta-lactam	2 µg	Access	semisynthetic
Chloramphenicol	–	30 µg	Access	semisynthetic
Gentamicin	Aminoglycoside	10 µg	Access	natural
Linezolid	Oxazolidinone	10 µg	Reserve	synthetic
Nitrofurantoin	Nitrofuran	100 µg	Access	synthetic
Novobiocin	Aminocoumarine	5 µg	Access	natural
Trimethoprim	Diaminopyrimidine	5 µg	Access	synthetic
Vancomycin	Glycopeptide	5 µg	Watch	natural

Table 3
Anthropogenic influence on snow and water samples based on CLC data and additional info available. Water quality was derived from Tiris Maps provided by the Amt der Tiroler Landesregierung (2023). Blue = lake samples, yellow = river samples, red = non-glacial snow samples.

Site	Anthropogenically used area* (%)	Additional Info	Relative level of anthropogenic influence
BAR	95.7	Public swimming lake	high
GKS	0	High altitude lake	low
KAI	0	Skiing area	low
WSL	42.3	Public swimming lake	medium
Fishpond	35.78	Pisciculture	medium
Inn	48.7	Water quality = 3	medium
Jambach	17.9	Water quality = 1.67	low
Melach	31.8	Water quality = 2	low
Sanna	36.1	Water quality = 5	medium
Trisanna	34.3	Water quality = 3	medium
Zirmbach	64.2	Water quality = 2	medium
Ellmau	100	Gas station	high
Falzthurn	6,09	Mountain area	low
Hafelekar	0 %	Mountain top	low
Hungerburg	33.42	Skiing area	medium
KAI	38.9	Skiing area	medium
Largoz	0	Mountain area	low
Seegrube	100	Skiing area	high
BGS	0	Mountain area	low

snow and ice samples at BGS (100 %), gas station Ellmau (100 %), and Tux Glacier (95.24 %) as well as from freshwater samples of two swimming lakes (BAR and WSL (71.43 %)) and two rivers (Melach and Trisanna (60 %)). These sites also showed the highest numbers of AR against all tested antibiotics (Fig. 3).

Contrary, no MDR bacteria have been isolated from snow sampled at Hafelekar and from freshwater samples taken at GKS and KAI.

We observed that the nature of antibiotics (synthetic, semisynthetic, or natural) did not have a significant effect on their overall effectiveness against environmental bacterial isolates ($p = 0.05$, Kruskal-Wallis).

3.5. Anthropogenic influence

The number of AR bacteria was significantly higher in areas with more anthropogenic influence ($p < 0.001$). Further analysis using a post hoc test indicated a significant difference in the presence of AR bacteria between low, medium, and high levels of human impact. AR in highly-impacted sampling sites was on average 18.5 % higher than in medium-impacted areas and 29.8 % higher than in regions with a comparably low anthropogenic influence.

The comparison of similar habitats confirmed the overall trend except for the following differences. The presence of AR in bacterial isolates from glacier habitats was significantly affected by the level of anthropogenic influence ($p < 0.01$). However, this effect was not evident when comparing sampling sites from medium to high levels of human influence ($p = 0.61$).

The anthropogenic impact also had a significant effect on AR in snow samples ($p < 0.01$), though a Tukey post hoc test indicated that there was no significant difference between areas with low and medium influence.

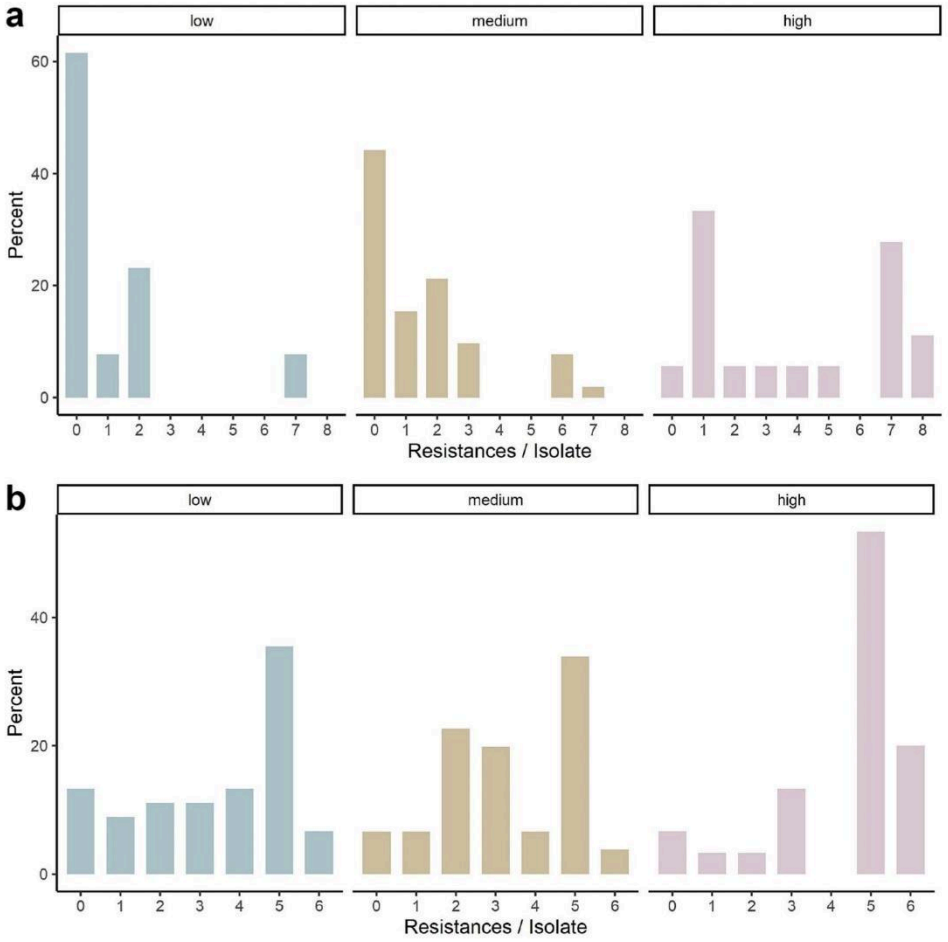

Fig. 2. Distribution of the number of AR within a single bacterial isolate in low, medium, and highly influenced sampling sites. Gram-positive bacteria (a) were tested against 8 different antibiotics including linezolid and vancomycin whereas gram-negative isolates (b) were tested for antibiotic resistance against 6 antimicrobial substances.

Table 4
Overview of resistant isolates (gram-positive and gram-negative) found against each of the 8 antibiotics. AMP = Ampicillin, C = Chloramphenicol, CN = Gentamicin, LZD = Linezolid, F = Nitrofurantoin, W = Trimethoprim, VA = Vancomycin, * = not effective against gram-negative bacteria.

	Antibiotic	AMP	C	CN	LZD*	F	NV	W	VA*
Gram-positive	Resistant Isolates	29	18	10	13	23	19	33	18
	Resistant Isolates [%]	34,52	21,43	11,90	15,48	27,38	22,62	39,29	21,43
Gram-negative	Resistant Isolates	130	77	33	–	121	137	132	–
	Resistant Isolates [%]	77,22	42,78	18,33	–	67,22	76,11	73,33	–

Lake samples significantly ($p < 0.001$; Kruskal-Wallis) differed regarding AR across varying levels of anthropogenic influence. Conversely, AR did not vary significantly between river samples from areas with low and medium levels of anthropogenic influence ($p = 0.051$; with Kruskal-Wallis). No river samples with high human impact were present (Fig. 4).

3.6. Sequencing

The average length of the trimmed sequences was 590 bp. A successful BLAST search could be performed in 214 sequences. *Pseudomonadaceae* was identified as the most abundant bacterial family throughout all samples corresponding to approximately 29 % of all sequenced isolates. Other abundant families include *Bacillaceae* (~16 %), *Oxalobacteraceae* (~11 %), *Flavobacteriaceae* (10 %), and *Micrococcaceae* (10 %). At species level, we observed a relatively high diversity within *Pseudomonadaceae* with 21 different species while species diversity was lower within other highly abundant bacterial families.

The two opportunistic pathogens *Escherichia coli* (BAR), and *Enterobacter cloacae* (WSL) have been isolated from swimming lake samples. Both species were MDR and showed enhanced resistance against Trimethoprim, Novobiocin, and Ampicillin (*E. coli*) as well as Nitrofurantoin (*E. cloacae*). On the other hand, the four different *Psychrobacter* species were only found in snow and ice samples and were resistant to up

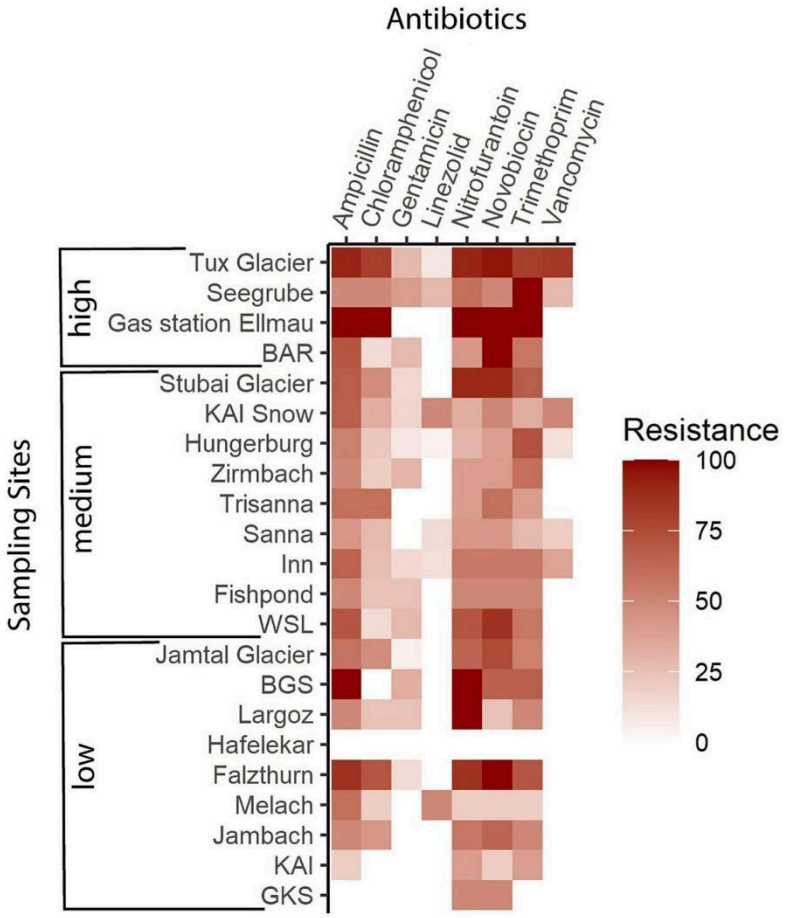

Fig. 3. Heatmap illustrating the distribution of AR throughout the different sampling sites while considering the anthropogenic influence in the respective environments.

to four antibiotics.

Janthinobacterium, a psychrophilic genus, was isolated from a variety of sampling sites including glacier ice, seasonal snow, high-altitude lakes, and flowing waters, and showed different susceptibility to the antibiotics depending on the sampling site.

4. Discussion

Antimicrobial resistance is a major global threat to human health (WHO Report, 2023), however, we still lack knowledge when it comes to AR in environmental samples.

In this study, we investigated the antibiotic susceptibility of culturable bacterial strains obtained from cryospheric and freshwater environments under different levels of anthropogenic influence. The aim was to explore the correlation between resistance and direct human presence to help closing this existing knowledge gap. Here, the Kirby-Bauer test allows for a comprehensive analysis of existing phenotypic resistance of culturable microorganisms. Although metagenomic or high-throughput approaches could further enhance the understanding of AR in environmental samples, cultivation-based approaches offer manifold advantages such as being well-proven, identifying resistance mechanisms with unknown ARGs, showing the actual viability of bacterial strains in the presence of antibiotics, and being fast and reliable.

4.1. Sequencing

Pseudomonadaceae emerged as the predominant bacterial family across all samples, constituting approximately 29 % of all sequenced isolates. This prevalence underscores the significance of Pseudomonas species in these environments, which are renowned for their intrinsic resistance mechanisms such as 12- to 100-fold reduced outer membrane permeability (Hancock and Brinkman, 2002), efflux pumps, and antibiotic-inactivating enzymes (Pang et al., 2019).

Overall, the culturable microbial community throughout all samples was dominated by gram-negative bacteria. A unique cell wall structure and ARGs like AmpC make many of these bacteria inherently resistant to some antibiotics like beta-lactam (Poole, 2011; Impey et al., 2020). This likely also contributes to the high numbers of AR found within the group of gram-negative bacteria compared to the gram-positive isolates (Table 4).

Bacterial species that are closely associated with humans, such as *Escherichia coli* and *Enterobacter cloacae* have been found in samples collected from swimming lakes, and therefore indicate the possible contamination of freshwater resources by direct anthropogenic influence. Both bacteria, isolated from medium and high anthropogenically influenced sampling sites, were found to be MDR. Antibiotic resistance in *E. coli* has become a challenge in clinical environments recently

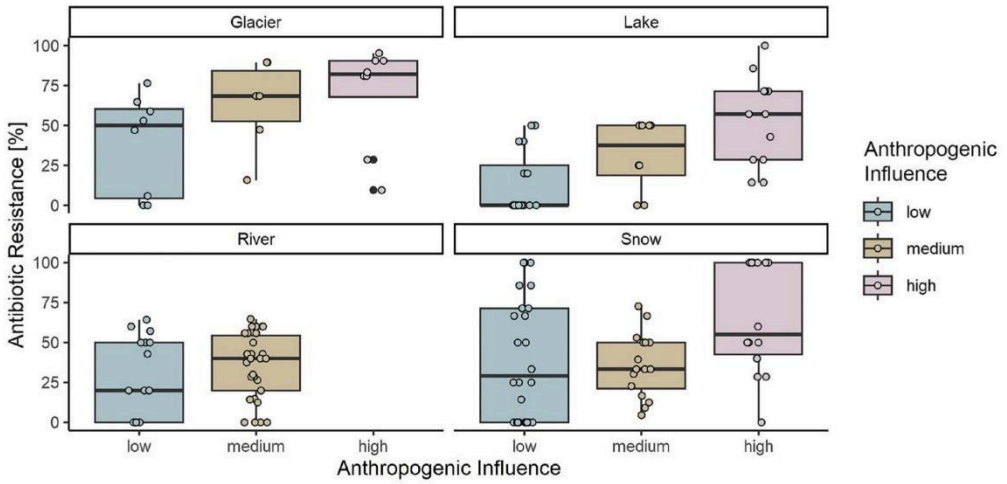

Fig. 4. Boxplot showing the difference in the percentage of AR bacteria in dependence of the anthropogenic influence. The samples were split up based on their origin (glacier, lake, river, snow). This increases comparability since human impact was determined on a relative basis with different methods for different sample types.

despite it being intrinsically susceptible to most relevant antibiotics (Poirel et al., 2018).

On the other hand, the identification of Psychrobacter species exclusively in snow and ice samples underscores their psychrophilic character, thriving in the extreme cold conditions characteristic of cryospheric environments.

However, since this sequencing was performed by culturable isolates from a variety of sampling sites, this does not reflect an accurate overview of the microbial composition of these habitats.

4.2. Agar disk diffusion test

Out of all successfully grown isolates (264), only 17.80 % were susceptible to all antibiotics tested. In contrast, 26.14 % of the bacterial isolates showed increased tolerance to five different antibiotics and over 50 % were MDR. This indicates the limited effectiveness of these eight antibiotics against the majority of the environmental bacterial strains analyzed here. Studies focusing on AR in soil have also shown that organisms can exhibit intrinsic MDR phenotypes to a variety of antibiotics independent of the antimicrobial nature (Dantas et al., 2008; D'Costa et al., 2006).

In general, AR is a highly complex topic especially when it comes to natural environments (Wright, 2007) because environmental bacteria harbor a significant prevalence of intrinsic resistance mechanisms like reduced outer membrane permeability or efflux pumps (Cox and Wright, 2013). This intrinsic resistance is likely contributing to a portion of the resistance found in this study. Additionally, AR organisms and antibiotics from anthropogenic sources that enter natural habitats through various sources can increase the selective pressure on indigenous bacteria (Baquero et al., 2008) which is a phenomenon not limited to habitats with proximity to human settlements (e.g. McCann et al., 2019; Depta and Niedźwiedzka-Rystwej, 2023). Previous studies revealed that specific AR mechanisms (e.g. vancomycin resistance) occurred long before the discovery of the first antimicrobial (D'Costa et al., 2011), emphasizing the complexity of AR in natural environments.

Although the cultivation-based approach used in this study does not allow to identify the original source for AR, a combination of intrinsic and acquired resistance seems most likely.

Resistance rates varied among the tested antibiotics. For example, Linezolid, a synthetic antibiotic classified as "Reserve" in the WHO AWaRe list, was the most active with only low levels of AR (15.66 %). It is considered an effective last-resort antibiotic to treat a variety of gram-positive infections (Theil et al., 2020). However, concerns about the rise of resistance against oxazolidinones are supported by recent studies in clinical (Bender et al., 2018), and even environmental isolates (Fioriti et al., 2021). Our results support these findings, as they reveal the presence of a certain level of linezolid resistance in environmental cryospheric samples.

Gentamicin and vancomycin also showed relatively high effectiveness in inhibiting the growth of isolated bacteria. Nevertheless, resistance to either of these antibiotics was prevalent in some bacterial strains in most of the sampling sites. ARGs that are widespread in environmental microbial communities, could be responsible for the observed gentamicin (Heuer et al., 2002) and vancomycin resistance (Guardabassi and Agersø, 2006).

Trimethoprim, ampicillin, and novobiocin demonstrated lower effectiveness, indicating a higher prevalence of resistance mechanisms against these drugs within the tested bacterial strains. The number of resistant isolates against broad-spectrum antibiotics like chloramphenicol was similar to other natural habitats like the Arctic (Mogrovejo et al., 2020) and Antarctic (Tam et al., 2015).

Resistances against all antibiotics were found independent of their nature (natural, semisynthetic, synthetic). Particularly, reduced susceptibility to synthetic antibiotics supports the assumption that AR in the environment is, to some extent, acquired and influenced by humans.

4.3. Antibiotic resistance and the anthropogenic influence

The results of our study offer compelling evidence of the substantial impact of the anthropogenic influence on AR in the examined environments. We observed a significant increase in AR and MDR bacteria when human impact was high. Similar results have been reported by other studies looking into the effect of human impact on sediment (Bhattacharyya et al., 2019), rivers (Guan et al., 2022), tropical (Pontes et al., 2009), and polar regions (Tan et al., 2018).

The long-established role of anthropogenic (mis-)use of antibacterial medication as a major driver for the development of AR in clinical settings (Austin et al., 1999; Kolár et al., 2001) is complemented by the drastic increase of the selective pressure on bacteria in natural environments due to mass production of antibiotics (Larsson, 2014; Tello et al., 2012; Xiong et al., 2015). Moreover, the enrichment and persistence of antimicrobial resistance can occur even at low antibiotic

concentrations (Gullberg et al., 2011) frequently encountered in natural environments.

A variety of mechanisms (e.g. horizontal gene transfer) can lead to the spread of ARGs and subsequent AR in the environment (Skandalis et al., 2021) potentially even leading to the transfer of resistances back to previously susceptible pathogenic bacteria in clinical settings (Maeusli et al., 2020; Wright, 2010).

The comparisons of similar environmental samples with varying levels of anthropogenic influence showed a substantial difference in the prevalence of AR bacteria.

Here, human impact can be exemplified by sampling sites like the NIP, where individuals often come into direct skin contact with snow and ice surfaces, and therefore potentially introducing and spreading (AR) microorganisms.

Similarly, the high number of frequent visitors of ski resorts at Stubai Glacier or Seegrube may also contribute to the dispersion of AR bacteria.

Furthermore, the swimming lakes WSL and BAR are both examples of water sampling sites with extensive direct human contact and therefore an increased likelihood of the spread of potential AR bacteria into freshwater ecosystems.

Highly-impacted cryospheric sampling sites like 'Gas Station Ellmau', had a significantly higher proportion of AR bacteria when compared to regions with low anthropogenic influence ($p < 0.001$).

This trend also remained consistent when focusing on glacier habitats. The uniqueness of Tux Glacier, being an ice cave open to the public with limited ventilation, led to a notably higher number of AR isolates compared to other glacier samples. Among the antibiotics tested, only Linezolid (<10 % resistant bacteria) and Gentamicin (<30 % resistant bacteria) proved to be effective antibiotics at this sampling site. Additionally, antibiotic resistance rates were among the highest across all sampling sites with 95 % of the isolates tested being MDR and > 80 % of the isolated bacteria being resistant to at least one of the other six antibiotics. Surprisingly, vancomycin was ineffective in 83.33 % of the tests which is comparably higher than reports from other glaciers (Segawa et al., 2013) and even hospital effluents (Nuñez et al., 2016).

In comparison, to the medium (Stubai) and highly influenced (Tux) glaciers, Jamtal Glacier lacks skiing infrastructure and is consequently less exposed to human activities. As a result, a significantly lower number of antibiotic-resistant microbes and MDR strains have been observed, suggesting that direct human presence is an important factor in the dissemination of AR in cryospheric habitats. This assumption is also supported by the susceptibility of respective bacterial isolates to the clinically relevant last-line-of-defense antibiotics linezolid and vancomycin.

Results of bacterial isolates sampled at glaciers in the Tyrolean Alpine space emphasize the significance of the anthropogenic influence on cold habitats which aligns with results from human-inhabited areas in Antarctica (Miller et al., 2009).

However, direct human impact represents just one facet among various factors influencing AR in cold habitats. The transport of ARGs through biological and physical mechanisms (Allen et al., 2010) presents a plausible explanation for the presence of AR bacteria in relatively remote regions, as observed in this study. Additionally, intrinsic resistance, arising from non-specific cellular processes not originally designed to protect bacteria from antibacterial substances (Tamae et al., 2008), possibly explains some of the identified resistances.

Freshwater samples showed a similar overall trend as snow and ice samples. Higher levels of anthropogenic influence corresponded to more MDR isolates and significantly higher numbers of overall AR. BAR and WSL, two popular swimming lakes in Tyrol largely surrounded by agricultural and urbanized areas and with substantial direct human contact, serve as examples of highly influenced sampling sites. Compared to regions with lower anthropogenic influence, such as the high alpine lakes GKS and KAI, the swimming lakes, BAR, and WSL, hosted a significantly higher number of culturable MDR bacteria. Notably, the two MDR opportunistic pathogens, *Escherichia coli* and *Enterobacter cloacae, were* isolated from these swimming lakes that have medium and high direct human impact. This underscores the intricate relationship between anthropogenic activities and the emergence of antibiotic resistance in environmental settings. The observed resistance of the *E. coli* strain against three different antibiotics highlights its capacity to accumulate ARGs despite being intrinsically susceptible to most antibiotics (Poirel et al., 2018). *E. cloacae* on the other hand is intrinsically resistant to Ampicillin (Davin-Regli and Pagès, 2015). Thus, our results support the concerns that it could potentially become an emerging global threat due to the rise in MDR strains (Annavajhala et al., 2019).

While the trend of increased numbers of AR and MDR at sampling sites with higher human influence was evident for lake samples, no significant difference could be observed in river ecosystems. However, it is essential to note that none of the studied rivers was categorized as strongly influenced by humans (based on urbanized and agricultural areas in their disposal areas).

5. Conclusion

In this study, we investigated AR in environmental cryospheric and freshwater samples and explored the correlation between resistance and direct human presence. Our findings shed new light on the significant impact of anthropogenic influence on AR in these habitats.

The Kirby-Bauer test revealed a concerning prevalence of culturable resistant bacterial isolates from various cryospheric environments. >50 % of MDR microorganisms indicate limited effectiveness of the tested antibiotics against the majority of environmental bacterial strains. Even so-called reserve antibiotics like linezolid were ineffective against some isolates (15.66 %) which supports the concerns about the decreased susceptibility against oxazolidinones.

Antibiotics, as key players, are essential for modern medicine, and their efficacy is threatened by the increasing prevalence of AR not only in clinical settings but also in environmental samples.

Furthermore, the results from our study emphasize the significance of the anthropogenic influence on AR. A trend towards reduced antibiotic susceptibility with increased human activity was observed even in cold habitats. This correlation was evident in glacier samples as well as seasonal snow and lake habitats.

Climate change-induced glacier melting possibly creates a pathway for AR bacterial strains to come in contact with urbanized areas and human activities. Transferring associated ARGs to pathogenic phenotypes through known mechanisms like horizontal gene transfer (Maeusli et al., 2020; Rhodes et al., 2000; Wright, 2010), could further intensify the rising problem of AR.

Therefore, our study highlights the urgent need to comprehensively monitor the spread of AR in natural and extreme environments like the cryosphere. Future research could further investigate non-culturable bacteria with a metagenomic approach to gain an even better understanding of AR in cryospheric habitats and the impact of anthropogenic influence on it.

Overall, our study contributes valuable insights into the complex dynamics of AR in environmental samples in the Tyrolean Alpine space and underlines the critical role of the human impact in shaping resistance patterns. Gaining insight into these factors and comprehending how resistant microorganisms spread in these habitats is crucial for understanding the extent of this global phenomenon.

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CRediT authorship contribution statement

Daniel Gattinger: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition. **Valentin Schlenz:** Data curation, Investigation, Methodology, Writing – review & editing. **Tobias Weil:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Birgit Sattler:** Conceptualization, Data curation, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Daniel Gattinger reports financial support was provided by University of Innsbruck. Birgit Sattler reports financial support was provided by Tawani Foundation. Tobias Weil reports financial support was provided by Autonomous Province of Trento. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary Material 1

From remote to urbanized: Dispersal of antibiotic-resistant bacteria under the aspect of anthropogenic influence

Daniel Gattinger, Valentin Schlenz, Tobias Weil, Birgit Sattler

Supplementary 1 Table 1 – Calculations of the average breakpoint based on the EUCAST breakpoint table for antibiotic susceptibility tests (eucast.org 2023).

Phyla	Antibiotic Family	Antibiotic	Disk content [µg]	S ≥ [mm]	R < [mm]
Aerococcus sanguinicola/urniae	Beta-lactam	Ampicillin	2	26	26
Enterococcus spp.	Beta-lactam	Ampicillin	2	10	8
Haemophilus influenzae	Beta-lactam	Ampicillin	2	16	16
Listeria monocytogenes	Beta-lactam	Ampicillin	2	16	16
Viridans group streptococci	Beta-lactam	Ampicillin	2	21	15
Calculated mean value				**17.80**	**16.20**
Enterobacteriaceae		Chloramphenicol	30	17	17
Haemophilus influenzae		Chloramphenicol	30	28	28
Moraxella catarrhalis		Chloramphenicol	30	30	30
Staphylococcus spp.		Chloramphenicol	30	18	18
Streptococcus groups A, B, C, D		Chloramphenicol	30	19	19
Streptococcus pneumoniae		Chloramphenicol	30	21	21
Calculated mean value				**22.17**	**22.17**
Acinetobacter spp.	Aminoglycosides	Gentamicin	10	17	17
Coagulase negative Staphylococcus	Aminoglycosides	Gentamicin	10	22	22
Corynebacterium spp.	Aminoglycosides	Gentamicin	10	23	23
Enterobacteriaceae	Aminoglycosides	Gentamicin	10	17	14
Pseudomonas spp.	Aminoglycosides	Gentamicin	10	18	18
Staphylococcus aureus	Aminoglycosides	Gentamicin	10	15	15
Calculated mean value				**18.67**	**18,17**
Corynebacterium spp.	Oxazolidinones	Linezolid	10	25	25
Streptococcus A, B, C and G	Oxazolidinones	Linezolid	10	19	16
Calculated mean value				**22.00**	**20.50**
Aerococcus sanguinicola/urniae	Nitrofuran	Nitrofurantoin	100	16	16
Enterobacteriaceae	Nitrofuran	Nitrofurantoin	100	11	11
Enterococcus spp.	Nitrofuran	Nitrofurantoin	100	15	15
Staphylococcus spp.	Nitrofuran	Nitrofurantoin	100	13	13
Streptococcus groups A, B, C, D	Nitrofuran	Nitrofurantoin	100	15	15
Calculated mean value				**14**	**14**
Enterobacteriaceae	Diaminopyrimidine	Trimethoprim	5	18	15
Enterococcus spp.	Diaminopyrimidine	Trimethoprim	5	50	21
Staphylococcus spp.	Diaminopyrimidine	Trimethoprim	5	17	14

Calculated mean value				**28.33**	**16.67**
Enterococcus spp.	Glycopeptides	Vancomycin	5	12	12
Streptococcus groups A, B, C, D	Glycopeptides	Vancomycin	5	13	13
Streptococcus pneumoniae	Glycopeptides	Vancomycin	5	16	16
Viridans group streptococci	Glycopeptides	Vancomycin	5	15	15
Corynebacterium spp.	Glycopeptides	Vancomycin	5	17	17
Aerococcus sanguinicola/urniae	Glycopeptides	Vancomycin	5	16	16
Calculated mean value				**14,83**	**14,83**
-	Aminocumarines	Novobiocin	5	**16***	**16***

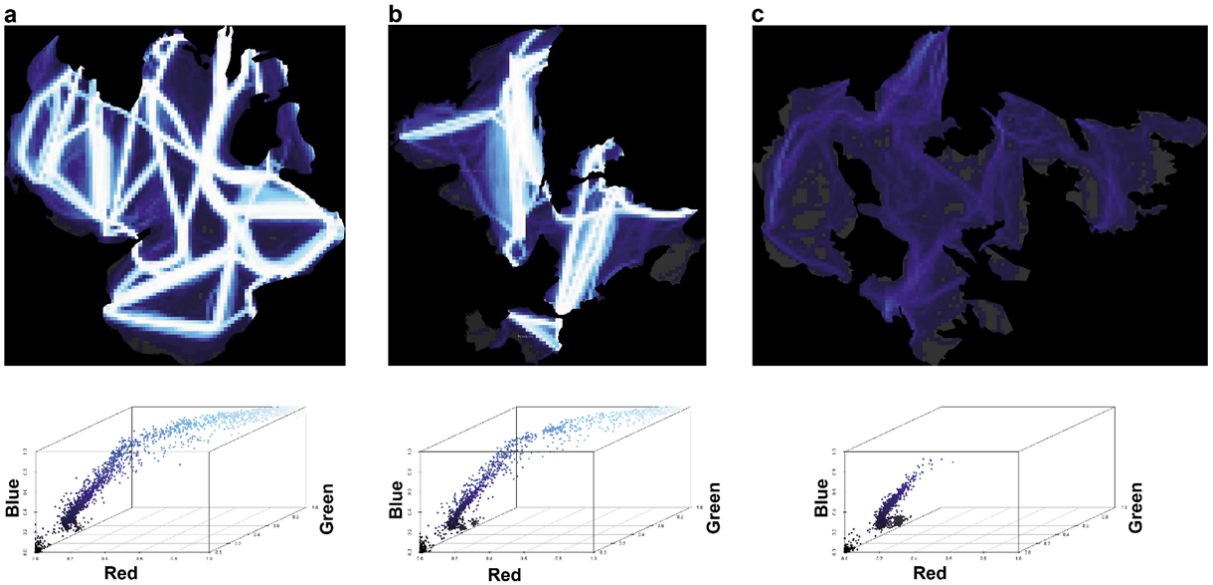
Supplementary 1 Table 2 – Overview of the sampling sites within this study

ID	Sample Site	Type	Additional Information
Mellach	Mellach	River	
GKS	Gossenkoellesee	Lake	Alpine lake
Zirmbach	Zirmbach	River	Alpine lake
KAI	KAI	Lake	
KAI Snow	KAI	Snow	
Forellenhof	Fishpond	Lake	Fishing pond
NIP 1	Tux Glacier	Glacier - Ice	Touristic ice cave
NIP 2	Tux Glacier	Glacier - Ice	Touristic ice cave
NIP 3	Tux Glacier	Glacier - Ice	Touristic ice cave
NIP 4	Tux Glacier	Glacier - Ice	Touristic ice cave
NIP 5	Tux Glacier	Glacier - Ice	Touristic ice cave
Hafelekar 1	Hafelekar	Snow	Mountain top
5	Seegrube	Snow	Skiing area
7	Seegrube	Snow	Skiing area
8	Seegrube	Snow	Skiing area
9	Hungerburg	Snow	Snow-clearance
10	Hungerburg	Snow	Snow-clearance
11	Hungerburg	Snow	Snow-clearance

12	Hungerburg	Snow	Snow-clearance
Windachferner	Stubai Glacier	Glacier - Snow	Skiing area
Windachferner	Stubai Glacier	Glacier - Snow	Skiing area
Daunkogelferner	Stubai Glacier	Glacier - Snow	Skiing area
Hafelekar 2	Hafelekar	Snow	Fresh snow
Jam1	Jambach	River	Glacier fed stream
Jam2	Jambach	River	Glacier fed stream
Jam3	Jambach	River	Glacier fed stream
Tri1	Trisanna	River	
Sanna1	Sanna	River	
Inn1	Inn	River	Near highway in Hatting
Jam Ice	Jamtalferner	Glacier - Ice	Most remote sampling site
Jam Snow	Jamtalferner	Glacier - Snow	Most remote sampling site
Jam bright	Jamtalferner	Glacier - Snow	Most remote sampling site
Jam dark	Jamtalferner	Glacier - Snow	Most remote sampling site
Jam Cryo1	Jamtalferner	Glacier - Cryoconite	Most remote sampling site
Jam Cryo2	Jamtalferner	Glacier - Cryoconite	Most remote sampling site
Stubai Snow 1	Stubaier Gletscher	Glacier - Snow	Skiing area
Stubai Snow 2	Stubaier Gletscher	Glacier - Snow	Skiing area
Stubai Snow 3	Stubaier Gletscher	Glacier - Snow	Skiing area
Stubai Water	Stubaier Gletscher	Glacier - Water	Skiing area
Stubai Ice 1	Stubaier Gletscher	Glacier - Ice	Ice Core (superficial layer)
Stubai Ice 2	Stubaier Gletscher	Glacier - Ice	Ice Core (2nd layer)
Stubai Ice 3	Stubaier Gletscher	Glacier - Ice	Ice Core (3rd layer)
Stubai Ice 4	Stubaier Gletscher	Glacier - Ice	Ice Core (innermost layer)
Tux Glacier M1	Tux Glacier	Glacier - Snow	Skiing area
Tux Glacier M2	Tux Glacier	Glacier - Snow	Skiing area

Tux Glacier M3	Tux Glacier	Glacier - Snow	Skiing area
Ellmau	Ellmau	Snow	
BGS	Berglsteinersee	Snow	
Falzturnalm	Falzturn	Snow	
Largoz	Largoz	Snow	
Inn2	Inn Zirl	River	Zirl, after sewage treatment plant
Inn3	Inn Mötz	River	Mötz
Inn4	Inn Rossau	River	Rossau, next to BAR
Inn5	Inn Fritzens	River	Fritzens, after sewage treatment plant
WSL	Weisslahn Lake	Lake	Swimming Lake
BAR	Baggersee	Lake	Swimming Lake

Supplementary 1 Figure 1 – Display of the Strava activity heatmaps on glacier areas on top and the color space at the bottom to see the color distribution of the maps. On a relative scale, Strava uses



brighter colors for higher human activity. Colors were split up into three color ranges (corresponding to three levels of human activity: low, medium, and high) and the pixel coverage of the glacier area was calculated for the glacier surface of the Tux Glacier (a), Stubai Glacier (b), and Jamtal Glacier (c).

Supplementary Material 2

From remote to urbanized: Dispersal of antibiotic-resistant bacteria under the aspect of anthropogenic influence

Daniel Gattinger, Valentin Schlenz, Tobias Weil, Birgit Sattler

Supplementary 2 Table 1 – Overview of all cultivated isolates tested for antibiotic resistance including BLAST results from the 16S sequencing data. ID = Sample ID, AMP = Ampicillin, C = Chloramphenicol, CN = Gentamicin, LZD = Linezolid, F = Nitrofurantoin, NV = Novobiocin, W = Trimethoprim, VA = Vancomycin, Temp = Initial incubation temperature and temperature to carry out the agar disk diffusion test. R = Resistant, S = Susceptible

ID	AMP	C	CN	LZD	F	NV	W	VA	Family	Genus	Species	Gram	Temp	Site	Type
1	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Melach	Water
2	R	R	S	R	R	R	R	R	*Bacillaceae*	*Bacillus*	*Bacillus subtilis EFB7*	positive	20°C	Melach	Water
4	S	S	S	NA	S	S	S	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fragi*	negative	20°C	Melach	Water
5	S	S	S	S	R	R	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis EFB7*	positive	4°C	GKS	Water
6	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis EFB7*	positive	20°C	GKS	Water
7	S	S	S	NA	S	S	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas reinekei*	negative	20°C	Zirmbach	Water
8	S	S	S	S	R	S	S	S	*Micrococcaceae*	*Paenarthrobacter*	*Paenarthrobacter ilicis*	positive	20°C	Zirmbach	Water
9	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis EFB7*	positive	20°C	Zirmbach	Water
10	S	S	S	R	R	R	S	S	*Micrococcaceae*	*Agreia*	*Agreia sp.*	positive	20°C	Zirmbach	Water
11	R	R	S	NA	R	R	R	NA			no blast result	negative	20°C	Zirmbach	Water
12	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus tequilensis*	positive	20°C	KAI	Water
14	S	S	S	S	R	S	R	S	*Microbacteriaceae*	*Plantibacter*	*Plantibacter flavus*	positive	20°C	KAI	Water
15	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	KAI	Water
17	R	S	S	NA	S	R	S	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium sp.*	negative	20°C	KAI	Snow
18	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	KAI	Snow
19	S	S	S	NA	S	S	S	NA	*Comamonadaceae*	*Acidovorax*	*Acidovorax facilis*	negative	20°C	KAI	Snow
20	R	R	S	NA	R	R	R	NA	*Paracoccaceae*	*Paracoccus*	*Paracoccus yeei*	negative	20°C	KAI	Snow
21	R	S	R	NA	R	R	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp.*	negative	20°C	Fishpond	Water
22	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus sp.*	positive	20°C	Fishpond	Water
23	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Fishpond	Water
24	R	R	S	R	R	R	S	R	*Bacillaceae*	*Lysinibacillus*	*Lysinibacillus sp.*	positive	4°C	KAI	Snow
25	R	S	R	NA	S	S	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas brenneri*	negative	4°C	KAI	Snow
26	S	S	S	NA	R	R	S	NA			no blast result	negative	4°C	Zirmbach	Water
27	R	R	R	NA	S	R	R	NA			83 % identity	negative	4°C	Zirmbach	Water
28	R	S	R	NA	S	S	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp.*	negative	4°C	Zirmbach	Water
30	R	S	R	NA	S	S	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp.*	negative	4°C	Zirmbach	Water
31	R	S	S	NA	S	S	R	NA			95.94% identity	negative	4°C	Zirmbach	Water

32	**11a6**	S	S	S	S	S	R	R	R	*Micrococcaceae*	*Paeniglutamicibacter*	*Paeniglutamicibacter sp.*	positive	4°C	Hungerburg	Snow
35	**11a7**	R	S	S	NA	S	S	R	NA			94% identity	negative	4°C	Hungerburg	Snow
36	**11a8**	R	S	S	S	S	S	S	S			no blast result	positive	4°C	Hungerburg	Snow
37	**11a9**	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas putida*	negative	4°C	Hungerburg	Snow
38	**11b1**	S	S	S	NA	R	R	R	NA			96% identity	negative	20°C	Hungerburg	Snow
	11b2	R	S	S	NA	S	S	R	NA			no blast result	negative	20°C	Hungerburg	Snow
10a	**11b3**	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Hungerburg	Snow
10a1	**11b4**	R	S	S	S	S	S	R	S			no blast result	positive	20°C	Hungerburg	Snow
10a1	**11b6**	R	S	S	NA	S	S	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*	negative	20°C	Hungerburg	Snow
10a1	**11b7**	R	S	S	S	S	S	R	S	*Bacillaceae*	*Bacillus*	*Bacillus sp.*	positive	20°C	Hungerburg	Snow
10a	**12a1**	S	S	S	NA	S	S	R	NA	*Moraxellaceae*	*Psychrobacter*	*Psychrobacter glaciei*	negative	4°C	Hungerburg	Snow
10a	**12a10**	R	S	S	S	S	S	S	S	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter alpinus*	positive	4°C	Hungerburg	Snow
10a	**12a11**	R	R	S	NA	R	R	R	NA			no blast result	negative	4°C	Hungerburg	Snow
10a	**12a7**	R	S	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas weihenstephanensis*	negative	4°C	Hungerburg	Snow
10a	**12a8**	S	S	S	S	S	S	S	S			93% identity	positive	4°C	Hungerburg	Snow
10a	**12a9**	R	S	S	R	S	S	R	S	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter alpinus*	positive	4°C	Hungerburg	Snow
10a	**12b1**	R	S	S	S	S	S	R	R	*Bacillaceae*	*Bacillus*	*Bacillus sp.*	positive	20°C	Hungerburg	Snow
10a	**12b2**	R	S	S	S	S	S	R	S	*Bacillaceae*	*Bacillus*	*Bacillus sp.*	positive	20°C	Hungerburg	Snow
10a	**12b3**	S	R	S	S	S	S	S	S	*Microbacteriaceae*	*Microbacterium*	*Microbacterium oxydans*	positive	20°C	Hungerburg	Snow
10b	**12b4**	R	S	S	S	S	S	S	S			96,47% identity	positive	20°C	Hungerburg	Snow
10b	**12b5**	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus pumilus*	positive	20°C	Hungerburg	Snow
11a	**12b6**	R	S	S	S	S	S	R	S			*no blast result*	positive	20°C	Hungerburg	Snow
11a1	**12b7**	R	S	S	S	S	S	R	S			*no blast result*	positive	20°C	Hungerburg	Snow
11a1	**12b8**	S	S	S	46	S	S	S	25			*no blast result*	positive	20°C	Hungerburg	Snow
11a1	**12b9**	R	S	S	S	S	S	R	S	*Bacillaceae*	*Bacillus*	*Bacillus sp.*	positive	20°C	Hungerburg	Snow
11a	**13a1**	R	S	S	NA	S	R	R	NA			no blast result	negative	4°C	Stubai Glacier	Snow
11a	**1a1**	S	S	S	50	S	S	S	S	*Microbacteriaceae*	*Subtercola*	*Agreia sp*	positive	4°C	Hafelekar	Snow
11a	**5a1**	S	S	S	S	S	S	R	S	*Micrococcaceae*	*Micrococcus*	*Micrococcus yunnanensis*	positive	20°C	Seegrube	Snow
11a	**5a2**	S	S	S	S	R	S	R	S	*Micrococcaceae*	*Micrococcus*	*Micrococcus yunnanensis*	positive	20°C	Seegrube	Snow

5a3	S	S	S	S	S	S	R	S			no blast result	positive	20°C	Seegrube	Snow
5a4	S	S	S	S	S	S	R	S	*Micrococcaceae*	*Micrococcus*	*Micrococcus yunnanensis*	positive	20°C	Seegrube	Snow
5a5	S	S	S	S	S	S	R	S	*Micrococcaceae*	*Micrococcus*	*Micrococcus luteus*	positive	20°C	Seegrube	Snow
7a2	R	R	R	NA	R	R	R	NA			96% identity	negative	4°C	Seegrube	Snow
7a6	R	R	R	R	R	R	R	R			no blast result	positive	4°C	Seegrube	Snow
7a7	R	R	S	R	R	R	R	R			no blast result	positive	4°C	Seegrube	Snow
8b2	R	R	R	NA	R	R	R	NA	*Burkholderiaceae*	*Cupriavidus*	*Cupriavidus sp*	negative	4°C	Seegrube	Snow
8b3	R	R	R	NA	R	R	R	NA	*Comamonadaceae*	*Acidovorax*	*Acidovorax sp.*	negative	4°C	Seegrube	Snow
9a1	S	S	S	S	S	S	S	S			no blast result	positive	20°C	Hungerburg	Snow
9a2	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus xiamenensis*	positive	20°C	Hungerburg	Snow
9a3	R	S	S	S	S	S	R	S	*Bacillaceae*	*Bacillus*	*Bacillus sp.*	positive	20°C	Hungerburg	Snow
9a4	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus pumilus*	positive	20°C	Hungerburg	Snow
9a6	S	S	S	S	S	S	S	S	*Bacillaceae*	*Lysinibacillus*	*Lysinibacillus sp.*	positive	20°C	Hungerburg	Snow
9a7	S	S	S	S	S	S	S	S			no blast result	positive	20°C	Hungerburg	Snow
9b1	S	S	S	S	R	S	S	S	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter alpinus*	positive	4°C	Hungerburg	Snow
9b10	R	S	S	NA	R	R	R	NA	*Moraxellaceae*	*Psychrobacter*	*Psychrobacter sp.*	negative	4°C	Hungerburg	Snow
9b11	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	4°C	Hungerburg	Snow
9b12	S	S	S	NA	S	R	R	NA	*Moraxellaceae*	*Psychrobacter*	*Psychrobacter alimentarius*	negative	4°C	Hungerburg	Snow
9b13	S	S	S	S	S	S	S	S	*Micrococcaceae*	*Glutamicibacter*	*Glutamicibacter sp.*	positive	4°C	Hungerburg	Snow
9b2	S	S	S	NA	S	R	R	NA	*Moraxellaceae*	*Psychrobacter*	*Psychrobacter cibarius*	negative	4°C	Hungerburg	Snow
9b3	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas brenneri*	negative	4°C	Hungerburg	Snow
9b4	S	S	S	NA	R	S	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas brenneri*	negative	4°C	Hungerburg	Snow
9b5	S	S	S	S	R	S	S	S	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter alpinus*	positive	4°C	Hungerburg	Snow
9b6	S	S	S	S	S	S	S	S	*Micrococcaceae*	*Paeniglutamicibacter*	*Paeniglutamicibacter sp.*	positive	4°C	Hungerburg	Snow
9b7	S	R	R	NA	R	R	R	NA	*Xanthomonadaceae*	*Stenotrophomonas*	*Stenotrophomonas rhizophila*	negative	4°C	Hungerburg	Snow
9b8	S	S	S	NA	R	S	R	NA	*Moraxellaceae*	*Psychrobacter*	*Psychrobacter sp.*	negative	4°C	Hungerburg	Snow
9b9	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas brenneri*	negative	4°C	Hungerburg	Snow

Berglsteiner See 2	R	S	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*	negative	20°C	BGS	Snow
Berglsteiner See 3	R	S	R	NA	R	S	S	NA	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas sp.*	negative	20°C	BGS	Snow
Berglsteiner See 4	R	S	S	NA	R	R	R	NA	*Comamonadaceae*	*Variovorax*	*Variovorax sp.*	negative	20°C	BGS	Snow
BP 1	R	R	S	NA	R	R	R	NA			no blast result	negative	20°C	Gas Station Ellmau	Snow
BP 2	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Gas Station Ellmau	Snow
BP 3	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Gas Station Ellmau	Snow
BP 4	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	4°C	Gas Station Ellmau	Snow
BS 1	R	R	R	NA	S	R	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium johnsoniae*	negative	20°C	BAR	Water
BS 2	R	S	S	NA	R	R	S	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	BAR	Water
BS 3	S	S	S	NA	R	R	R	NA	*Sphaerotilaceae*	*Roseateles*	*Roseateles terrae*	negative	20°C	BAR	Water
BS 4	R	S	R	NA	R	R	R	NA			no blast result	negative	20°C	BAR	Water
BS 5	S	S	S	NA	S	R	S	NA	*Pectobacteriaceae*	*Pectobacterium*	*Pectobacterium carotovorum*	negative	20°C	BAR	Water
BS 6	R	S	S	NA	S	R	S	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	BAR	Water
BS 8	R	S	S	NA	S	R	R	NA	*Enterobacteriaceae*	*Escherichia*	*Eschericha coli*	negative	20°C	BAR	Water
C. nivalis 1	S	S	R	S	S	S	S	S	*Enterococcaceae*	*Enterococcus*	*Enterococcus casseliflavus*	positive	20°C	Tux Glacier	
C. nivalis 2	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Tux Glacier	
C. nivalis 3	S	S	R	S	S	S	S	S			no blast result	positive	20°C	Tux Glacier	
C. nivalis 4	S	S	S	NA	S	S	S	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp.*	negative	20°C	Tux Glacier	
C. nivalis 5	R	S	R	S	S	S	R	S			no blast result	positive	20°C	Tux Glacier	
C. nivalis 6	R	R	S	R	R	R	R	R			80% identity	positive	20°C	Tux Glacier	
Falzturn 1	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Falzturn	Snow
Falzturn 2	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Falzturn	Snow
Falzturn 3	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Falzturn	Snow
Falzturn 4	R	S	R	NA	R	R	S	NA	*Sphingobacteriaceae*	*Pedobacter*	*Pedobacter sp.*	negative	20°C	Falzturn	Snow
Falzturn 5	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Falzturn	Snow
Falzturn 6	S	S	S	NA	R	R	S	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium lividum*	negative	4°C	Falzturn	Snow

Falzturn 7	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	4°C	Falzturn	Snow
HT1	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas massiliensis*	negative	20°C	Tux Glacier	Ice
HT10	R	S	S	NA	R	R	S	NA	*Yersiniaceae*	*Yersinia*	*Yersinia intermedia*	negative	20°C	Tux Glacier	Ice
HT11	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*	negative	20°C	Tux Glacier	Ice
HT12	R	R	S	R	R	R	R	R	*Bacillaceae*	*Bacillus*	*Bacillus altitudinis*	positive	20°C	Tux Glacier	Ice
HT13	R	R	S	NA	R	R	R	NA	*Oxalobacteraceae*	*Massilia*	*Massilia sp.*	negative	20°C	Tux Glacier	Ice
HT14	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas deceptionensis*	negative	20°C	Tux Glacier	Ice
HT15	R	R	R	NA	R	R	R	NA	*Yersiniaceae*	*Yersinia*	*Yersinia intermedia*	negative	20°C	Tux Glacier	Ice
HT16	R	R	R	R	R	R	R	R	*Bacillaceae*	*Bacillus*	*Bacillus pumilus*	positive	20°C	Tux Glacier	Ice
HT17	R	S	S	NA	R	R	S	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium lividum*	negative	4°C	Tux Glacier	Ice
HT18	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	4°C	Tux Glacier	Ice
HT19	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas brenneri*	negative	4°C	Tux Glacier	Ice
HT2	S	S	S	NA	S	S	S	NA	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas sp.*	negative	20°C	Tux Glacier	Ice
HT20	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas yamanorum*	negative	4°C	Tux Glacier	Ice
HT21	R	R	S	NA	R	R	R	NA			no blast result	negative	4°C	Tux Glacier	Ice
HT3	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fragi*	negative	20°C	Tux Glacier	Ice
HT4	R	R	R	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas asplenii*	negative	20°C	Tux Glacier	Ice
HT5	R	R	S	R	R	R	R	R			*no blast result*	positive	20°C	Tux Glacier	Ice
HT6	R	S	R	S	S	R	R	R			no blast result	positive	20°C	Tux Glacier	Ice
HT7	R	R	R	S	R	R	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Tux Glacier	Ice
HT8	R	R	S	R	R	R	R	R	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus epidermidis*	positive	20°C	Tux Glacier	Ice
HT9	R	R	R	NA	R	R	R	NA	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas sp.*	negative	20°C	Tux Glacier	Ice
Inn 1	R	R	S	R	R	R	R	R			no blast result	positive	20°C	Inn	Water
Inn 10	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Inn	Water
Inn 11	R	S	S	NA	S	R	S	NA	*Aeromonadaceae*	*Aeromonas*	*Aeromonas salmonicida*	negative	20°C	Inn	Water
Inn 12	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Inn	Water
Inn 13	S	S	S	NA	R	R	S	NA			no blast result	negative	20°C	Inn	Water

Inn 14	S	S	S	S	R	S	R	S			*NA*	positive	20°C	Inn	Water
Inn 15	R	S	R	NA	R	S	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium johnsoniae*	negative	20°C	Inn	Water
Inn 16	R	S	S	S	S	S	S	R			96% identity	positive	20°C	Inn	Water
Inn 18	S	S	S	S	S	S	S	S	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter oryzae*	positive	20°C	Inn	Water
Inn 19	S	S	S	S	R	S	S	S	*Micrococcaceae*	*Arthrobacter*	*Arthrobacter sp.*	positive	20°C	Inn	Water
Inn 2	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Inn	Water
Inn 20	S	S	S	NA	S	S	S	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp.*	negative	20°C	Inn	Water
Inn 21	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*	negative	20°C	Inn	Water
Inn 22	S	S	S	S	S	S	S	S			no blast result	positive	20°C	Inn	Water
Inn 23	R	S	S	NA	S	S	R	NA	*Actinomycetes*	*Actinomycetes*	*Actinobacterium*	positive	20°C	Inn	Water
Inn 24	R	R	S	NA	R	R	R	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium lividum*	negative	20°C	Inn	Water
Inn 25	S	S	S	NA	R	R	R	NA	*Sphingobacteriaceae*	*Mucilaginibacter*	*Mucilaginibacter sp.*	negative	20°C	Inn	Water
Inn 27	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Inn	Water
Inn 28	S	S	S	NA	R	R	R	NA	*Sphingomonadaceae*	*Sphingopyxis*	*Sphingopyxis sp.*	negative	20°C	Inn	Water
Inn 29	R	S	S	NA	S	R	R	NA	*Aeromonadaceae*	*Aeromonas*	*Aeromonas salmonicida*	negative	20°C	Inn	Water
Inn 3	S	S	S	NA	S	S	S	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Inn	Water
Inn 31	S	S	S	NA	S	S	S	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp*	negative	20°C	Inn	Water
Inn 32	R	R	S	NA	R	R	R	NA	*Xanthomonadaceae*	*Stenotrophomonas*	*Stenotrophomonas rhizophila*	negative	20°C	Inn	Water
Inn 33	S	S	S	NA	R	S	S	NA	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas sp.*	negative	20°C	Inn	Water
Inn 34	R	S	R	NA	R	S	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp.*	negative	20°C	Inn	Water
Inn 35	R	S	S	NA	R	R	S	NA	*Oxalobacteraceae*	*Massilia*	*Massilia aurea*	negative	20°C	Inn	Water
Inn 36	R	S	R	NA	R	R	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp.*	negative	20°C	Inn	Water
Inn 37	R	S	R	NA	S	S	R	NA			*no blast result*	negative	20°C	Inn	Water
Inn 38	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas azotoformans*	negative	20°C	Inn	Water
Inn 39	R	S	R	NA	R	R	S	NA			no blast result	negative	20°C	Inn	Water
Inn 41	R	S	S	NA	S	R	S	NA	*Budviciaceae*	*Budvicia*	*Budvicia aquatica*	negative	20°C	Inn	Water
Inn 5	R	S	S	NA	S	R	S	NA	*Erwiniaceae*	*Erwinia*	*Erwinia rhapontici*	negative	20°C	Inn	Water
Inn 6	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Inn	Water
Inn 9	R	S	S	NA	S	S	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium piscis*	negative	20°C	Inn	Water

Jam Ice 1	S	R	S	S	S	R	S	S	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	positive	20°C	Jamtal Glacier	Ice
Jam Ice 2	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas frederiksbergensis*	negative	20°C	Jamtal Glacier	Ice
Jam Ice 3	S	S	S	NA	S	R	S	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Jamtal Glacier	Ice
Jam Ice 4	R	S	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas frederiksbergensis*	negative	20°C	Jamtal Glacier	Ice
Jam Ice 5	S	S	S	NA	R	R	S	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium sp.*	negative	4°C	Jamtal Glacier	Ice
Jam Ice 6	R	S	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas frederiksbergensis*	negative	4°C	Jamtal Glacier	Ice
Jam Ice 8	S	S	S	NA	S	S	S	NA			no blast result	negative	4°C	Jamtal Glacier	Ice
Jam K2	R	S	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Jamtal Glacier	Cryoconite
Jam K3	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas frederiksbergensis*	negative	20°C	Jamtal Glacier	Cryoconite
Jam K4	R	S	S	NA	R	R	S	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium sp.*	negative	20°C	Jamtal Glacier	Cryoconite
Jam Snow 1	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Jamtal Glacier	Ice
Jam Snow 2	S	S	S	NA	S	S	S	NA	*Burkholderiales*	*Paucibacter*	*Paucibacter toxinivorans*	negative	20°C	Jamtal Glacier	Ice
Jam Snow 3	S	R	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus stratosphericus*	positive	20°C	Jamtal Glacier	Ice
Jam Snow 4	R	R	R	NA	R	R	R	NA	*Oxalobacteraceae*	*Massilia*	*Massilia sp.*	negative	4°C	Jamtal Glacier	Snow
Jam Snow 7	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Jamtal Glacier	Snow
Jam Snow 8	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Jamtal Glacier	Snow
Jam Snow 9	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas kairouanensis*	negative	20°C	Jamtal Glacier	Snow
Jam1	S	S	S	NA	S	S	S	NA			84 % identity	negative	20°C	Jambach	Water
Jam10	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas extremaustralis*	negative	20°C	Jambach	Water
Jam11	R	S	S	NA	R	R	R	NA			no blast result	negative	20°C	Jambach	Water

Jam12	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Jambach	Water
Jam13	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Jambach	Water
Jam14	S	S	S	NA	R	R	S	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium sp.*	negative	20°C	Jambach	Water
Jam2	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Jambach	Water
Jam3	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas mandelii*	negative	20°C	Jambach	Water
Jam4	S	S	S	NA	S	S	S	NA			no blast result	negative	20°C	Jambach	Water
Jam5	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas psychrophilia*	negative	20°C	Jambach	Water
Jam6	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas orientalis*	negative	20°C	Jambach	Water
Jam7	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas orientalis*	negative	20°C	Jambach	Water
Jam8	S	S	S	NA	S	S	S	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp .*	negative	20°C	Jambach	Water
Jam9	S	S	S	NA	S	R	S	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium sp.*	negative	20°C	Jambach	Water
Largoz 2	S	S	S	NA	R	S	S	NA	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas sp.*	negative	4°C	Largoz	Snow
Largoz 6	R	S	S	NA	R	S	R	NA	*Sphingomonadaceae*	*Sphingomonas*	*Sphingomonas sp.*	negative	20°C	Largoz	Snow
Largoz 7	R	R	R	NA	R	R	R	NA	*Nitrobacteraceae*	*Rhodopseudomonas*	*Rhodopseudomonas sp.*	negative	20°C	Largoz	Snow
Largoz 8	S	S	S	NA	R	S	S	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium lividum*	negative	20°C	Largoz	Snow
Sanna 1	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Sanna	Water
Sanna 2	R	R	S	R	R	R	S	R	*Bacillaceae*	*Bacillus*	*Bacillus tequilensis*	positive	20°C	Sanna	Water
Sanna 3	R	R	S	NA	R	R	R	NA	*Enterobacteriaceae*	*Eschericha*	*Eschericha marmotae*	negative	20°C	Sanna	Water
Sanna 4	R	S	S	NA	R	R	R	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium sp.*	negative	20°C	Sanna	Water
Sanna 5	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Sanna	Water
Sanna 6	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus subtilis*	positive	20°C	Sanna	Water
Sanna 7	S	S	S	S	S	S	S	S	*Bacillaceae*	*Bacillus*	*Bacillus tequilensis*	positive	20°C	Sanna	Water
Stubai 10	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas kairouanensis*	negative	20°C	Stubai Glacier	Water
Stubai 11	R	R	R	NA	R	R	R	NA	*Burkholderiales*	*Burkholderiales*	*Burkholderiales bacterium*	negative	20°C	Stubai Glacier	Ice
Stubai 12	S	S	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Stubai Glacier	Ice

Stubai 13	S	S	S	NA	S	S	S	NA			no blast result	negative	20°C	Stubai Glacier	Ice
Stubai 15	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Stubai Glacier	Ice
Stubai 16	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas kairouanensis*	negative	20°C	Stubai Glacier	Ice
Stubai 18	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	Stubai Glacier	Ice
Stubai 19	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*	negative	20°C	Stubai Glacier	Ice
Stubai 20	S	S	S	NA	R	R	S	NA			87% identity	negative	4°C	Stubai Glacier	Snow
Stubai 21	R	S	S	NA	R	R	S	NA			no blast result	negative	4°C	Stubai Glacier	Water
Stubai 22	R	R	R	NA	R	S	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium frigidimaris*	negative	4°C	Stubai Glacier	Snow
Stubai 23	S	S	S	NA	R	R	S	NA	*Chromobacteriaceae*	*Aquaspirillum*	*Aquaspirillum arcticum*	negative	4°C	Stubai Glacier	Ice
Stubai 24	R	S	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas frederiksbergensis*	negative	4°C	Stubai Glacier	Ice
Stubai 25	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*	negative	4°C	Stubai Glacier	Ice
Stubai 26	S	S	S	NA	R	R	S	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium sp.*	negative	4°C	Stubai Glacier	Ice
Stubai 27	R	S	R	NA	R	R	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium piscis*	negative	4°C	Stubai Glacier	Ice
Stubai 5	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas frederiksbergensis*	negative	20°C	Stubai Glacier	Snow
Stubai 9	S	S	S	NA	R	R	S	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium lividum*	negative	20°C	Stubai Glacier	Water
Tri 1	S	S	S	NA	S	S	S	NA	*Burkholderiaceae*	*Burkholderiaceae*	*Burkholderiaceae bacterium*	negative	20°C	Trisanna	Water
Tri 2	R	R	S	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas fluorescens*	negative	20°C	Trisanna	Water
Tri 4	R	R	S	NA	R	R	R	NA	*Oxalobacteraceae*	*Janthinobacterium*	*Janthinobacterium lividum*	negative	20°C	Trisanna	Water
Tri 5	S	S	S	NA	S	S	S	NA			91 % identity	negative	20°C	Trisanna	Water
Tri 6	R	R	S	NA	S	R	S	NA	*Yersiniaceae*	*Rahnella*	*Rahnella aquatilis*	negative	20°C	Trisanna	Water

WL 2	R	S	S	NA	R	R	S	NA	*Chromobacteriaceae*	*Iodobacter*	*Iodobacter limnosediminis*	negative	20°C	WSL	Water
WL 3	S	S	S	NA	R	R	S	NA	*Alteromonadaceae*	*Alishewanella*	*Alishewanella sp.*	negative	20°C	WSL	Water
WL 4	R	R	R	NA	R	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas putida*	negative	20°C	WSL	Water
WL 5	S	S	S	NA	R	R	S	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas psychrotolerans*	negative	20°C	WSL	Water
WL 6	R	S	S	NA	S	R	R	NA	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas sp.*	negative	20°C	WSL	Water
WL 7	R	S	R	NA	S	S	R	NA	*Flavobacteriaceae*	*Flavobacterium*	*Flavobacterium piscis*	negative	20°C	WSL	Water
WL 8	R	S	S	NA	R	R	R	NA	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter cloacae*	negative	20°C	WSL	Water

Paper III

Exploring Antibiotic Resistance in Earth's Coldest Regions

(submitted to Frontiers for Young Minds)

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Abstract

Antibiotics, important medicines that fight harmful bacteria, face a big problem: supervillains - bacteria that became resistant to antibiotics - are on the rise. Once only a concern in hospitals, antibiotic resistance now reaches Earth's coldest places, like glaciers and the Arctic. This article explores how bacteria in these icy places learned to withstand antibiotics and how human activities, like tourism, worsen this problem. Join us on a fascinating journey into the ice to understand how antibiotic resistance spreads in these faraway places, how strong these bacteria can be, and how human behavior affects this. Together, we'll find ways to keep antibiotics working well and protect people's health everywhere.

Keywords: antibiotic resistance, cold environment, glacier bacteria, human impact, snow and ice

Shaping antibiotic resistance: Human influence in Earth's coldest realms

Have you ever heard of antibiotics? You can think of them as superheroes, that help us deal with annoying bacteria trying to make us sick. Chances are your doctors gave you some to free you from these nasty one-celled organisms that are tiny and therefore can only be seen under a microscope. Most bacteria are harmless and rather helpful because they perform important tasks like helping us to digest our food. Yet, some of them cause serious diseases like pneumonia and need to be treated with antibiotics. Antibiotics come in two main types: natural and synthetic. Natural antibiotics, like penicillin, are made by living organisms such as fungi and bacteria. These organisms produce antibiotics to protect themselves from other bacteria that try to harm them. On the other hand, synthetic antibiotics are created in laboratories to mimic or enhance the effects of natural ones.

All these different types of antibiotics have saved millions of lives, but it can happen that taking them does not help make us feel better. This is when our superheroes must deal with supervillains - bacteria with antibiotic resistance (read more here: https://kids.frontiersin.org/articles/10.3389/frym.2020.00005). Antibiotics do not work against these troublemakers because these bacteria have learned new ways to defend themselves. We are currently concerned about the increasing frequency of cases in which our antibiotics cannot defeat these supervillains. But there are even more reasons giving scientists a headache.

For a long time, we thought that antibiotic resistance is a phenomenon that only occurs where and when we treat sick people having infections with antibiotics. However, scientists found traces of these supervillains in various places on the Earth including the North [1] and South Pole [2] even though nobody is living there.

The North and South Poles are part of the so-called cryosphere where the Greek words “kryos” and “sphaira” mean “cold” and “globe” respectively hence, comprising all areas on Earth where water exists in its solid form as snow or ice. We humans generally consider these regions as harsh to live in. However, many microorganisms prefer conditions that are dominated by low temperatures, no or too much sunlight (depending if it is polar night or day), and very low food availability. However, it was a surprise that bacteria living there

learned to resist some antibiotics. How these microbes slowly transformed into supervillains is largely unknown, as is the actual extent of this phenomenon.

Learning to withstand antibiotics - How bacteria become supervillains

In principle, there are several different ways bacteria can become resistant to antibiotics. While specific defensive mechanisms are often learned by the microbes after prolonged contact with the antibiotic, nonspecific or basic defense strategies usually involve things like “pumps” that remove all harmful substances from the bacterial cell (Figure 1).

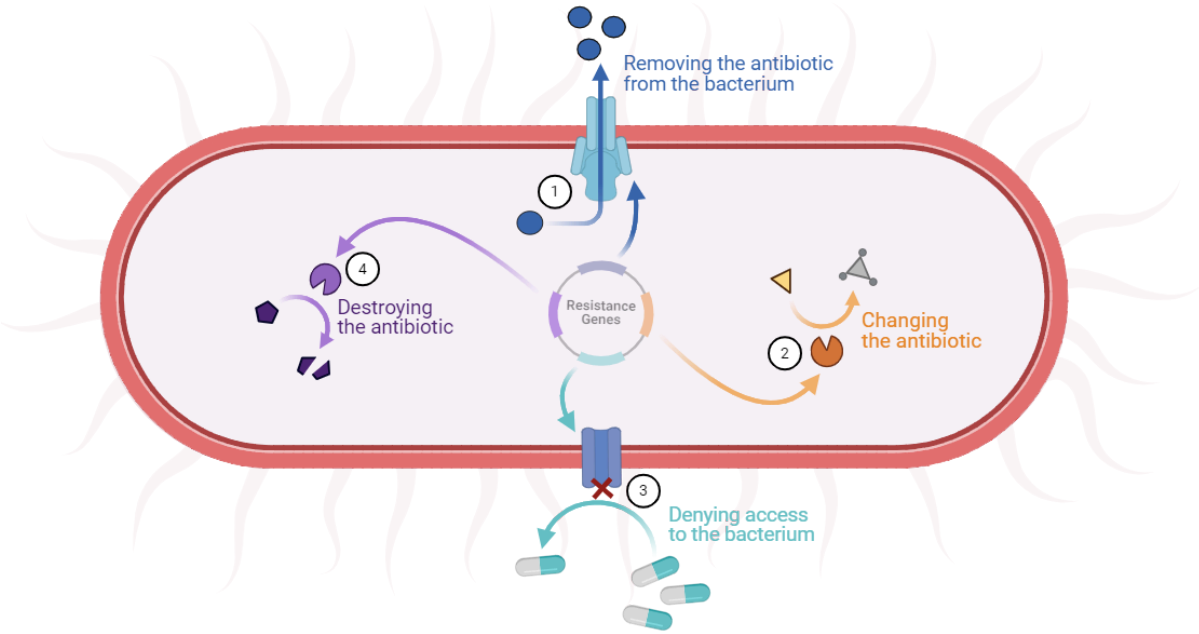

Figure 1 - Possible defensive strategies that help the bacterium to withstand antibiotics. Some of the main mechanisms include 1) pumping out the toxic substance 2) changing the antibiotic into a harmless substance 3) limiting access for the harmful molecule 4) destroying it. Created with BioRender.com.

While non-specific antibiotic resistance can occur in any bacterial species and help protect them from toxic substances, specific defensive strategies usually only develop when microbes are exposed to antibiotics in their environment. Of course, this can also be the case in the cryosphere. Additionally, microorganisms including these supervillains can travel with animals (e.g. migrating birds), global winds (think about Saharan dust that can travel over the sea to other continents), and of course, us humans (e.g. traveling) [3]. All of this combined leads to something that we call the cryospheric resistome - which is the sum of all antibiotic resistance mechanisms within snow and ice habitats.

Scientists could already show that the transport of antibiotic-resistant bacteria is an important factor when we try to understand these supervillains in the cryosphere [4].

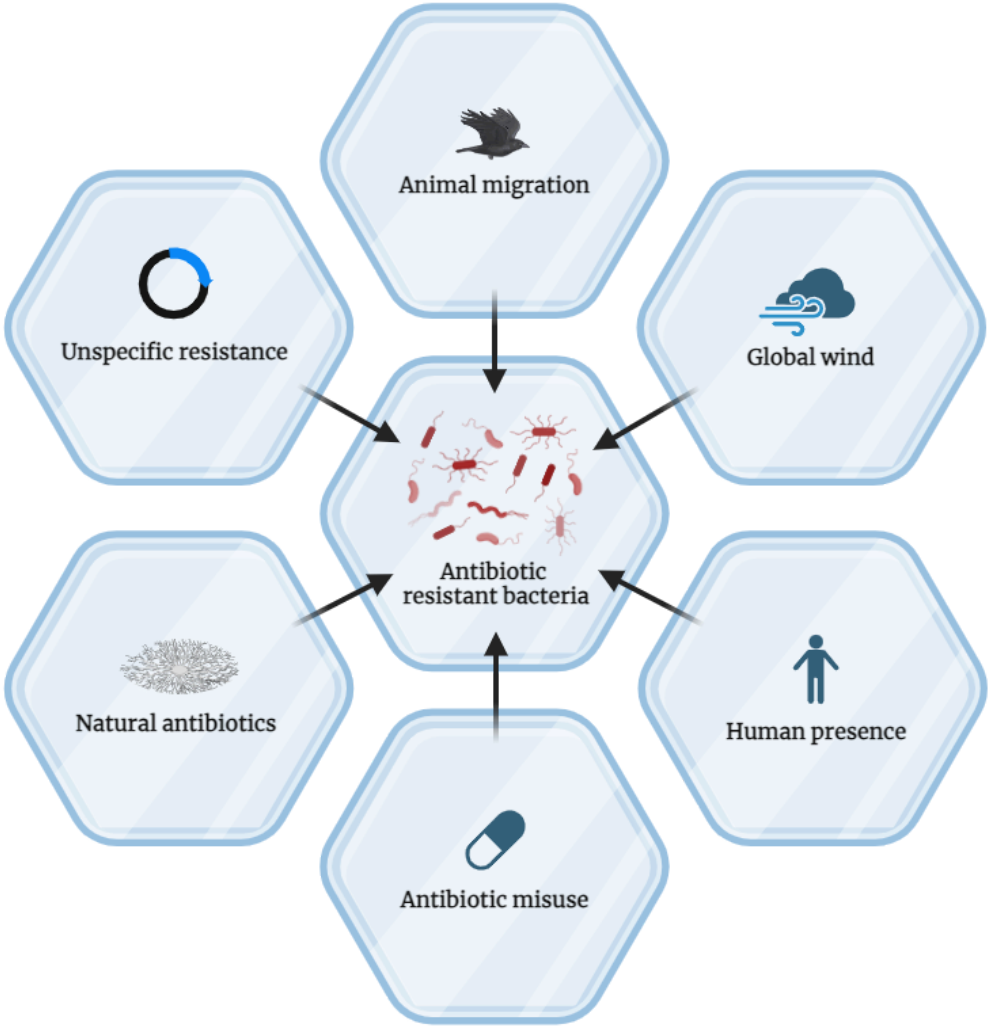

Figure 2 - How bacteria living in snow and ice can become resistant to antibiotics. The main drivers are nonspecific resistance mechanisms like pumps, the presence of antibacterial substances, and various transport pathways leading to the distribution of supervillains into remote areas. Created with BioRender.com.

Human impact on ice supervillains - antibiotic resistance in Earth's coldest regions

After learning the principles of antibiotic resistance, let us take a deep dive into the supervillains of the cryosphere. Imagine, that these bacteria could resist not just one or two, but up to eight different antibiotics! That is what we discovered in our study across multiple cryospheric spots in the Alps. For our work, we looked at samples with more and less human presence in mountainous regions.

Interestingly, we found that humans also play a major role in antibiotic resistance in icy regions, especially when these regions are easily accessible. For example, the Nature Ice Palace, a popular ice cave on top of an Austrian mountain with many daily visitors, showed some of the highest levels of antibiotic resistance in all of our samples. Here we found 20 out of 21 bacteria being resistant to more than 3 antibiotics while on the comparatively remote glacier named “Jamtal glacier” within the Austrian Alps, only about half of the microorganisms could not be harmed by at least 3 different antibacterial substances. In addition, even powerful antibiotics like vancomycin and linezolid, which are often used to treat sick people when other medications fail, could only fight very few of the microbes present in the ice cave. In fact, the number of supervillains that could resist the effects of vancomycin within the Nature Ice Palace was higher than what scientists found in some hospital surroundings [5]! Again, bacteria at the Jamtal glacier, which can only be reached by a long hike, also had some resistance to these precious antibiotics, but the number of supervillains was nowhere close to that of the touristic ice cave.

We found this trend to be true in all our almost 300 samples. Regardless of whether samples came from glaciers, lakes, or seasonal snow collected on top of some remote mountains and then compared with snow from large cities. All of these samples had one thing in common: The higher the human activity, the higher the number of supervillains. The same was true for the number of bacteria with resistance against synthetic antibiotics. These antibacterial substances completely designed in the lab are usually a good indicator of antibiotic resistance caused by human (mis-)use. Humans can introduce potentially antibiotic-resistant bacteria into the environment by simply breathing or having skin contact with the ice. And as you might know: humans like to touch what they see. So it is the case with beautiful ice features. Together with our results, this shows that human presence is likely one of the main reasons for the spread of supervillains in unexpected environments like the cryosphere.

Exploring the secrets of the toughest bacteria in cold areas

Let’s take a step back and take another look at some really remote places. Places far from cities or busy ski resorts. Locations with very few daily visitors and no hospitals or animal farms close by. Although we have already mentioned that antibiotic resistance in bacteria is not as common there, we could still find quite a few superbugs. While most antibiotics

worked to some extent, others could only kill 2-5 out of 10 bacteria we tested. Fortunately, these organisms do not pose any danger to us humans.

A possible reason why these supervillains are so tough can be explained by special adaptations such as thick cell walls that protect them from harsh environments in which they occur. Also, do not forget that bacteria can spread even without humans, using different ways we have discussed previously (Figure 2). One thing is clear though: We need much more research to understand better. During summer seasons (and increasingly since the Earth warms up), ice and snow in these remote places melt and the bacteria living there can find their way into human surroundings by melt flows (Figure 3). Although they do not harm us, they can potentially teach other bacteria how to survive antibiotics and thus increase the already existing problem of antibiotic resistance. Therefore. it is important to continue studying these clever bacteria in all parts of the world to stay one step ahead.

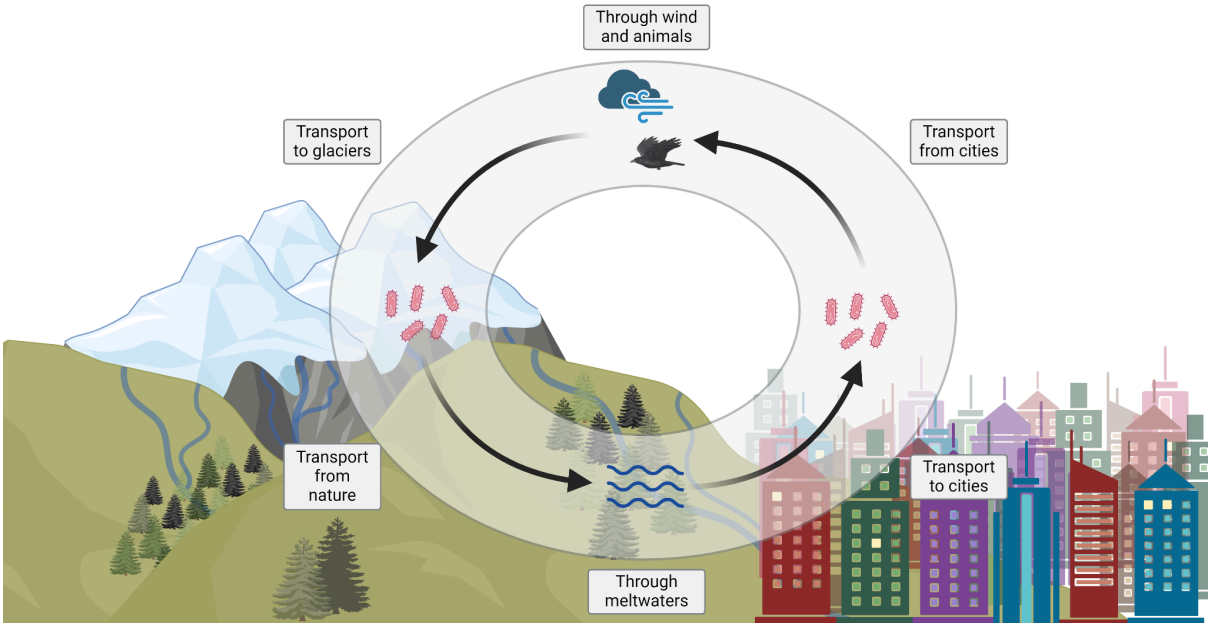

Figure 3 - The potential cycle of antibiotic-resistant bacteria between our cities and our environment (e.g. glaciers). Created with BioRender.com.

All in all, we learned that even in the mountains, human activity can shape the world of bacteria, turning some of them into antibiotic-resistant supervillains. Our study provided some insight into this topic but there is still much to learn. And while science is doing its best to find out more, you can already make a small difference today! By making sure not to consume valuable antibiotics too much, or, if necessary, only as prescribed by your doctor

and by raising awareness for the topic, we can stay one step ahead of the supervillains together.

If you want to learn more about antibiotic resistance - we have a whole website centered around that topic. Explore our blog or play around on our interactive map to learn more about these supervillains.

Glossary

Antibiotics - Medicines that help our bodies fight harmful bacteria and get rid of infections.

Antibiotic Resistance - When bacteria change in a way that makes antibiotics no longer able to kill them.

Bacteria - Tiny organisms that can only be seen under a microscope. Some are helpful, but others can cause diseases.

Cryosphere - The parts of Earth where water exists in solid form, like snow and ice (e.g. North and South Poles).

Supervillains - Bacteria that have become resistant to antibiotics and are very hard to kill.

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