



Original Articles

Quantification of class 1 integrons and characterization of the associated gene cassettes in the high Arctic – Interplay of humans and glaciers in shaping the aquatic resistome

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ABSTRACT

The Arctic is one of the regions most affected by global climate change, and is subjected to changes linked with a melting cryosphere and increasing anthropopressure. Although antibiotic resistance is a global problem, the diversity and spread of antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs) and integrons in the Arctic are strongly understudied. Therefore, the main aims of this study are the (1) determination of the type and frequency of integron-integrase genes and characterization of incorporated gene cassettes in the genomes of culturable bacteria and (2) quantitative analysis of class 1 integron-integrase gene and human mitochondrial DNA (*Hmt*-DNA) in the metagenome as markers of anthropogenic impact on the high Arctic environments of the Svalbard Archipelago. Samples of ice, water and sediments were collected in the most populated area of Svalbard, Longyearbyen and its vicinity. Sampling was conducted along an environmental gradient with varying levels of human activity. The environmental gradient started from glaciers, following the proglacial river, the seashore, and the fjord bottom water, including untreated wastewater outflow to the sea. Class 1 integrons were detected in ARB isolated from glacial environments, freshwater and seawater, including wastewater outflow. Moreover, in the variable regions of integrons, genes determining different functions, including antibiotic resistance, virulence and physiological traits were found. These genes play crucial roles in the adaptation of bacteria to cold and dynamic environments. The relative abundance of *int1* genes were reported in metagenomes with different relationships to human activity (ice cores vs wastewater outflow), with the highest mean values observed in the wastewater outflow, and was positively correlated with abundance of the *Hmt* gene, revealing both natural and human roles in shaping the polar aquatic resistome.

1. Introduction

Warming in polar regions influences deep changes in both abiotic and biotic environments, including landscape, atmosphere, biogeochemistry and biodiversity (CAFF, 2017; IPCC, 2019; Ignatiuk et al., 2022; Pries et al., 2022). Additionally, increasing anthropopressure in remote sites related to the opening of new fishing grounds and transport routes, tourism industry and scientific activity as well as a growing

number of citizens is the next most important factor contributing to negative changes (AMAP, 2018). One such example is the spread of antibiotic resistance, which poses an important threat to public health worldwide and is still poorly studied in the Arctic, which is changing (e. g., warming) faster than other regions of the world (Segawa et al., 2013; Tan et al., 2018; Makowska et al., 2020).

Human activity has led to global changes that resulted in the coining of a new term: Anthropocene (epoch of human, Head et al., 2022).

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Anthropogenic activities lead to the presence of pollution that select for antibiotic resistance genes (ARGs) which in turn are associated with and disseminated by integrons (Hayward et al., 2018; Uyaguari-Díaz et al., 2018; Makowska et al., 2020). The overuse and misuse of antibiotics not only for human therapy but also for livestock breeding and agriculture have led to the emergence and spread of antibiotic-resistant bacteria (ARB) and ARGs in the environment. Environmental factors such as wind, ocean currents and animal migration may accelerate the transfer of ARB and ARGs (Sjölund et al., 2008; Zhu et al., 2021). Therefore, even remote ecosystems can no longer be considered pristine, as they are all in fact affected by anthropogenic pressure (McConnell et al., 2018).

It is believed that environmental bacteria play an important role in the spread of antibiotic resistance. Antibiotic resistance is thought to have existed in nature for millennia, long before the first commercial use of antibiotics (Chen et al., 2013). In recent years, glaciers and permafrost have been discussed as repositories of potential threats to humans, including ARB and ARGs (Sajjad et al., 2020; Beard et al., 2022; Łokas et al., 2022). The melting cryosphere is thought to be a source of free DNA and ancient genes, including ARGs, release from ice into relatively young geological (i.e., only recently ice free) and fragile polar ecosystems (Rogers et al., 2004; Sajjad et al., 2020). Therefore, the resistome in the Arctic might be enriched with potential resistance determinants, which could be transported with glacial rivers to terrestrial and marine ecosystems. Glaciers are melting as the climate progressively changes, and this results in an increase in microbial diversity (Rogers et al., 2004). Because of the increasing accessibility to remote places, tourism and growing human settlements in Arctic regions, studies on the antibiotic resistance load released from glaciers to the environment through melting are of high importance.

The role of wastewater in the emergence and acceleration of the dissemination of ARB and ARGs is crucial. Wastewater is characterized by a high abundance of bacteria from various hosts and by nutrient-rich environments that support bacteria as the source of energy. Heavy metals, detergents and antibiotics are also present in the wastewater. This leads to the selection of bacterial strains resistant to antibiotics, and increases the prevalence of class 1 integrons and antibiotic resistance gene exchange through horizontal gene transfer (HGT) between strains of the same or different species (Gillings et al., 2015; Bengtsson-Palme et al., 2017). Although extremely important, the effects of wastewater on the environmental resistome are still poorly studied in the Arctic. In the many Arctic regions, the human settlements deliver to the ecosystems wastewater containing various pollutants (Gunnarsdóttir et al., 2013; Daley et al., 2018) which could potentially mix with genetic material released from glaciers (Sajjad et al., 2020), forming a new combination of threats. Thus, the interplay between glaciers and wastewater to the spread of antibiotic resistance in the Arctic is of global importance in the identification of unknown and unexplored determinants of antibiotic resistance.

In ARB, ARGs may be located on different genetic structures, including transposons, conjugative plasmids, and integrons as gene cassettes (Zhang et al., 2009). Integrons are DNA fragments that are capable of capturing gene cassettes coding for proteins mediating resistance to antibiotics, disinfectants and heavy metals. Additionally, they capture genes coding for proteins of physiological significance that may promote the adaptation of bacteria to extreme environments (Gillings, 2014; 2017; Makowska et al., 2020). Consequently, integron systems can create genetic novelty during periods of stress or environmental change (Gillings, 2014). Integrons are evolutionarily old genetic elements and occur in the genomes of bacteria in various environments (Ghaly et al., 2021). It is essential that integrons are embedded in mobile genetic elements (MGEs) and that they can be transferred within a microbial population through HGT. There are five classes of integrons associated with ARGs. Class 1 integrons are particularly important in the emergence and spread of multidrug resistance among bacteria (Gillings, 2014; 2017; 2018). Due to their widespread presence in the genomes of commensal bacteria of humans and domestic animals, their high

frequency of occurrence in wastewater and their highly conserved DNA sequence, class 1 integrons are recognized as environmental markers of biotic pollution associated with human activity (Gillings et al., 2015).

Polar regions are characterized by remoteness from overpopulated areas and farms and ecosystem simplicity e.g., simple trophic webs, low biodiversity, few sources of nutrients for terrestrial and aquatic environments, small human population. Therefore, these areas offer a unique environment for studies on how the melting cryosphere and human settlements increase the gene pool of the resistome in these specific ecosystems. In this study, we focused on integrons in the European part of the Arctic, the Svalbard Archipelago. Using human mitochondrial DNA as a human-specific molecular marker, the effect of anthropogenic activity on enriching the environmental resistome with *intI1* genes was evaluated. Understanding the mechanisms and pathways of ARGs dissemination is critical for global action against the problem of constantly increasing antibiotic resistance in natural environments. Specifically, the question of how glacier meltwater and wastewater discharged directly into Arctic fjords affects the gene pool of the polar aquatic resistome, has never been addressed. Therefore, the objectives of this study were (1) to determine the type and frequency of the integrase gene, and characterize associated gene cassettes in the genomes of culturable bacteria and (2) to quantitatively analyze class 1 integron-integrase gene and human mitochondrial DNA (*Hmt*-DNA) in the metagenome of ice cores, freshwater, seawater, sediments and wastewater outflows from high Arctic environments as markers of pollution.

2. Materials and methods

2.1. Sampling

Samples were collected from Spitsbergen, the largest island in the Svalbard Archipelago (located in European part of the Arctic) during two summer seasons (2020, 2021) (Supplementary Table S1). The sampling was conducted in the most populated, industrialized and highly touristic area of Svalbard, specifically in the proximity of Longyearbyen (central Spitsbergen), capitol of Svalbard (78° 13'N, 15° 33'E). This region is characterized by presence of simple and young ecosystems such as geologically young soils and water bodies, new formed ecosystems in proglacial fields, explicitly-two seasons during the year (summer and winter), short food chain, and low biodiversity. Owing to this simplicity and different anthropopressure (glaciers-city-fjord), we chose this area for studies on the effects of the melting cryosphere and human activities on the resistome in freshwater and marine environments.

Longyearbyen was built in a valley located at the small Arctic fjord Adventfjorden. Two small, cold-base, valley glaciers are located in the southern part of the Longyearbyen area, which melt and form a small Arctic river, Longyearrelva, that crosses the town and flows into the fjord. The city releases untreated wastewater 2 km from the shore, ca. 50–60 m b.s.l. in Adventfjorden (Granberg et al., 2017). Samples were collected along the environmental gradient starting from the glaciers (Larsbreen and Longyearbreen, the latter is commonly visited by tourists), following the catchment (Longyearrelva), the seashore, and wastewater outflow, then crossing the Adventfjorden until reaching the Isfjorden system – the largest fjord on western Spitsbergen (Fig. 1, a detailed description of the sampling and study area is provided in Supplementary material S1, Table S1). The sampling reflects ecosystem heterogeneity, a) the oligotrophic glacial ecosystem that can be a source of ARB, ARGs and integrons for the proglacial river, b) the river crossing the small Arctic settlement Longyearbyen, c) the seashore where the river outflows to the fjord and where tourists have a space for picnicking, d) the bottom water from the fjord close to the discharge of untreated wastewater from the city Longyearbyen, and e) the bottom water collected in increasing distances from the wastewater discharge, toward the border between small Adventfjorden and the much larger and open Isfjorden.

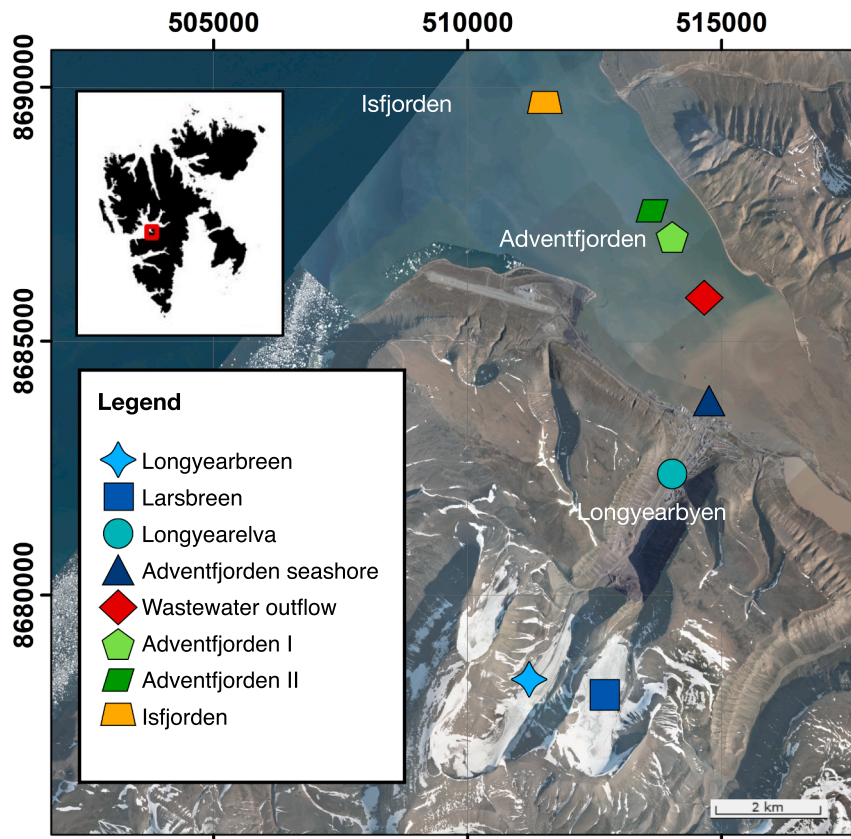


Fig. 1. Map indicating sampling sites.

2.2. Bacterial cultures

The total number of culturable heterotrophic bacteria from the Longyearrelva, Adventfjorden, wastewater outflow and Isfjorden was determined on brain heart infusion BHI agar (bioMerieux) and from glaciers Larsbreen as well as Longyearbreen on R2A agar, in duplicate technical repetitions (Thermo Scientific). R2A possesses a low nutrient concentration and is used for culturing bacteria from nutrient-limited ecosystems as glacial ecosystems, especially ice, are nutrient poor (Poniecka, et al., 2020). In order to select bacterial strains with integrons, heterotrophic bacteria resistant to sulfonamides were selected on BHI and R2A agar supplemented with sulfamethoxazole (350 µg/ml). Samples of water were inoculated onto the media in a volume of 1 ml and 0.1 ml. Three grams of sediment from Longyearrelva, Adventfjorden were suspended in 3 ml of sterile saline, vortexed 15 min, then the mixture was spun down and 0.1 ml was inoculated onto media. Moreover, for sediment samples and samples from wastewater outflow a series of logarithmic dilutions were prepared in sterile saline and in duplicate technical repetitions, 0.1 ml inoculated onto the media. The plates were then incubated at 8 °C for 10–21 days in light conditions. The bacterial isolates were stored at –80 °C in brain heart infusion broth containing 50 % (vol/vol) glycerol.

2.3. DNA template preparation

Genomic DNA from the culturable bacteria was isolated by heating (95 °C for 2 min) according to Dillon et al. (2005), and lysates were stored at –20 °C.

For isolation of the metagenomic DNA, water samples were filtered through 0.45-µm and then 0.2-µm cellulose nitrate filters (Sartorius Stedim): Adventfjorden seashore (2 l), Longyearrelva (2 l), wastewater outflow (1 l), Adventfjorden I, II and Isfjorden, as well as from Larsbreen

and Longyearbreen (each 0.8 l, after thawing at 3 °C). Filters were washed with sterile water, and after centrifuging the pellet, total DNA was extracted by heat lysis and with a Genomic Mini Kit (A&A Biotechnology) according to the manufacturer's instructions. Metagenomic DNA from sediment samples (Longyearrelva, Adventfjorden seashore) was isolated from 1 g (wet weight) of each sample and centrifuged, and total DNA was extracted from the pellet as described above. Quality of the obtained DNA samples was assessed by spectrophotometry and electrophoresis.

2.4. Qualitative PCR with the use of DNA of bacterial isolates

To assess the frequency of class 1, 2 and 3 integron integrases and genes determining resistance to sulfonamides, bacterial colony lysates were prepared in 96-well plates. Amplification of integrase genes (*int1*, *int2* and *int3*) and three genes conferring resistance to sulfonamides (*sul1*, *sul2*, *sul3*) was conducted in a 25-µl volume containing 1 × PCR reaction buffer (1.5 mM MgCl₂), 0.25 µM each primer, 0.2 mM dNTP mix, 1.25 U of OptiTaQ DNA Polymerase (EURx), and 2 µl of genomic DNA. PCR conditions consisted of initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, annealing (varied temperature; 55–60 °C) for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. Primer sequences and annealing temperatures are shown in Supplementary Table S2. All PCR amplifications were performed in a Mastercycler® nexus (Eppendorf). The amplicons were separated in a 1.5 % agarose gel. The molecular weight of the PCR products was determined with an Alliance Q9 Mini (Uvitec). Amplicons were purified and sequenced in a 3130xl Genetic Analyzer (Applied Biosystems).

2.5. Detection and analysis of the variable regions of integrons in DNA of bacterial isolates by conserved segment (CS)-PCR

The variable regions of the integrons were amplified according to Lévesque et al. (1995). PCR conditions consisted of initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 5 min, and a final elongation at 72 °C for 8 min. PCRs were performed in a Mastercycler® nexus (Eppendorf) and C1000 Thermal Cycler (Bio–Rad). The amplicons of the variable regions of integrons were purified and sequenced. When two or more amplicons were present, the PCR products were cloned using the pGEM-T Easy vector (Promega). Individual products obtained were sequenced and aligned with available GenBank data by using nucleotide BLAST (Basic Local Alignment Search Tool). A gene was identified if the similarity with GenBank data was equal to or higher than 98 %.

2.6. Antimicrobial susceptibility

To determine the resistance profile of the *intI1*-positive strains, a disc diffusion test was performed using seven drugs representing six classes of antimicrobials according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021). The antimicrobials included tobramycin (10 µg), penicillin (10 µg), ciprofloxacin (5 µg), tetracycline (30 µg), sulfamethoxazole (25 µg) (for the unselected strains on the medium with sulfamethoxazole), co-trimoxazole (25 µg), and chloramphenicol (30 µg). The antimicrobial resistance profile of the tested strains was expressed as the number of antimicrobial classes to which these strains were resistant. The strains were considered multidrug resistant when they exhibited resistance to at least three different classes of antimicrobials.

2.7. Identification of bacteria by sequencing the 16S rRNA gene

Bacteria harboring integrons were identified by sequencing the 456-bp-long fragment of the 16S rRNA gene, which was PCR amplified with the universal primers 343F and 798R according to Nossa (2010). The 343F/798R primer set, producing amplicons encompassing bases 361–784 and hypervariable regions (HVRs) 3 and 4 of the 16S rRNA gene, has a broad taxonomic coverage of common bacterial species and gives maximum accuracy of taxonomical classification. The partial 16S rRNA gene sequences were subjected to a BLASTn sequence similarity search (Altschul et al., 1990).

2.8. Droplet digital PCR

Droplet digital PCR (ddPCR) was used to determine the copy number and relative abundance of the class 1 integron-integrase gene *intI1* in the metagenomic DNA. Gene quantities were normalized to the 16S rRNA gene copy number, and relative abundance values were expressed as percentages and calculated as follows: $[(\text{the gene copy number}/16\text{S rRNA gene copy number}) \times 4 \times 100\%]$ with four being the average number of copies of the gene encoding 16S rRNA per bacterial cell, according to the ribosomal RNA database (Stalder et al., 2012). Human mitochondrial DNA (*Hmt*-DNA) was also quantified to ascertain the degree of contamination by human activity. The presence of PCR inhibitors in template DNA was checked by conventional PCR amplification of bacterial 16S rRNA gene fragments using DNA templates at dilutions from 1:5 to 1:200. Primer sequences are shown in Supplementary Table S2. Each ddPCR was prepared in three technical replications. Reactions were conducted in a QX200 Droplet Digital PCR System (Bio–Rad) with QX200 ddPCR EvaGreen Supermix (Bio–Rad).

2.9. Statistical analysis

Associations among integron-bearing bacteria, frequencies of integrons, gene copy numbers, relative abundances of integrons in the

metagenome, sampling sites and various types of ecosystems were assessed with the Kruskal–Wallis test and Mann–Whitney *U* test. Correlations among the frequencies of ARGs, integrons, gene copy numbers, and the relative abundances of genes in the metagenome were determined with Spearman's rank correlation coefficient. Calculations were performed with Statistica 13.1 software (TIBCO) (Statsoft, 2016). $p < 0.05$ was used to reflect statistical significance.

3. Results

3.1. Culturable bacteria

The average total number of bacteria ranged from 2.0×10^1 in the ice core from Longyearbreen to 8.1×10^3 CFU/ml in the sediment of the Adventfjorden seashore. The average number of sulfamethoxazole-resistant heterotrophic bacteria ranged from 0.2×10^1 in the ice core from Larsbreen to 1.3×10^3 CFU/ml in the sediment of the Adventfjorden seashore (Fig. 2A). There were no significant differences in the total number of heterotrophic bacteria among the materials collected from the three types of ecosystems: glacial, freshwater and marine. There were significant differences in the number of sulfamethoxazole-resistant heterotrophic bacteria among samples collected in various ecosystems; specifically, from ice cores and sediments ($p = 0.04$).

The frequency of culturable sulfamethoxazole-resistant heterotrophic bacteria normalized to the total number of heterotrophs was the lowest in water of the Adventfjorden seashore (0.8 %) and the highest in wastewater outflow (17 %). Moreover, the frequency of sulfamethoxazole-resistant heterotrophic bacteria in ice cores ranged from 6.9 % in Larsbreen to 15 % in Longyearbreen (Fig. 2B). There were no significant differences between the materials (ice vs water vs sediment) and ecosystems (glacial vs freshwater vs marine).

3.2. PCR detection of integrons and *sul* genes in bacterial isolates

The average frequency of the integron-positive heterotrophic isolates ranged from 2.4 % in sediment of the Adventfjorden seashore to 11.2 % of isolates recovered from water at the same sampling site (Fig. 3). We did not find class 2 or 3 integrons in any of the samples. Sulfamethoxazole-resistant heterotrophic bacteria carried class 1 integrons with an average frequency ranging from 7.7 % in the ice of Larsbreen to 100 % in the sediment of the Adventfjorden seashore (Fig. 3). There were no significant differences in the frequencies of *intI1* genes in the genomes of culturable bacteria between the material and ecosystems.

Of all the isolates (837 strains, including 498 strains of heterotrophic bacteria and 339 heterotrophic strains resistant to sulfamethoxazole), 48 had class 1 integrons: three originated from Larsbreen (ice core), 12 from water of the Longyearbreen, seven from sediment of the Longyearbreen, 10 from the water and two from sediment of the Adventfjorden seashore, and 14 from wastewater outflow. Samples of Longyearbreen ice and bottom water from Adventfjorden I, II and Isfjorden did not contain bacteria with *intI1*. Of all the *intI1*-positive strains, 85.4 % (41 isolates) carried the *sul1* gene. The *sul2* and *sul3* genes were not detected. There was a strong correlation between the frequency of *sul1* and *intI1* detected in both heterotrophs and sulfamethoxazole-resistant heterotrophic strains ($r = 0.90$, $r = 0.96$, respectively).

3.3. Analysis of the variable regions of integrons

The analysis of the variable region of integrons yielded amplicons for 17 isolates (five and three from water and sediment of the Longyearbreen, respectively, two from water of the Adventfjorden seashore and seven from wastewater outflow).

The size of the variable regions of *intI1*-positive strains from water of the Longyearbreen ranged from 0.6 to 3.7 kbp. The variable regions included genes encoding proteins of different functions, including

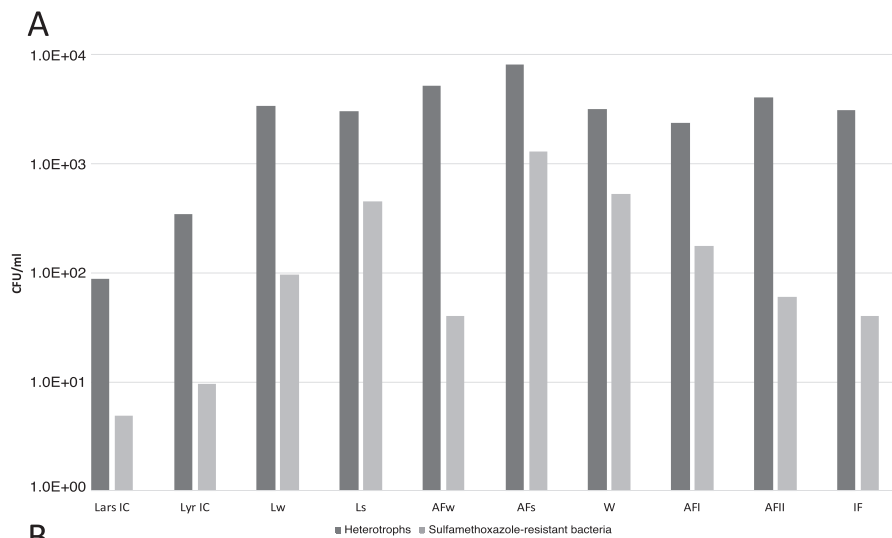


Fig. 2. A) Total number of heterotrophic bacteria and sulfamethoxazole-resistant heterotrophic bacteria in ice, freshwater and seawater, sediment samples, wastewater outflow and bottom seawater. B) Frequency of culturable sulfamethoxazole-resistant heterotrophic bacteria normalized to the total number of heterotrophs in samples from Svalbard. Legend: Lars IC – Larsbreen ice core, Lyr IC – Longyearbreen ice core, Lw – water of Longyearlva, Ls – sediment of Longyearlva, AFw – water of Adventfjorden seashore, AFs – sediment of Adventfjorden seashore, W – wastewater outflow, AFI – bottom water of Adventfjorden I, AFII – bottom water of Adventfjorden II, IF – bottom water of Isfjorden.

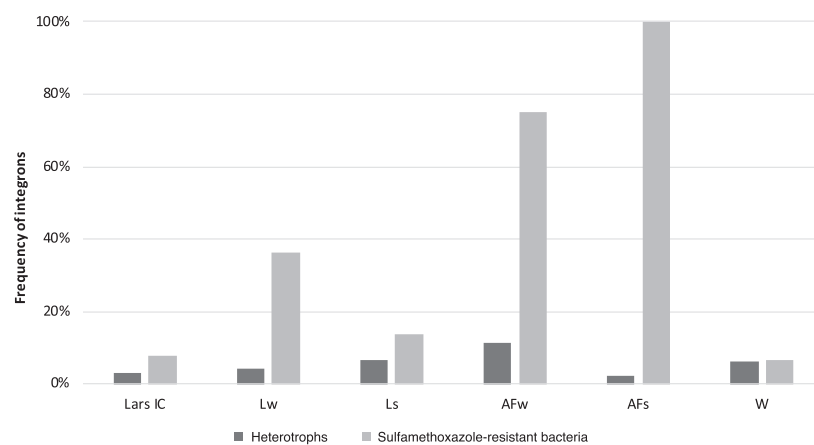
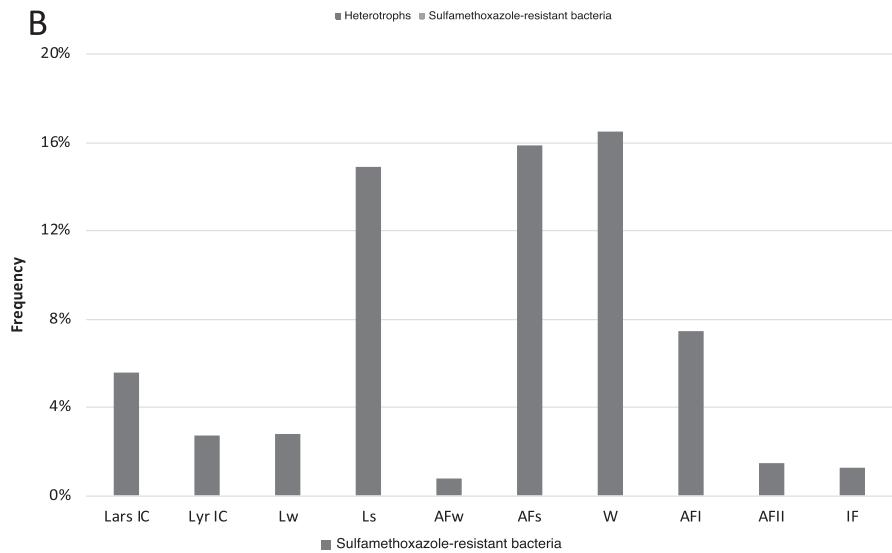


Fig. 3. Frequency of integrons in heterotrophic and sulfamethoxazole-resistant heterotrophic bacteria cultured from ice, freshwater and seawater, sediment samples and wastewater outflow. Legend: Lars IC – Larsbreen ice core, Lw – water of Longyearlva, Ls – sediment of Longyearlva, AFw – water of Adventfjorden seashore, AFs – sediment of Adventfjorden seashore, W – wastewater outflow.

antibiotic resistance, virulence and physiology (Table S3). Gene cassettes determining resistance to antibiotics were found in the cassette *fusC*, (GCN5)-related *N*-acetyltransferase (GNAT), and hemerythrin-like

protein. Additionally, a direct link of virulence was found in the cassette *tamB*. The variable region of integrons also included other genes directly or indirectly responsible for the production of membrane transporters

and forming efflux pumps, such as cassette arrays containing genes encoding ABC transporter-like proteins, homoserine/homoserine lactone efflux protein (*rhtB*), sodium:dicarboxylate symporter, TRAP transporter (*dctM*), L-asparagine permease (*ansP*) and GABA permease (*gabP*).

Two strains from the sediment of the Longyearelva had a variable region of 180 bp, which indicates a lack of integrated gene cassettes, whereas the remaining strain had a variable region gene encoding the DUF1405 protein of unknown function (Table S3). One strain isolated from Adventfjorden seashore water had an empty integron without inserted gene cassettes, while the second strain had the gene cassette containing a phosphoenolpyruvate-protein phosphotransferase-encoding gene (*ptsI*) (Table S3).

The variable region of *intI1*-positive strains from wastewater outflow ranged from 0.8 to 3.2 kbp and had genes encoding proteins of different functions (Table S3). Directly determining resistance to antibiotics were genes encoding the multidrug efflux MFS transporter MdtM and multidrug efflux RND transporter periplasmic adaptor subunit AdeG. The variable region of these integrons also included genes encoding other proteins, such as the Tol/Pal system protein YbgF, phosphoenolpyruvate-protein phosphotransferase (*ptsI*), methyl-accepting chemotaxis protein (TAR) and protease II PtrB.

3.4. Antimicrobial susceptibility

All strains were resistant to antimicrobials belonging to classes 2–6. Among all *intI1*-positive strains, 93.3 % were resistant to at least three classes of antibiotics (i.e., multidrug resistant). All tested strains were resistant to sulfamethoxazole. A high percentage of resistance was found for tobramycin and chloramphenicol (each 87.1 %). The lowest frequency of resistant strains was noted for tetracycline (19.4 %) (Supplementary Figure S1). The greatest variation in the resistance profile was observed for the strains isolated from the water and sediment of the Longyearelva and Adventfjorden seashore (classes 3–6 and 2–5, respectively), whereas the smallest variation was observed from the ice of Larsbreen (4 classes) (Fig. 4). There were no significant differences among the antimicrobial resistance profiles of bacteria isolated from

different materials and ecosystems.

3.5. Quantification of class 1 integron-integrase and *Hmt* genes by ddPCR

The average number of 16S rDNA gene copies varied from 2.2×10^2 /ml in ice of Longyearbreen to 2.0×10^6 /ml in wastewater outflow (Table S4, Fig. 5). The occurrence of class 1 integron integrase gene (*intI1*) and *Hmt* gene was noted in all of the analyzed metagenome samples; the average copy number per 1 ml was the lowest in ice of Longyearbreen (0.6×10^1 and 0.2×10^1 , respectively) and the highest in wastewater outflow (4.4×10^4 and 1.2×10^6 , respectively) (Table S4, Fig. 5). There were significant differences in 16S rDNA, *intI1* and *Hmt* copy number/ml among the materials collected in the various types of ecosystems, specifically, from ice cores and sediments ($p = 0.03$). There were also significant differences in *Hmt* copy number/ml among samples from ice cores of glaciers, the bottom water from the fjord close to the discharge of untreated wastewater and the bottom water collected in increasing distances from the wastewater discharge ($p = 0.03$). A strong correlation between the *intI1* and *Hmt* copy number/ml ($r = 0.9$) was found.

To determine the relative abundance of class 1 integron-integrase genes in metagenomes, copy numbers of the *intI1* in each site were normalized to the copy number of the bacterial 16S rRNA gene. The highest mean values of the relative abundances of the *intI1* genes were observed in the wastewater outflow (88.0 %), and the lowest values were observed in the water of Isfjorden (2.4 %) (Table S4, Fig. 6). The metagenome of ice from Longyearbreen represented a higher frequency of the *intI1* (7.1 %) than ice from Larsbreen (4.1 %) (Table S4, Fig. 6).

4. Discussion

One of the negative effects of the development of civilization and the growing human population is the appearance of various pollutants in the environment, such as pesticides, heavy metals, detergents, wastewater, antibiotics, ARB and ARGs, the last of which has been gaining increasing attention over the course of few decades due to global health risks (WHO, 2019; Stanton et al., 2022). The effects of human activity on the

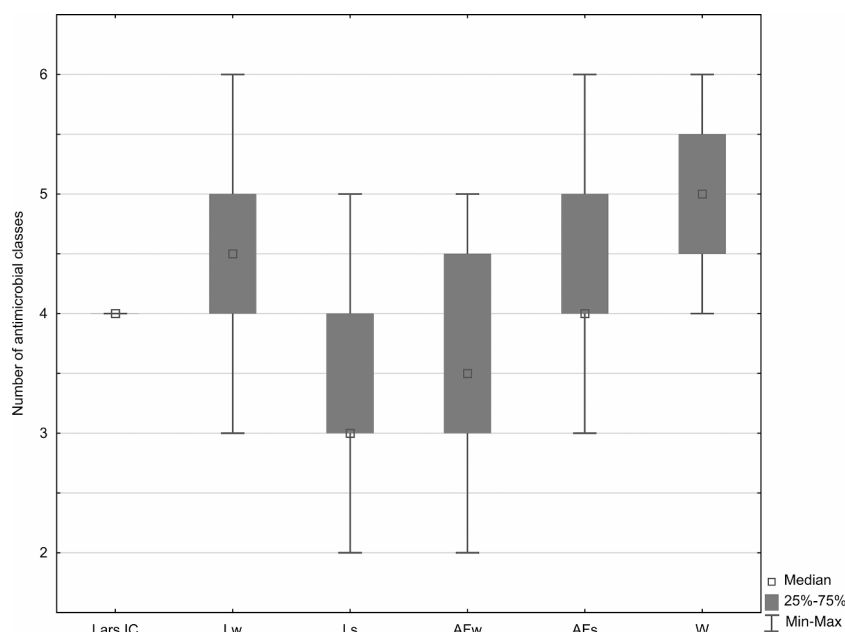


Fig. 4. The antimicrobial resistance profile (defined as the number of antimicrobial classes to which the strain was resistant) of *intI1*-positive strains isolated from ice, freshwater and seawater, sediment samples and wastewater outflow. Legend: Lars IC – Larsbreen ice core, Lw – water of Longyearelva, Ls – sediment of Longyearelva, AFw – water of Adventfjorden seashore, AFs – sediment of Adventfjorden seashore, W – wastewater outflow.

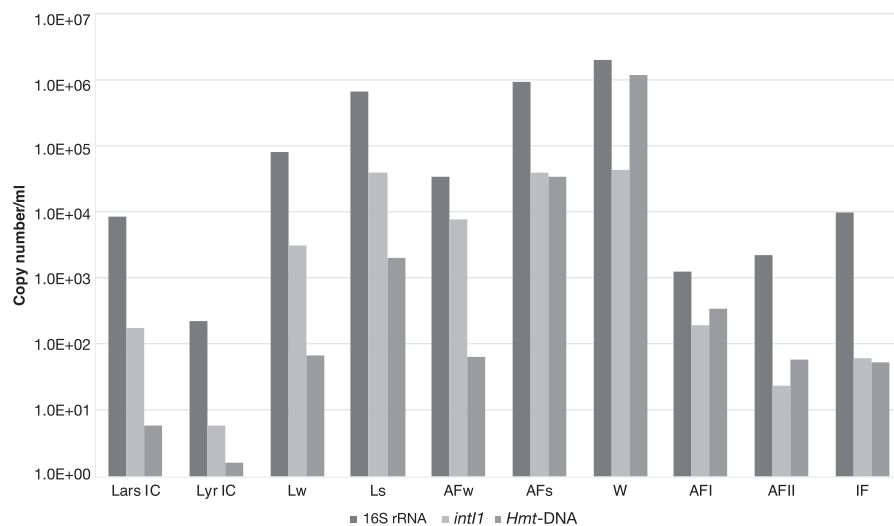


Fig. 5. Copy numbers of the 16S rRNA, *int11* and *Hmt* genes in the metagenome of ice, freshwater and seawater, sediment samples, wastewater outflow and bottom seawater. Legend: Lars IC – Larsbreen ice core, Lyr IC – Longyearbreen ice core, Lw – water of Longyearlva, Ls – sediment of Longyearlva, AFw – water of Adventfjorden seashore, AFs – sediment of Adventfjorden seashore, W – wastewater outflow, AFI – bottom water of Adventfjorden I, AFII – bottom water of Adventfjorden II, IF – bottom water of Isfjorden.

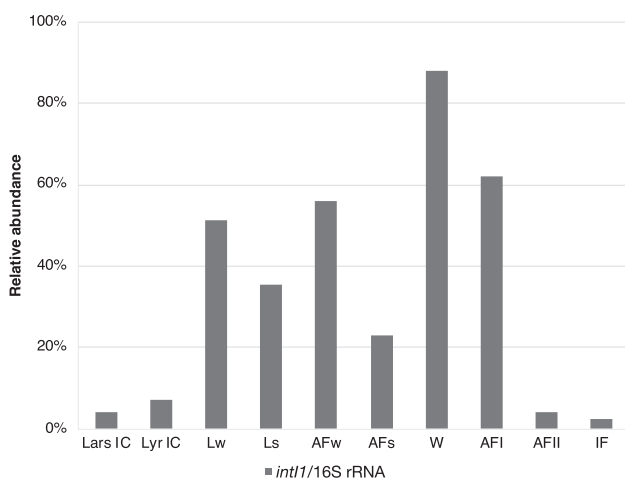


Fig. 6. The relative abundances of the *int11* gene in the metagenomes of ice, freshwater and seawater, sediment samples, wastewater outflow and bottom seawater. Legend: Lars IC – Larsbreen ice core, Lyr IC – Longyearbreen ice core, Lw – water of Longyearlva, Ls – sediment of Longyearlva, AFw – water of Adventfjorden seashore, AFs – sediment of Adventfjorden seashore, W – wastewater outflow, AFI – bottom water of Adventfjorden I, AFII – bottom water of Adventfjorden II, IF – bottom water of Isfjorden.

transfer and dissemination of antibiotic resistance are undisputable (Gillings et al., 2015; Tan et al., 2018). The emergence of a new phenotypes, and the impact on natural selection, microbial biogeochemistry and the animal microbiome are the core of the microbiological effects of the Anthropocene (Gillings and Paulsen, 2014). However, the links between natural resistance and resistance affected by humans remain unclear. Therefore, the essence of this research was to investigate the occurrence and characteristics of integrons in remote and fragile Arctic environments, which are characterized by relatively low anthropopressure and simplicity of ecosystems compared to those of strongly industrialized areas. Here, we used an integrative approach combining culture-based methods with metagenomic studies and revealed the previously unknown diversity and abundance of integrons in the polar environmental resistome. We investigated ice, sediments, freshwater, marine water along with wastewater outflow to the sea as a proxy for ARGs and integron dissemination.

Untreated wastewater with all the contaminants, including microbiological pollution from the city of Longyearbyen, is discharged

directly into Adventfjorden, posing immediate danger to human and marine animal health. Water flowing from melting glaciers may result in an increase in the gene pool of Arctic rivers and fjords at broad geographical scales, contributing to an increase in the gene pool of the Arctic aquatic resistome. Consequently, the delivery of integrons from melting glaciers, as well as sewage, to marine ecosystems is a health hazard due to the generation of new resistance phenotypes among opportunistic pathogens, regardless of the original source (Gillings, 2014; 2017; 2018; Ghaly et al., 2021).

Regardless of the strength of the anthropopressure in our sampling sites, we determined the presence of integrons, integron-associated gene cassettes and genes conferring resistance to sulfonamides in the genomes of bacteria, as well as class 1 integron-integrase and *Hmt* genes in the metagenomic DNA.

4.1. Heterotrophic communities

Glaciers are known as repositories of both inactive and active microbial species, among which heterotrophic bacterial species are rich and abundant (Makowska et al., 2016; Margesin and Collins, 2019; Liu et al., 2022). Therefore, meltwater originating from glaciers may increase the total numbers of heterotrophic bacteria and sulfamethoxazole-resistant heterotrophic bacteria downstream. The highest average total numbers of heterotrophs (5.2×10^3 in water, 8.1×10^3 CFU/ml) and sulfamethoxazole-resistant heterotrophic bacteria (1.3×10^3 CFU/ml) were recorded in sediment samples collected from the seashore; however, this increase was already observed in the Longyearlva, a glacier-fed river outflowing to the fjord. The total number of heterotrophs was 3.4×10^3 in water and 3.0×10^3 CFU/ml in sediment, while sulfamethoxazole-resistant heterotrophic bacteria 9.5×10^1 in water and 4.5×10^2 CFU/ml in sediment of Longyearlva. Our results indicate that glaciers release enormous microbial genomic diversity, including ARB (Makowska et al., 2020; Sajjad et al., 2020). However, it could be also the interplay between glaciers and extra “payload” of heterotrophs from soils in the river catchment.

The literature has reported that at least 10^{17} – 10^{21} viable microbes (including fungi, bacteria and viruses) are released annually from melting glaciers worldwide, which is equivalent to 10^3 – 10^7 metric tons of microbial biomass (Rogers et al., 2004). Moreover, subglacial environments are the source of organic carbon and ancient organic matter for glacial rivers and seas (Bardgett et al., 2007; Koziol et al., 2018). This ancient matter might crop out from the ice or be removed from the basal glacial environments during ice movements and in subglacial outflows, which can contribute to the resistome of river water and sediment.

Nevertheless, the state of knowledge about the released ARB and determinants of resistance is completely unknown. The total number of culturable heterotrophic bacteria was one to three orders lower than the estimated number of bacteria calculated on the basis of the bacterial 16S rRNA gene copy number (Table S4, Fig. 5), which corroborates that most microorganisms originating from polar regions are nonculturable under laboratory conditions using standard culturing techniques. However, culture-dependent methods are necessary for full insight into environmental resistomes, including profiling of both resistance phenotypes and genotypes of bacteria and further assessment of their potential risks to human health (McLain et al., 2016; Su et al., 2017).

4.2. Integrons in bacterial isolates

Initially, integrons were considered to play a key role in the spread of multidrug resistance primarily in clinical settings (Cambray et al., 2010; Lupo et al., 2012). Here, for the first time, we provide data on the frequency of class 1 integron-integrase genes in bacterial strains isolated from glacial ice and aquatic environments of Svalbard. Among the isolates, 5.7 % (48 isolates) had class 1 integrons. An increase in the frequency of integrons in heterotrophs (4.3 % isolated from water and 6.7 % from sediment) and in heterotrophic bacteria resistant to sulfamethoxazole was already observed in the Longyearlva. Only Makowska et al. (2020) have shown the presence of strains with class 1 integrons in cryoconite (biogenic sediment on the glacier surface, Rozwalak et al. (2022)) on glaciers in Svalbard, the Caucasus and Greenland. Glaciers in Svalbard (Longyearbreen) and Greenland (Russel Glacier) are impacted by both tourists and scientists, however, the integrons were detected even on the low human-impacted Adishi Glacier in the Caucasus (Makowska et al., 2020). Although other studies investigated ARGs and ARB in snow and ice of Svalbard, no data on integrons were provided (Segawa et al., 2013). We show that not only the surface of glaciers but also glacial ice can be a reservoir of bacteria with integrons, which may spread via water from melting glaciers. The diversity of integrons in bacteria, including ARB that we found in ice cores only, corroborates the hypothesis that ARB, ARGs and *intI1* might accumulate in glacial ice in low abundance; however, glacier melting accelerates and accumulates all the antibiotic resistance cargo in meltwater released into the glacial river. Additional anthropic input, although minor, may have occurred in the river samples due to activity of tourists and citizens, especially in the area of the city. At the same time, through large masses of organic sludge, the river greatly enriches the water of Adventfjorden in *intI1*-positive bacteria. Furthermore, a strong correlation between the occurrence of the *sul1* gene and class 1 integrons indicates the association of the *sul1* gene with the structure of class 1 integrons and suggests the *sul1* gene is a marker of integrons in various environments (Partridge et al., 2009; de los Santos et al., 2021), including polar ecosystems.

4.3. The variable regions of integrons

The variable regions of integrons revealed the presence of genes encoding proteins of different functions, including antibiotic resistance, virulence and physiology, most of which may increase adaptation to harsh, nutrient-limited polar ecosystems (Chek et al., 2017; Gillings, 2014; 2017; Mitra et al., 2021).

In the variable regions of *intI1*-positive strains from water of the Longyearlva gene cassettes involved in antibiotic resistance is *fusC* carried on mobile elements and encodes a protein-mediating resistance to fusidic acid (Monecke et al., 2021). Moreover, the (GCN5)-related *N*-acetyltransferase (GNAT)-encoding gene determined resistance to aminoglycosides (Srivastava et al., 2014), and hemerythrin-like protein-encoding genes, which affects oxidation–reduction regulation and resistance to erythromycin (Li et al., 2015). The gene cassette *tamB* determined virulence. The *tamB* gene encodes the translocation and assembly module (TAM), a protein channel mediating the secretion of virulence factors during pathogen infection (Li et al., 2020). The

variable region of integrons also included other genes directly or indirectly responsible for the production of membrane transporters, e.g., cassette array-containing genes encoding ABC transporter-like proteins forming efflux pumps. Moreover, in the variable region genes encoding histidine kinase, and an AMP-binding protein, playing a role in cAMP signaling, along with several proteins of unknown functions were found (Khorchid and Ikura, 2006; Endoh and Engel, 2009). Most of them may increase adaptation to the environment, but some might have an impact on bacterial resistance. One of the gene cassettes found in this study contained the *phaC₁* and *phaC₂* genes encoding type II polyhydroxyalkanoate (PHA) synthases. PHA synthase is a key enzyme in the polymerization of polyhydroxyalkanoate (PHA), a biopolymer produced by various microorganisms as carbon and energy storage components (Reddy et al., 2003). Cells accumulate PHA when the carbon source is available in excess, with a simultaneous deficiency of other nutrients, such as nitrogen or phosphorus, the amount of which is limited in the water from melting glaciers (Chek et al., 2017; Mitra et al., 2021).

Variable regions of class 1 integrons of bacteria from wastewater outflow contained gene cassettes coding for the multidrug efflux MFS transporter MdtM and multidrug efflux RND transporter periplasmic adaptor subunit AdeG, directly conferring antibiotic resistance. Drug efflux is a common resistance mechanism in pathogens and relies on overexpressed multidrug resistance transporters, which may group into the major facilitator superfamily (MFS) and resistance-nodulation-division (RND) superfamily. MdtM belongs to the clinically relevant MFS superfamily of efflux pumps and determines resistance to chloramphenicol, cationic antimicrobials, and biocides (quaternary ammonium compounds) (Nishino and Yamaguchi, 2001; Holdsworth and Law, 2012). RND efflux pumps are widespread, especially among gram-negative bacteria and determine resistance to many antibiotics and chemotherapeutic agents (Alav et al., 2018). Taking into account all identified gene cassettes in this study, the efflux pumps are the most dominant resistance mechanism in bacteria isolated from Arctic aquatic environments, also reported by McCann et al. (2019) in soil cores collected from Spitsbergen. The remaining integrons carry genes coding for proteins of physiological significance, which may increase the adaptation of bacteria to extreme environments (Gillings, 2014; 2017; Makowska et al., 2020). Within the variable parts of class 1 integrons, we found genes encoding the Tol/Pal system protein YbgF, which is necessary for colicin A uptake and maintains cell envelope integrity, sigma factor RpoS and sigma factor RpoD, whose regulation in response to environmental stressors, such as temperature changes and exposure to UV light, osmotic shock, oxidative stress and nutrient deprivation, enables bacterial cell survival (Walburger et al., 2002; Fu et al., 2015). Furthermore, RpoD has also been found responsible for genes that confer tetracycline resistance, whereas the RpoS sigma factor is crucial for virulence in many bacterial pathogens (Meena et al., 2019). Taking into account the low temperature of aquatic environments in the Arctic, strong seasonality, frequent freeze–thaw cycles, and high doses of UV radiation (especially on ice surfaces), adaptation to harsh environments is of crucial importance. Moreover, some mechanisms may trigger the environmental versatility of bacteria (such as adaptation to osmotic stress), especially when they are thawed from ice and then transported through suspension-rich freshwater rivers to marine ecosystems in fjords.

Class 1 integrons of bacteria from water and sediment of the Longyearlva as well as water of the Adventfjorden seashore had empty integrons with no genes inserted into the variable region. Recent studies have shown that the presence of empty integrons may also be associated with highly human-impacted environments such as wastewater (Quintela-Baluja et al., 2021). Moreover, the presence of integrons with no genes inserted into the variable region in the environment may indicate that gene cassettes are excised from the structure in the absence of antibiotic selective pressure (Rosser and Young, 1999) or that they represent ancestral elements that have not yet acquired gene cassette inserts, representing great potential for the acquisition and creation of

novel gene combinations (Stokes et al., 2006).

4.4. Antimicrobial susceptibility

Almost all *intI1*-positive strains were multidrug resistant (93.3 %). Although there are no typical gene cassettes in the structure of integrons that determine resistance to specific antibiotics, multidrug antibiotic resistance of bacteria is closely associated with the presence of class 1 integrons (Gillings, 2014; 2017). Our results did not show significant differences in resistance profiles among strains isolated from the various Arctic environments. This finding suggests that integron-associated multidrug resistance is a phenomenon that widely occurs in the environment, regardless of the level of human activity.

4.5. Metagenomic analysis of integrons and *Hmt*-DNA

The *intI1* and *Hmt* were recorded in all analyzed samples. The highest mean copy number was obtained for *intI1* in wastewater outflow (4.4×10^4 /ml and 1.2×10^6 , respectively) and the lowest in ice from Longyearbreen (0.6×10^1 /ml and 0.2×10^1 , respectively). Indeed the wastewater in Svalbard contributes to the spread of contaminants (Kalinowska et al., 2020; Herzke et al., 2021) effectively increasing the gene pool of the resistome.

The use of human mitochondrial DNA (*Hmt*-DNA) is a promising molecular marker to assess the impact of anthropogenic activity. The source of *Hmt*-DNA is each cell originating from human (e.g., skin or intestinal tract cells) (He et al., 2015; Tan et al., 2018). The presence of *Hmt*-DNA in Arctic glacial ecosystems or glacial-fed rivers indicate that even unoccupied human systems are not free from human-related contaminants. As shown in Fig. 5, *Hmt* gene abundance was different between heavily impacted wastewater outflow samples (1.2×10^6 /ml) and less impacted ice core samples (1.0×10^1 /ml for Larsbreen and 0.1×10^1 /ml for Longyearbreen), still present in glaciers. Similar results were observed by Tan et al. (2018) who found higher abundance of *Hmt* in heavily impacted regions, including the Haihe River (1.10×10^6 copies/g sediment) and the Tianjin Water Park (8.03×10^5 copies/g sediment) in China, and it was more than two to three orders of magnitude higher than those of sediment samples from sub-Arctic and Arctic regions (4.37×10^2 to 2.35×10^3 copies/g sediment). Moreover, a very strong correlation between *intI1* and *Hmt* copy numbers in metagenomic DNA supports the use of *Hmt*-DNA as a suitable indicator for evaluating anthropogenic pollutants such as *intI1*. Our results suggest that human activities play an important role in the accumulation and dissemination of integrons in Arctic environments, which corroborate other studies conducted in relatively pristine environments (McCann et al., 2019; Sajjad et al., 2020; Sanchez-Cid et al., 2022). In metagenomic studies, McCann et al. (2019) found *intI1* in high Arctic soil ecosystems, which indicates the development of antibiotic resistance and accumulate in even the most remote locations.

The relative abundance of *intI1*, calculated by normalization to the copy number of the 16S rRNA gene, was the highest in wastewater outflow (88.0 %). The lowest relative abundance of *intI1* was found in Isfjorden (2.4 %), which is a large and open fjord system with a strong impact on the Greenland Sea and marine currents, most likely influenced by dilution. Moreover, there was an increase in the relative abundance of *intI1* in water (51.1 %) and sediment (35.6 %) of Longyearbreen compared to those of Larsbreen (4.1 %) and Longyearbreen (7.1 %) glaciers. Thus, glaciers may enrich rivers in integrons by releasing biological material during each melting season, which then potentially accumulates in sediments or is transported and diluted in the fjord. However, we cannot exclude that soils in the catchment or human traffic in area above Longyearbyen city center could be an additional source of the *intI1* in the river.

The relative abundance of the studied genes was higher on Longyearbreen than on Larsbreen, suggesting that Longyearbreen is a more polluted glacier and may constitute a larger reservoir of integrons.

Longyearbreen, due to its relatively easy access, is commonly visited by tourists, hikers and students. Thus, even a very minor amount of contaminated water that possibly percolates ice could increase the presence of integrons found in ice cores. There is little information in the literature on the abundance of class 1 integron-integrase genes determined by metagenomic approaches in aquatic environments of polar regions and glacial habitats worldwide (Makowska et al., 2020). Our results suggest that glacial ice may be a reservoir of integrons and associated genes and may accumulate contaminants currently considered biotic pollutants, which mix with water from melting glaciers and may disseminate into the environment (Hauptmann et al., 2017; Makowska et al. 2020).

5. Conclusion

Our results indicate the existence of pathways for the spread of integrons in Svalbard leading from the glacial environment through the proglacial river and municipal sewage to the marine environment, posing a threat to human and animal health. The presence of ARB and integrons in the marine environment may potentially cause infections in marine vertebrates affecting their microbiome, and thus posing a risk of transferring ARBs and integrons back into humans and the environment. We focused on integrons as markers of anthropogenic pollution and characterized associated gene cassettes in the genomes of culturable bacteria, as well as class 1 integron-integrase genes in the metagenome of ice, freshwater and seawater, sediment samples, wastewater outflow and bottom seawater from high Arctic environments of Svalbard. We detected ARGs, class 1 integrons in ARB isolated from wastewater outflow, glacial environments, water of Longyearbreen and Adventfjorden. Our findings show that even a low and legal transport of wastewater to the marine environment leaves a human fingerprint on the Arctic ecosystem. Moreover, in the variable regions of integrons, the occurrence of genes determining different functions, including antibiotic resistance, virulence and physiology was noted, highlighting the crucial role of these genes in adaptation and versatility to stress (osmotic, light, temperature) in Arctic environments. The relative abundance of the *intI1* gene was reported in metagenomes with different effects of human activity (ice cores vs wastewater outflow) and was positively correlated with abundance of the *Hmt* gene, mirroring both natural and human roles in shaping the polar aquatic resistome. Thus, natural factors such as water from melting glaciers as well as human activity such as wastewater discharged directly from Longyearbyen to Adventfjorden, contribute to the enrichment of the marine resistome of this Arctic region. Potentially, similar effects may exist in other Arctic regions where the terrestrial cryosphere is melting and human settlements exist.

6. Data availability statement

All data generated or analyzed during this study are included in this published article (and its supplementary information files) and are deposited in the public database GenBank (accession numbers ON795922-ON795938 and ON886241-ON886277).

CRedit authorship contribution statement

Nicoletta Makowska-Zawierucha: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Joanna Mokracka:** Formal analysis. **Marcelina Małacka:** Formal analysis. **Piotr Balazy:** Investigation, Writing – review & editing. **Maciej Chelchowski:** Investigation. **Dariusz Ignatiuk:** Investigation, Methodology, Visualization. **Krzysztof Zawierucha:** Conceptualization, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2022.109633>.

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