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Recreational hot springs as environmental reservoir of potential multidrug-resistant pathogens

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1. Introduction

The past decade of research on the interdependence of human, animal, and environmental health has highlighted the importance of investigating and interpreting the spread of pathogens at the ecosystem level, in a "One Health" framework (Destoumieux-Garzón et al., 2018). The connection between environmental status and human health appears even stronger in the wake of the recent SARS-CoV2 pandemic, which likely emerged from human interactions with the environment and unsafe food handling (De Sadeleer and Godfroid, 2020). In this context, antibiotic resistance has become a serious problem in clinical settings (French, 2005), with the development of new multidrug resistance in several groups of pathogens representing a public health threat (Tanwar et al., 2014). According to the most recent Center for Disease Control (CDC) report, more than 2.8 million antibiotic-resistant infections occur every year CDC 2019, 2023), with trends projected to increase for the next decade.

Some clinical resistance genes have also been found to originate from environmental strains (Wright, 2010). The myriad interactions between pathogens and environmental bacterial communities favors the acquisition of new resistance (Amalfitano et al., 2015). Ecosystems that come into contact with contamination vectors (for example sewage runoffs) may become reservoirs for the spreading and the evolution of new emerging drug resistant pathogens (Harris et al., 2012; Wang et al., 2021) that subsequently make their way back to the general population (Kraemer et al., 2019). Ingestion or contact with water contaminated by bacteria, viruses or protozoa can lead to several infections (Leclerc et al., 2002). In this way, new outbreaks of emerging opportunistic pathogens may occur, like those reported in Central Europe where the emerging pathogenic genus *Arcobacter* has been shown to be one of the main causes of enteric disease (Pérez-Cataluña et al., 2017; Prouzet-Mauléon et al., 2006). Together with emerging strains, the main healthcare-associated pathogens are represented by *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Motbainor et al., 2020), which

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cause a broad spectrum of infections from skin and wounds (Fleming et al., 2017; Kim et al., 2015), and in rare cases necrotizing fasciitis (Charnot-Katsikas et al., 2009; Reisman et al., 2012). *Pseudomonas aeruginosa* alone is responsible for 10–20% of nosocomial infections in intensive care units (Nicastri et al., 2003), and a high prevalence of these infections are caused by drug resistant phenotypes (Gill et al., 2016).

The capacity of several groups of pathogens to persist in different kinds of environments and spread through them remains a worldwide concern (Exner et al., 2005). Often, the discovery of environmental pathogens is associated with water bodies like alpine rivers and lakes (Di Cesare et al., 2017; Eckert et al., 2018), urban rivers (Cui et al., 2019) and coastal regions where fecal bacteria can diffuse out from areas of major concentrations of human land-based activities (Manini et al., 2022; Walters et al., 2011; Wang et al., 2017). Intense adverse weather events, likely to increase in frequency and intensity due to the current climate crisis (Ebi et al., 2021), are also correlated with increased pathogens and microbial resistance genes in the environment (Di Cesare et al., 2017; Manini et al., 2022). Intensive agriculture also contributes to water quality impairment due to animal feeding operation or land applied manure (Rosen et al., 2000). Berg et al. (2005) showed that the soil can host a huge number of well known and emerging pathogens which cover a broad spectrum of clinical syndromes, underscoring the need to understand more about the colonization and transmission strategies of the opportunistic human pathogens through the rhizosphere. Recent studies focused their attention on potential reservoirs like hospital sewage where pathogens can acquire antibiotic resistance (Zhang et al., 2013). In the same way, the release of sewage sludge as soil fertilizer containing antibiotics, antibiotic-resistant bacteria, and antibiotic resistance genes increase the environmental pool of antibiotic resistance genes and consequently favor the horizontal gene transfer of resistance among environmental microorganisms, constituting a significant reservoir of potential antibiotic-resistant pathogens (Bondarczuk et al., 2016; Calero-Cáceres et al., 2014).

Besides soils, recreational water can be an important source of disease caused by pathogenic organisms, (Purnell et al., 2020). Water quality monitoring and management are the main approaches to reduce the potential infection risk, but the high variety of potential pathogenic organisms complicates the quantification and detection procedures. The most common indicator of recreational water contamination are fecal bacteria like *E. coli* or Enterococci, which can be tracked with conventional or alternative methods (Manini et al., 2022; Rodrigues and Cunha, 2017). However, the efficacy of water treatment to reduce the infection rate by pathogens is not always clear; according to the US Center for Disease Control, 41% of outbreaks related to recreational water that had been treated to decrease pathogens were associated with hot tubs/spas and can be caused by pathogens from the genera *Cryptosporidium*, *Legionella* and *Pseudomonas*. On the other hand, outbreaks related to untreated recreational waters like those of lakes, rivers or oceans are mostly caused by enteric pathogens when fecally contaminated water is ingested. Sources of contamination can be stormwater runoff, sewage treatment plant discharge, and animal or waste associated with boating (Graciaa et al., 2018; Hlavsa et al., 2018).

In this context, water monitoring strategies are of primary importance to protect the public from infection risks due to pathogens harbored in recreational environments. The World Health Organization (WHO) has established specific guidelines for the monitoring of water quality (World Health Organization, 2003), which have been adopted with specific legislations by numerous countries. Water types monitored worldwide include drinking water and treated (often chlorinated) or untreated recreational waters. According to the WHO, the levels of bacteria like *E. coli*, *P. aeruginosa* and *Legionella* spp. in public pools should be less than 1 colony forming unit (CFU) per 100 mL of water (World Health Organization, 2006).

Hot springs constitute terrestrial hydrothermal systems that origin from geothermally-heated groundwater which arises from deep Earth's crust as a consequence of tectonic and magmatic processes (Kresic, 2010). These hydrothermal systems are globally distributed and there are evidences for the important role that hot springs played in human society since Greek and Roman times, when these were not only used for recreational and social purposes but were also considered sacred sites with powerful healing properties (Des Marais and Walter, 2019; Erfurt, 2021; Lamoreaux, 2005; van Tubergen and van der Linden, 2002). Currently, the recreational use of hot springs supports a growing sector of tourism worldwide with an estimated industry worth in excess of 40 billion US dollars (Global Wellness Economy Monitor, 2023). Unrestrained commercial tourism, however, can lead to an irresponsible use of natural resources (Erfurt, 2021; Mavridou et al., 2018). Land over-exploitation for large-scale resort developments, wrong disposal of waste water and septic systems can bring to deterioration of groundwater aquifers, including hydrothermal reservoirs (Erfurt, 2021; Jack et al., 2013; Page et al., 2014). Given the hundreds of millions of people that use recreational, naturally-occurring hot springs, and spas annually, it does not come as a surprise that hot springs might be connected with infections (Mavridou et al., 2018). Several cases have been reported in the last decades, frequently linked to bacteria belonging to genus *Bacillus* (Pandey et al., 2015), *Legionella* (James et al., 2022; Ji et al., 2014) *Pseudomonas* (Mukherjee et al., 2012; Rahel et al., 2021), and free-living protozoa (Fabros et al., 2021). The presence of *P. aeruginosa* as a cause of folliculitis linked to recreational water was described for the first time in 1975 (McCausland and Cox, 1975). Since then, several cases of infection related to *P. aeruginosa and* recreational water usage have been reported (Tate et al., 2003). Infections caused by the free-living amoebae, *Naegleria fowleri*, have made news due to deadly cases of meningoencephalitis linked to hot spring use (Abrahams-Sandí et al., 2015; Vugia et al., 2019). Despite growing reports of infection cases linked with the recreational use of hot springs, information relative to the diversity and distribution of potential pathogens in naturally-occurring hot springs are limited (Ghilamicael et al., 2018; Jardine et al., 2017; Mavridou et al., 2018).

Here, we investigated the distribution of potential pathogens and antimicrobial resistance genes in several Costa Rican hot springs, comparing the ones used for recreational purposes in resorts and spas with natural-occurring hot springs located in more remote areas. Culture independent analyses based on 16S rRNA amplicons and shotgun metagenomic sequencing were used to: i) detect the springs with higher abundance of putative pathogenic bacteria; and ii) study the presence of antimicrobial resistance genes. In addition, a group of recreational hot springs were investigated through culture dependent analyses to isolate putative pathogens and test their antibiotic susceptibility. Our results show the presence of diverse potential pathogens linked with the recreational hot springs as well as the occurrence of multidrug-resistant pathogens. Omics analyses and pure cultures isolated from the springs suggest that hot springs represent a major environmental reservoir of potential multidrug-resistant pathogens and emphasize that additional management measures need to be introduced to increase public safety.

2. Methods

2.1. Sampling approach

During two sampling campaigns in 2017 (CR17) and 2019 (CR19), a total of 22 individual hot springs were sampled spanning the volcanic areas of Costa Rica (Table 1). Two types of pools were sampled: naturally occurring hot springs (n = 15), hot springs which had been developed for recreational use (n = 6) located within spas and resorts, and one boiling mud pond located within a resort (Fig. 1). Several of the natural hot springs had a detectable amount of anthropization, presumably due to occasional bathing of the local populations or due to use for washing, cleaning, and other household activities. However, the number of people accessing the natural systems was presumably limited compared to recreational springs. In 2017, hot spring hydrothermal fluids and sediments (Table 1) were collected following the protocols

Table 1
Location and main environmental features of the hot springs sampled.

Hot spring	Sampling year	Sample type[a]	Hot spring type	Altitude (m)	Temperature (°C)	pH	Salinity (g/L)
S1	2017	S	Recreational/Natural	535	88.9	2.11	2.7
S2	2017/2019	F/S	Recreational	437	59	6.16	3.3
S3	2017/2019	F/S	Recreational	434	53.8	5.87	3.1
S4	2017/2019	F	Recreational	557	42.7	6.19	29.3
S5	2017/2019	F/S	Recreational	368	41.4	6.19	0
S6	2017	F/S	Recreational	553	60	6.24	1.8
S7	2017	F	Recreational	166	59.1	6.32	3.4
S8	2017	F/S	Natural	184	72	6.31	3.2
S9	2017	F/S	Natural	NA	26.4	9.99	0.1
S10	2017	F	Natural	122	27.9	9.75	0.1
S11	2017	F/S	Natural	109	55.2	5.93	3
S12	2017	F/S	Natural	765	87.9	1.82	3.3
S13	2017	F	Natural	53	28.7	5.81	2
S14	2017	F/S	Natural	298	48.7	8.53	2.2
S15	2017	F/S	Natural	300	36.7	8.69	1.4
S16	2017	F/S	Natural	429	22.9	5.6	0.1
S17	2017	F/S	Natural	82	29.4	9.96	0.1
S18	2017	F/S	Natural	36	35.9	9.83	1.8
S19	2017	F/S	Natural	165	57	6.12	1.4
S20	2017	F	Natural	173	31.8	9.25	0.1
S21	2017	F/S	Natural	2209	55.8	4.51	2.98
S22	2017	F/S	Natural	436	59.8	5	7.19

[a] Sample type: 'F' indicate hot spring water fluids while 'S' indicate hot spring sediments.

previously described (Barry et al., 2019; Fullerton et al., 2021; Rogers et al., 2022) and using the rationale described in detail in Giovannelli et al. (2022). Briefly, at each sampling site, hot spring hydrothermal fluids (0.5–1L) were filtered from the spring inlet through Sterivex 0.22 μm filter cartridges (MilliporeSigma) and 15 mL falcon tubes were filled with surficial sediments. Both filters and sediment-filled tubes were instantaneously frozen onsite at 196 °C in a cryogenic dry shipper (Termo Fisher Scientifc, Arctic Express 20) for transport back to the home laboratory. Samples for cell counts were fixed in 3% formaldehyde (Hayat, 2012) and kept at 4 °C. In 2019, hydrothermal fluids from four recreational hot springs already sampled in 2017 (Table 1) were collected in sterile 50 mL falcon tubes storing a part of samples directly at 4 °C for isolation purposes (see Table 2).

2.2. Colony forming units

Hot spring hydrothermal fluids for colony forming unit (CFU) analysis were kept at 4 °C until processing. Briefly, 7–10 mL per sample were filtered on a 47 mm diameter 0.22 μm filter using a sterile filtering apparatus. In order to restrict the growth of non-target bacteria, the filter was placed on top of a defined agar plate, either using selective media such as Leeds Acinetobacter Medium (Jawad et al., 1994; McConnell et al., 2011) and Pseudomonas Selective Agar CN media (Goto and Enomoto, 1970; Weiser et al., 2014). For each condition, two replicate samples were incubated overnight at 37 °C in accordance with standardized protocols (APHA, 2017). CFUs were manually counted on a colony counter and normalized to the fluid volume filtered (2020).

2.3. Genomic DNA extraction and sequencing

Genomic DNA was extracted from Sterivex filters and from sediment following a modified protocol from (Giovannelli et al., 2016). Genomic DNA was visualized on 1.2% w/v agarose gel and quantified using a Nanodrop spectrophotometer. In 2018, extracted DNA was subjected to both amplicon sequencing and shotgun metagenome sequencing. Amplicon sequencing was carried out after amplifying the bacteria-specific V4-V5 region of the 16S rRNA gene using primers 518F (AATTGGANTCAACGCCGG) and 926R (CCGYCAATTYMTTTRAGTTT) (Parada et al., 2016). Amplicon and shotgun metagenome sequencing was performed as part of the Census of Deep Life initiative with the Deep Carbon Observatory and performed at the Marine Biological Laboratory sequencing facility (https://www.mbl.edu/) on an Illumina MiSeq platform for samples from 2017. In both cases, the Illumina Nextera Flex kit for MiSeq + NextSeq, which requires a very small amount of starting material (1 ng) was used. Obtained shotgun metagenomes varied from 25 to 150 million base pairs. Amplicon sequencing data is available from the NCBI SRA archive with accession numbers PRJNA579365, while the shotgun metagenomes are deposited in PRJNA627197.

2.4. Strain isolation and testing

Small aliquots of hydrothermal fluids from recreational hot springs sampled in 2019 (Table 1) were inoculated in nutrient broth (BD, Difco) and incubated at 37 °C for several days. Afterward, dilutions to extinction were performed, following a further incubation at 37 °C. Small volumes of enrichment showing positive growth were transferred on a Nutrient -agar plate and incubated at 37 °C for 12–18 h. From them, singular bacterial colonies were systematically picked and streaked on Nutrient - agar plates in order to obtain isolated colonies. To identify *Pseudomonas* spp. strains, DNA was extracted and the 16S rRNA gene was amplified by PCR with universal primers 16S-8F (AGA GTT TGA TCC TGG CTC AG) and 16S-1517R (ACG GCT ACC TTG TTA CGA CTT). PCR products were sequenced with Bio-Fab Research, and identities were confirmed by blasting DNA sequences against the NCBI database. Sequences of the *Pseudomonas* spp. strains isolated and analyzed in this study are available through the European Nucleotide Archive (ENA) under project accession PRJEB61745. Small aliquots of hydrothermal fluids from naturally occurring hot springs were tested directly on selective media such as Leeds Acinetobacter Medium and Pseudomonas Selective Agar CN media and incubated at 37 °C.

2.5. Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed on all the *Pseudomonas* spp. identified strains, according to the European Committee and Antimicrobial Susceptibility Testing (EUCAST) Poole, 2011 and Clinical and Laboratory Standards Institute (CLSI, 2018) providing tables that list breakpoint guidelines to interpret antimicrobial resistance. *Pseudomonas* PAO1 was included in the analysis as a reference strain. Pure cultures were obtained on Mueller-Hinton agar plates after 12 h at 37 °C and individual colonies were used to prepare the suspension for inoculation in liquid Mueller-Hinton media, two replicates for each sample.

Table 2
Results of the antibiogram on the isolated *P. aeruginosa*.

Hot spring	Sample	EUCAST				CLSI			
		Meropenem (M, 10 μg)	Ciprofloxacin (Cp, 5 μg)	Gentamicin (G, 10 μg)	Cefepime (Cf, 30 μg)	Meropenem (M, 10 μg)	Ciprofloxacin (Cp, 5 μg)	Gentamicin (G, 10 μg)	Cefepime (Cf, 30 μg)
S5	36	I*	S	R	S	I	S	R	S
S5	38	I	S	S	S	S	S	S	S
S5	40	S	S	S	S	S	S	S	S
S5	41	S	S	S	R	S	S	S	S
S5	42	S	S	S	S	S	S	S	S
S5	43	S	S	S	S	S	S	S	S
S5	44	S	S	S	S	S	S	S	S
S5	45	S	S	S	R	S	S	S	S
S5	46	S	S	S	R	S	S	S	S
S5	47	R	R	R	S	R	S	I	S
S5	48	I	R	R	R	S	S	I	S
S5	49	R	R	R	R	R	S	I	I
S5	50	I	R	R	R	S	S	I	S
S5	51	I	R	R	R	S	S	I	S
S5	52	R	R	R	R	R	S	I	S
S5	53	I	S	S	R	S	S	S	S
S5	54	I	S	R	R	S	S	I	S
S5	55	I	R	S	S	S	S	S	S
S5	56	I	R	R	R	S	S	R	S
S5	57	S	S	R	R	S	S	I	S
S5	58	I	S	S	R	S	S	S	S
S5	59	I	S	R	R	S	S	I	S
S5	60	I	R	R	R	I	S	I	S
S5	61	S	S	R	R	S	S	I	S
S4	62	S	S	R	S	S	S	R	S
S4	63	S	S	R	R	S	S	I	S
S4	64	I	S	R	S	S	S	I	S
S4	65	I	S	S	S	S	S	S	S
S4	66	S	S	R	S	S	S	I	S
S4	67	I	S	S	S	S	S	S	S
S4	68	S	R	R	S	S	S	I	S
S4	69	I	S	R	R	S	S	I	S
S4	70	S	S	S	S	S	S	S	S
S4	71	S	S	R	S	S	S	I	S
S4	72	I	S	R	S	S	S	R	S
S4	73	I	R	R	R	S	S	I	S
S2	84	S	S	R	S	S	S	I	S
S2	85	I	R	R	S	S	S	I	S
S2	86	I	S	R	S	S	S	I	S
S2	87	I	R	R	R	S	S	R	S
S2	88	I	R	R	S	S	S	R	S
S2	89	S	R	R	S	S	S	R	S
S3	90	S	R	R	S	S	S	I	S
S3	91	I	R	S	S	S	S	S	S
S3	92	I	R	R	R	S	I	I	S
S3	93	I	R	R	R	S	S	I	S
S3	94	S	R	R	R	S	S	I	S
S3	95	S	R	S	R	S	S	S	S

Note: R=Resistant, S=Sensitive, I=Intermediate. EUCAST Zone diameter breakpoint: Meropenem S ≥ 24, R < 18. Ciprofloxacin S ≥ 26, R < 26. Gentamicin S ≥ 15, R < 15. Cefepime S ≥ 21, R < 21. * The Meropenem intermediate value has been arbitrarily defined because it is absent in the EUCAST breakpoint table. CLSI Zone diameter breakpoint: Meropenem S ≥ 19, I 16–18, R ≤ 15. Ciprofloxacin S ≥ 21, I 16–20, R ≤ 15. Gentamicin S ≥ 15, I 13–14, R ≤ 12. Cefepime S ≥ 18, I 15–17, R ≤ 14.

After an overnight incubation, an aliquot of the liquid culture was measured on the UV/VIS Spectrophotometer at the absorbance of 625 nm, until reaching 0.5 McFarland turbidity standard. Then, the suspension was inoculated on Mueller-Hinton agar plates and test disks were applied over the surface. The antibiotics were organized in two groups, specific and generic, the first group refers to recommended antibiotics for antimicrobial susceptibility testing by EUCAST and CLSI instead the second group refers to generic antibiotics of common use. Meropenem (M, 10 μg), Ciprofloxacin (Cp, 5 μg), Gentamicin (G, 10 μg), Cefepime (Cf, 30 μg) are part of the specific group. Erythromycin (E, 30 μg), Cefsulodin (C, 30 μg), Ampicillin (A, 10 μg), Kanamycin (K, 30 μg) are part of the generic group. After overnight incubation at 37 °C, the inhibition zone diameter was measured according to the EUCAST and CLSI BreakPoint Table (http://www.eucast.org/clinical_breakpoints/, https://clsi.org/). Finally, relative abundance of antibiotic resistance was calculated as the number of bacterial strains resistant per hot spring tested.

2.6. Bioinformatic and statistical approaches

Amplicon sequencing analysis was previously described in Fullerton et al. (2021). Briefly, obtained 16S rRNA reads were processed using MOTHUR (Schloss et al., 2009), following the Miseq standard operating procedure for the identification of Amplicon Sequencing Variants (ASVs). Taxonomy was assigned using the RDP naive Bayesian classifier against the Silva v132 release (Quast et al., 2013). All statistical analyses, data processing and plotting were carried out in the R statistical software (Core, 2021), using the phyloseq (McMurdie and Holmes, 2013) and ggplot2 (Wickham, 2011) packages, as previously reported (Barosa et al., 2023; Cordone et al., 2022, 2023). Briefly, the obtained count table, taxonomy assignment and phylogenetic tree were combined together with the environmental variables into a phyloseq object. Low

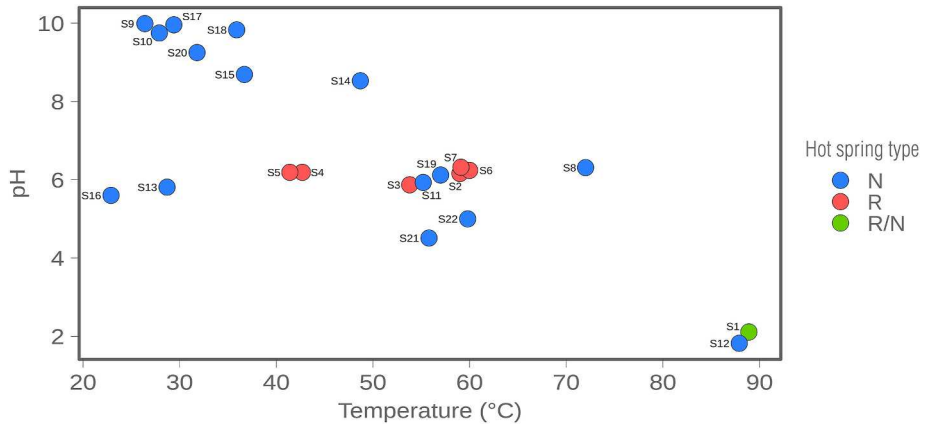

Fig. 1. Temperature and pH of the sampled hot springs. Recreational hot springs placed within spa and resort are colored in blue while natural hot springs are in red. Spa/resort hot springs not linked with recreational waters usage are indicated in green.

prevalence ASVs, mitochondria and chloroplast related sequences were removed. In both fluids and sediments, common laboratory contaminants from DNA processing, feces, and skin (Sheik et al., 2018) were largely absent (<0.04% in the entire dataset and less than 0.01% in any individual library), and no ASV was shared by all samples. After QC and filtering a total of 2,346,695 reads comprising 40,690 ASVs were obtained. Normalization of the ASVs count was carried out using the median library size across the dataset. Genera from putative pathogenic species were used for downstream analysis. A list of the putative pathogenic genera searched in the libraries is available as Supplementary Table 1. Beta-diversity was investigated through the Weighted Jaccard dissimilarity index implemented in the Vegan package (Oksanen et al., 2018). The resultant matrix was displayed through a Non-metric Multidimensional Scaling (NMDS) with the aim to understand any relationship between the distribution of the putative pathogens and the sampled hot springs. Vector fitting analysis (Vegan package) was used as well to estimate any effect of the environmental parameters on the hot spring putative pathogens. Shotgun metagenomic assembly, binning, and annotation were previously described in Rogers et al. (2022), Briefly, raw reads were quality checked and trimmed using Trimmomatic v 0.38 (Bolger et al., 2014) using default settings. De novo assembly was carried out with metaSPAdes (Nurk et al., 2017) with standard parameters and a minimum contig lenght of 1.5 kb. Assemblies were used to build 404 metagenomic assembled genomes (known as MAGS/bins) with ≥70% completeness and <5% contamination (Bowers et al., 2017; Grettenberger and Hamilton, 2021) through the MetaWRAP pipeline (Uritskiy et al., 2018) and checked using CheckM (Parks et al., 2015). Further details on the obtained MAGs are available in Rogers et al. (2022). Post QC raw reads were used to assess the taxonomic composition with Kaiju (Menzel et al., 2016). A small group of assembled contigs were searched against the Comprehensive Antibiotic Resistance Database (CARD; Alcock et al., 2023) to identify antimicrobial resistance genes. From the BLAST, only the hits with almost 70% of coverage and 70% of similarity were retained. Complete 16S rRNA genes from the MAGs identified as *Acinetobacter* spp. and 16S rRNA partial sequences with individual abundance above 0.1% from the amplicon libraries were used for phylogenetic analyses. Representative sequences of *Acinetobacter* spp. type strains were downloaded from NCBI. Partial sequences of 16S rRNA from *Pseudomonas* spp. isolates and 16S rRNA partial sequences with individual abundance above 0.05% from the amplicon libraries were used for phylogenetic analyses while the representative sequences of *Pseudomonas* spp. type strains were downloaded from NCBI. Both the trees were processed separately. Alignment of the 16S rRNA sequences was carried out using MAFFT (Katoh et al., 2019) while the trimming step was performed with ClipKit (Steenwyk et al., 2020). Phylogenetic distances matrix were calculated using the GRT model and the maximum-likelihood trees were constructed with IQ-TREE (Minh et al., 2020). The branch support was estimated using the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006). All the figures were finalized using the open-source vector graphics editor Inkscape (https://inkscape.org/). A complete R script containing all the steps to reproduce our analysis is available at https://github.com/giovannellilab/Selci_et_al_Hot_springs_pathogens and released as a permanent version using Zenodo under the DOI: https://DOI:10.5281/zenodo.8274180.

3. Results

3.1. Abundance and distribution of putative pathogenic sequences in Costa Rica hot springs

Putative pathogens were identified in nearly all sites that yielded amplifiable DNA (n = 13 sites) with the exception of S1, S12, S17, and S18. Sequences associated with known putative pathogens constitute the 6% of the whole ASVs identified (Fig. 2A). Among them, the number of ASVs related to known pathogenic taxa accounted for more groups in fluid samples compared to the sediments (Kruskal test, p value < 0.001; Supplementary Fig. 1). In hot spring hydrothermal fluids (Fig. 2A), putative pathogens were represented, on average, by ASVs assigned to the genus *Acinetobacter* (52.8%) followed by *Flavobacterium* (9.5%) and *Pseudomonas* (5.6%). Less abundant sequences were related to the genera *Bacillus* (5.5%), *Halomonas* (3.9%), *Treponema* (3.2%), *Roseomonas* (3.1%), and *Comamonas* (3.0%). They were followed by genera like *Legionella* (2.3%), *Deinococcus* (1.8%), *Staphylococcus and* Enterococcus (1.6%), while *Leptospira*, *Stenotrophomonas*, and *Coxiella* had an abundance of 1.0%. Sampled fluids of recreational hot springs were characterized by higher concentrations of putative pathogens when compared to natural hot springs located in more remote locations (Kruskal test, p value < 0.05).

Acinetobacter was the main represented genus with a relative abundance of 94.3% in S3, 91.5% in S5, 88.2% in S6, 83.2% in S2, and 71.1% in S7. The genus *Pseudomonas* was second in abundance with 12% in S2 and S7, while in S3 was around 5%. These were followed by S5 and S6 fluids with a lower abundance of *Pseudomonas* (0.3% and 1.7%, respectively) but a higher abundance of *Comamonas* (~8% for both). In natural hot springs not associated with spas and recreational centers, on the other hand, *Acinetobacter* was found with a high relative abundance only in S13 (86.7%), probably due to the presence of a farm next to the spring. Other natural hot springs like S10, S11, and S16 showed an average abundance of 51%, while for sites like S8, S9, S15, and S19 the abundance of *Acinetobacter* was 4.25% on average. *Pseudomonas* was present in natural hot springs with an abundance of 8.73% and 8.33% for S8 and S9, while for S11, S16, S10, and S19 the abundance had an average of 6.1%. The remaining springs (S13 and S15) were under 1%.

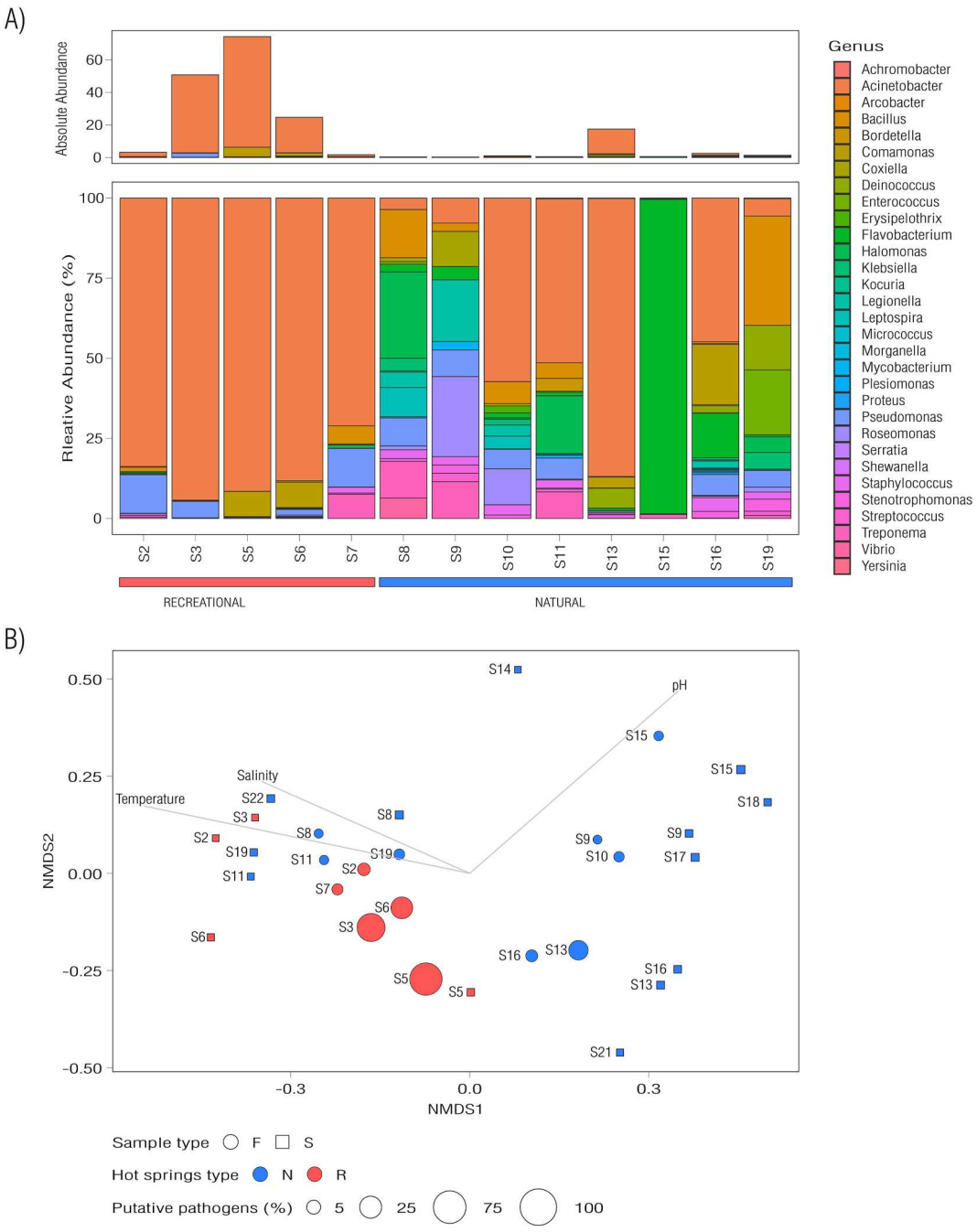

Fig. 2. (A) Barplot showing the absolute (above) and relative abundance (below) of putative pathogens in the 16S rRNA libraries of hot spring fluid samples (Supplementary Table 2). Recreational and natural hot springs are indicated by the red and the blue bars, respectively. (B) Non-metric multidimensional scaling (NMDS, stress 0.16) plot of the 16S rRNA gene amplicon microbial diversity based on the weighted Jaccard dissimilarity index. The colors 'red' and 'blue' indicate the hot spring type (R = recreational, N = natural), the shapes 'circle' and 'squares' indicate the sample type (F = fluid, S = sediment), and the size indicate the relative abundance of the putative pathogens listed in Supplementary Table 2.

The beta-diversity of the hot springs microbial community was investigated through an Non-metric Multidimensional Scaling (NMDS) analysis based on the weighted Jaccard dissimilarity index (Fig. 2B). The sample type (hot spring fluids, F; hot spring sediment, S) showed a statistical separation (Adonis, p value < 0.05) as well as the hot spring type where the microbial community of the recreational hot springs differed from the natural ones (Adonis, p value < 0.01). Distribution of fluid samples from recreational hot springs indicated a similarity in terms of community composition, showing the highest abundance of putative pathogens, as previously mentioned. Vector fitting analysis was used to understand the role of environmental parameters in explaining the distribution of the samples within the ordination. Microbial communities from hot springs at higher temperature and salinity distributed on the top-left side of the ordination while hot springs with a more alkaline pH distributed on the top-right side. This suggested a potential effect of the tested parameters on the distribution of the samples, pushing the communities adapted at more extreme conditions toward the borders of the ordination and leaving the ones able to thrive at more moderate conditions suitable for mesophilic pathogenic bacteria growth closer to the center of the ordination.

The presence of putative pathogens was also confirmed using shotgun metagenomic data. Reads classified as *Acinetobacter* were

mainly present in recreational hot springs metagenomes, especially in S4 (50%), S3 (23.5%), S6 (2.2%), and S7 (0.65% of the total classified reads) while they were in low abundance or absent in natural hot springs, with abundances of 1.64% in S16. Reads assigned to *Comamonas* were found only in S6 (0.5%) while *Pseudomonas* was identified in all the investigated recreational hot springs with a relative abundance of 5% in S3, 1.42% in S6, 1.14% in S4, and 0.74% in S2, while it was not found in the natural springs (Supplementary Fig. 2). The viability of the putative pathogens identified through sequencing and the colony forming units (CFU) were tested on Pseudomonas Selective Agar CN media (PCN agar) and Leeds Acinetobacter Medium (LCM). Putative pathogen isolations on PCN agar showed bacterial growth for all the isolates from the recreational hot springs tested (S2, S3, S4, and S5), while LCM did not show any growth of bacterial colonies for the same spring samples. Hot spring hydrothermal fluids from naturally occurring hot springs were tested on both selective media (PCN and LCM) without getting any colony. CFUs found on PCN agar for the spring S5 showed the highest number of colonies, with a bacterial load equal to 27 CFU/mL (Fig. 3A), while no CFUs were obtained on LCM.

Phylogenetic analyses focused on the two most abundant genera identified in the 16S rRNA analysis, *Acinetobacter* and *Pseudomonas*. DNA sequences from the CR17 campaign were used to extract complete *Acinetobacter* spp. 16S rRNA genes (MAGs) and 16S rRNA gene partial sequences (amplicon libraries). The phylogenetic tree of *Acinetobacter* spp. (Fig. 4) revealed that almost all the 16S rRNA ASVs fall close to *Acinetobacter* type strains known as human pathogens. In particular, *Acinetobacter* sp. CR1 clusters with *Acinetobacter nosocomialis* HQ180192, *Acinetobacter seifertii* FJ860878, and *Acinetobacter pittii* HQ180184, while *Acinetobacter* sp. CR10 is positioned close to *Acinetobacter baumannii* X81660. Moreover, all the complete 16S rRNA genes recovered from the MAGs and 104 ASVs fall within the *Acinetobacter junii* X81664 cluster. The abundance of amplicon sequence variants related to *Acinetobacter* spp. was also evaluated for hot spring type, with more ASVs in recreational hot springs relative to natural ones (Kruskal test, p value < 0.01). A *Pseudomonas* phylogenetic tree was constructed with the same approach as described above (Fig. 5). Most of the 16S rRNA ASVs are related to *Pseudomonas* type strains known in literature as human pathogens, while all the isolated type strains fall in a cluster related to *Pseudomonas aeruginosa*. The abundance of ASVs related to *Pseudomonas* spp. was evaluated for hot spring type, with more ASVs in recreational hot springs compared to natural ones (Kruskal test, p value < 0.01).

3.2. Antibiotic resistance of the putative pathogens

Antibiotic resistance profiles were determined for *P. aeruginosa* strains (48) isolated from four different recreational springs. All 48 identified strains showed different antibiotic susceptibility to the two groups of antibiotics tested, specific and generic, respectively (Table 2). In accordance to EUCAST, the distribution of the antibiotic resistance profiles obtained against Meropenem (M, 10 μg), Gentamicin (G, 10 μg), Ciprofloxacin (Cp, 5 μg), and Cefepime (Cf, 30 μg) (specific group) showed different patterns both in the fluids coming from the recreational hot spring and in comparison with PAO1 reference strain (Fig. 6). More than 12% of the *P. aeruginosa* type strains identified in each study site showed resistance against Gentamicin, Ciprofloxacin, and Cefepime while only the strains isolated from S5 showed resistance against Meropenem. Multi-drug resistances (MDRs) were found in all the study sites with a relative abundance higher than 30% in S5 and S3. PAO1 reference strain showed resistance only against the Meropenem and Cefepime and no MDR was detected. In accordance with CLSI, antibiotic resistance profiles obtained against Meropenem, Gentamicin, Ciprofloxacin, and Cefepime (specific group) showed a higher sensitivity of the *P. aeruginosa* type strains identified (Supplementary Fig. 3). Only the strains coming from S5 showed resistance against two antibiotics while strains from S4 and S2 were resistant against Gentamicin. Strains isolated from S3 were completely sensitive to the antibiotics tested while PAO1 reference strain showed resistance only against the Meropenem.

In all the investigated sites, 100% of the *Pseudomonas* sp. isolates were resistant against Ampicillin (A, 10 μg) and Erythromycin (E, 30 μg), while a lower percentage were resistant against Cefsulodin (C, 30 μg) and Kanamycin (K, 30 μg) (Supplementary Fig. 4). Also for the generic group of antibiotics, MDR were found in each study site, and S5 showed the highest relative abundance in MDR strains (62.5% of 24 total strains were MDR) while S3 showed the lowest (16.7% of 6 total strains were MDR). PAO1 reference strain showed resistance only against the Cefsulodin and Kanamycin, and no MDR were detected.

The occurrence of Antimicrobial Resistance Genes (ARGs) in natural and recreational hot springs was investigated by blasting the assembled DNA contigs against the Comprehensive Antibiotic Resistance Database (CARD; Fig. 7). Natural hot springs fluids and sediments (S8, S13, S16, and S18) were characterized by a low number of ARGs that primarily belong to the group of *mux* and *sme* which codify for resistance against, while almost all recreational hot springs displayed a major diversity of ARGs (Fig. 7). The fluids of S3, S4, and S2 showed the higher number of resistance genes which comprised the categories of *OXA*, *ADC*, and *ade*. In S5 and S6 fluids, in contrast, only a *nov* gene and a *ade* gene were found, respectively.

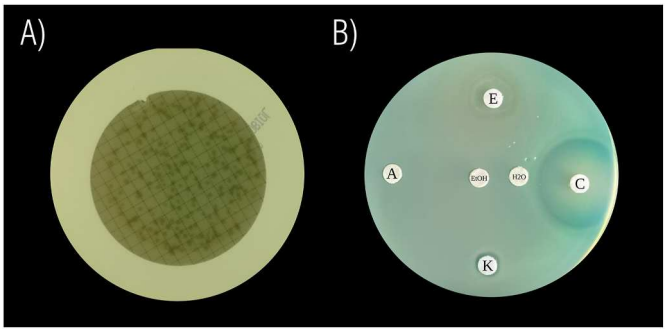

Fig. 3. (A) Colony forming units of *Pseudomonas aeruginosa* (as identified by 16S rRNA sequencing) from the hot spring waters on a plate of PCN agar selective media. (B) Example of antimicrobial susceptibility test on *Pseudomonas* spp. strains isolated from a recreational hot spring showing multidrug resistance. The antibiotics shown include: A (Ampicillin, 10 μg), E (Erythromycin, 30 μg), C (Cefsulodin, 30 μg), and K (Kanamycin, 30 μg).

4. Discussion

Environmental reservoirs of antibiotic resistance represent a growing concern for global public health as potential sources for human infection (Barrett, 2012; Mills and Lee, 2019; Tello et al., 2012). Remote environments as well as recreational waters have been identified as current reservoirs of antibiotic resistance bacteria and genes (Eckert et al., 2018; Knapp et al., 2012; Overbey et al., 2015; Segawa et al., 2013; Zhang et al., 2022). The increased antimicrobial resistance of opportunistic pathogens together with increased human population densities in areas subject to habitat deterioration represent a global threat to public health (Myers and Patz, 2009).

Hot springs harbor a plethora of microorganisms that include not only naturally-occurring microbes but also introduced opportunistic pathogens. Previous studies reported the presence of several infective microorganisms including free living protozoa (Abrahams-Sandí et al., 2015; De Jonckheere, 2005; Montalbano Di Filippo et al., 2017) and bacteria (Aburto-Medina et al., 2020; Ghilamicael et al., 2018; Mukherjee et al., 2012; Rupasinghe et al., 2022; Sheehan et al., 2005; Thorolfsdottir and Marteinsson, 2013) in hot springs and spas. Recreational use of hot springs and their association to infective agents have been reported worldwide (Barben et al., 2005; Falkinham et al., 2015; Lutz and Lee, 2011; Martins et al., 1995; Moore et al., 2002; Niewolak

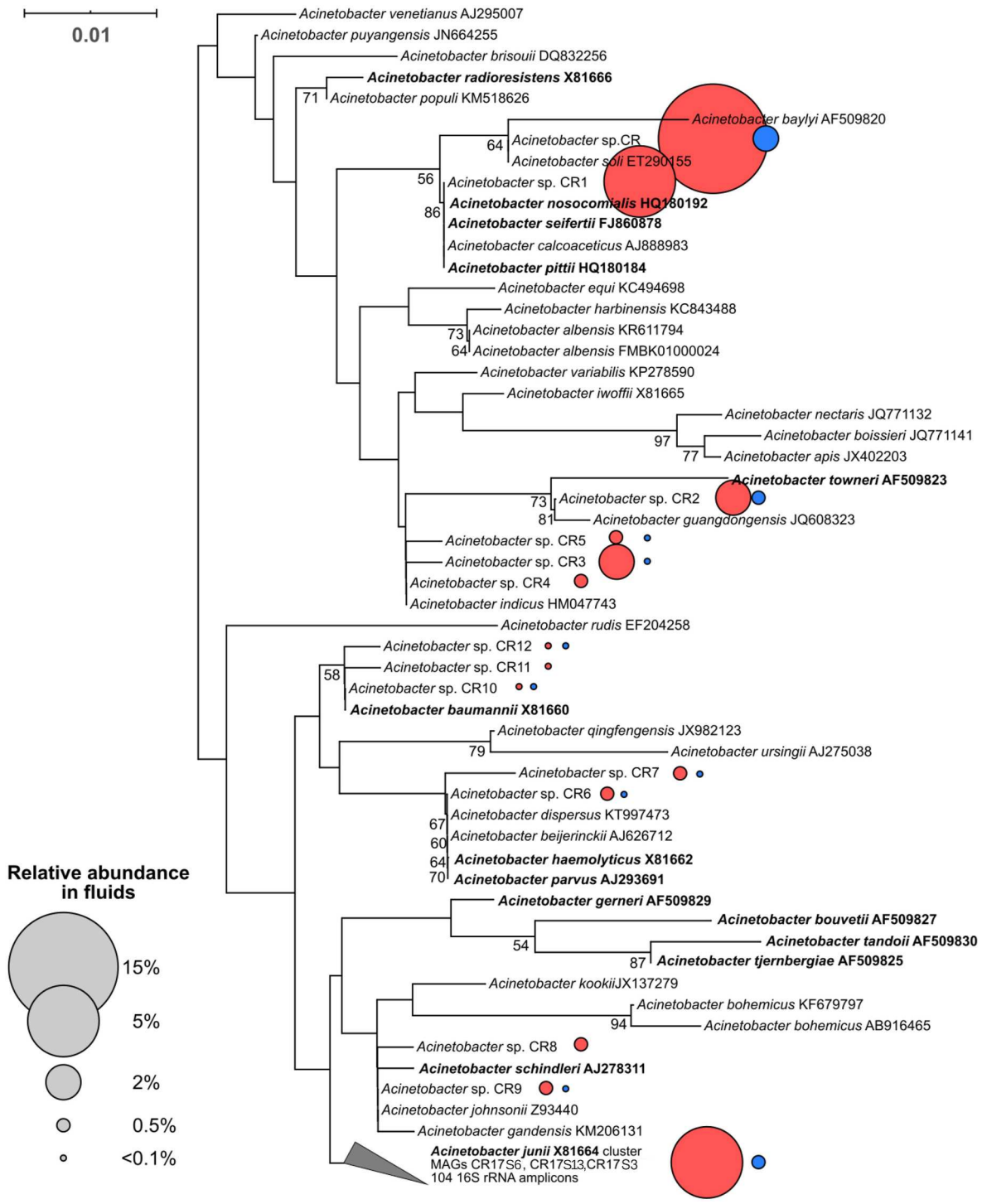

Fig. 4. Neighbor-joining phylogenetic tree showing the relative position of the ASVs and the MAGs' 16S rRNA genes related to the genus *Acinetobacter* relative to known *Acinetobacter* species. The reference sequences of known human pathogens are displayed in bold and 16s rRNA library sequences from this study are followed by CR#. The average relative 16S rRNA gene amplicon abundance in the fluid 16S rRNA libraries is reported for the natural (blue) and recreational (red) hot springs. The *A. junii* cluster also contains the sequences of *A. gyllenbergii* AJ293694, *A. proteolyticus* KT997475, *Acinetobacter* sp. X81659, *A. modestus* KT997474, *A. viviani* KT997477, *A. courvalinii* KT997472. Tree based on 1000 replicated bootstrap.

and Opieka, 2000). However, while their presence is well documented (Rahel et al., 2021), information related to their origin, distribution and antimicrobial resistance profiles are still limited (Ghilamicael et al., 2018; Lutz and Lee, 2011).

In the last years, most of the microbiological large-scale investigations on the Costa Rican hot springs focused on the role of subsurface chemolithoautotrophic communities in affecting the carbon cycling (Arce-Rodríguez et al., 2019; Barry et al., 2019; Crespo-Medina et al., 2017; Fullerton et al., 2021; Rogers et al., 2022) with the addition of a few studies on microbial mats community composition (Uribe-Lorío et al., 2019; Brenes-Guillén et al., 2021; Finsinger et al., 2008) and microbial heterotrophic pathways across the surficial geothermal systems (Paul et al., 2023). The only studies conducted on Costa Rican waterborne pathogens were targeted on cases of amoebic

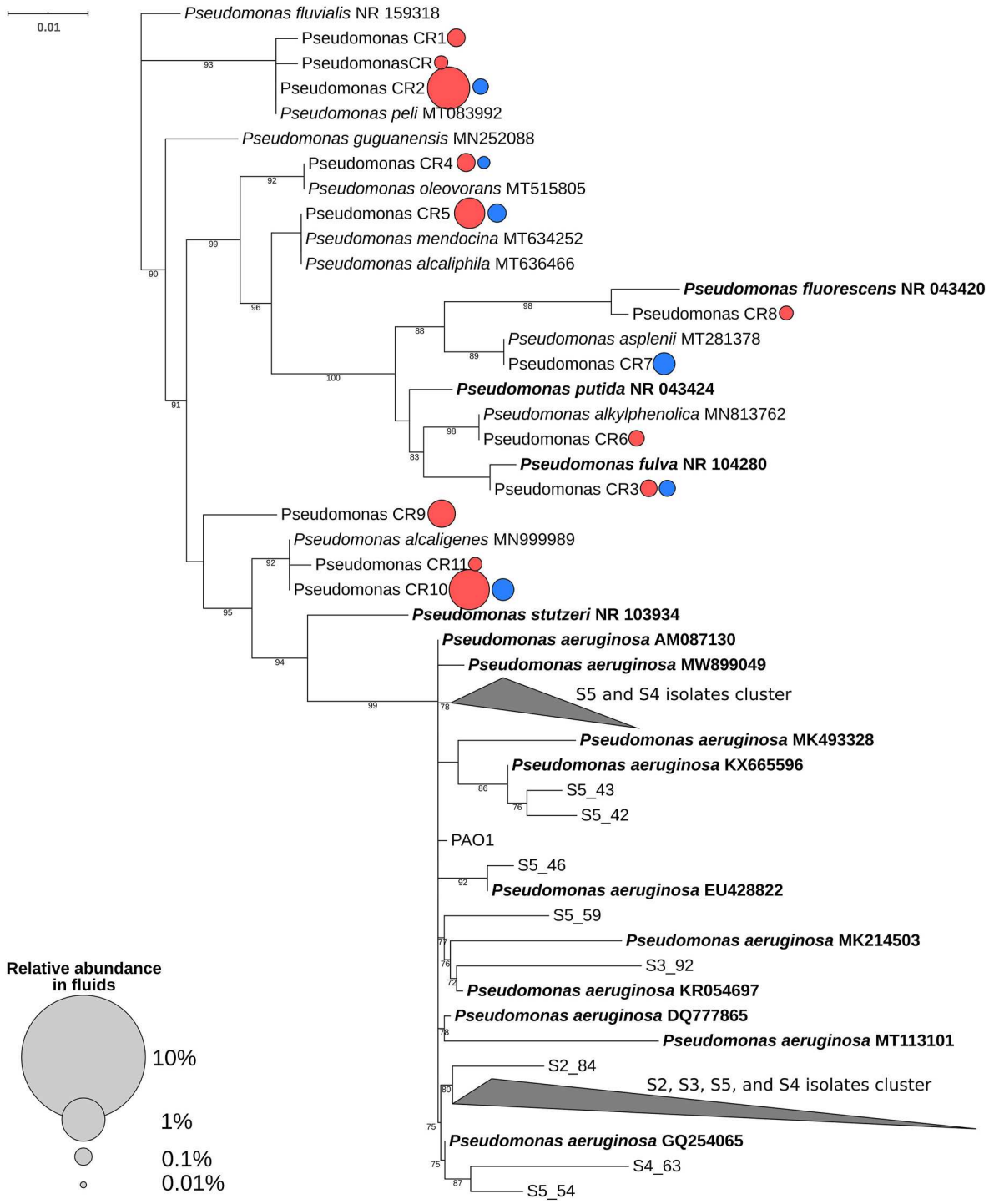

Fig. 5. Neighbor-joining phylogenetic tree showing the relative position of the ASVs and the 16S rRNA genes of isolated type strains related to the genus *Pseudomonas* relative to known *Pseudomonas* species. The reference sequences of known human pathogens are displayed in bold while 16S rRNA library sequences from this study are followed by CR#. Average relative abundances in the fluid 16S rRNA libraries are reported for the natural (blue) and recreational (red) hot springs. Tree based on 1000 replicated bootstraps.

meningoencephalitis from *Naegleria fowleri* infections found in hot spring waters (Abrahams-Sandí et al., 2015; Barrantes et al., 2022) and surveillance monitoring of drinking water aimed to alert the authorities for potential disease outbreaks (Barrantes et al., 2022).

Here, we report for the first time, sequencing and isolation data from 22 Costa Rican hot springs divided into recreational hot springs (e.g. resorts and spas) and natural hot springs from remote areas. Our results from 16S rRNA amplicon sequencing showed recreational hot springs waters (resorts and spas) with moderate-high temperature (40–60 °C) as the principal reservoir for the distribution of opportunistic pathogens. High temperatures are known to have a limiting effect on pathogenic bacterial growth (Spinks et al., 2006) which is in line with the low average abundance of potential pathogenic taxa we found within the 16S rRNA libraries. However, ASVs related to *Acinetobacter* and *Pseudomonas* were dominant in the recreational hot springs investigated, suggesting the presence of moderate thermotolerant species for both

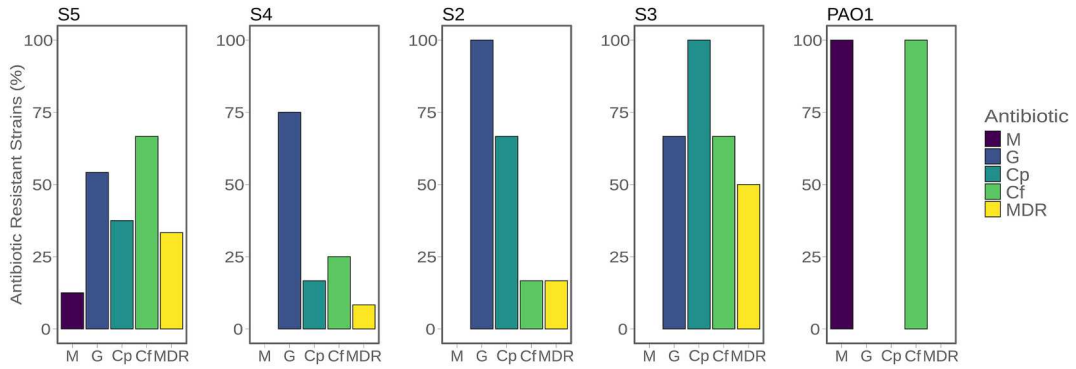

Fig. 6. Antibiogram for *P.aeruginosa* isolates according to EUCAST. Antibiotic resistance profile was developed by testing *P. aeruginosa* against Meropenem (M, 10 μg), Gentamicin (G, 10 μg), Ciprofloxacin (Cp, 5 μg), and Cefepime (Cf, 30 μg). Strains of *P. aeruginosa* with more than two antibiotic resistances were indicated as MDR (Multi-Drugs Resistance).

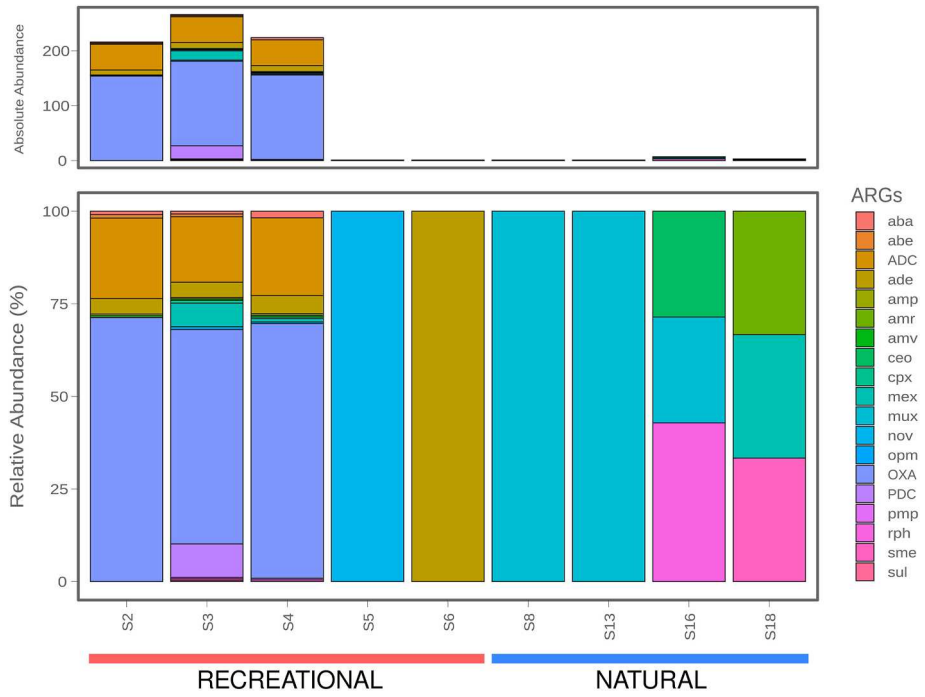

Fig. 7. Absolute and relative abundances of Antimicrobial Resistance Genes (ARGs) found in Costa Rica (2017) sampled sites. Recreational and natural hot springs are indicated by the red and the blue bars, respectively.

genera, as observed in hot springs isolates from other studies (Kumar et al., 2023, p.; Obeidat and Al-Shomali, 2023; Saini et al., 2023; Obeidat and Al-Shomali, 2023; Saini et al., 2023). In addition, the occurrence of *Acinetobacter* and *Pseudomonas* genera were recorded in other hot springs from developing countries where anthropogenic impact is more pronounced (Adjeroud et al., 2020; Moussard et al., 2004; Najar et al., 2018; Rawat and Joshi, 2019; Wu et al., 2023). Among the naturally occurring hot springs, only one site showed a high abundance of sequences associated with *Acinetobacter*. The presence of a livestock farming area next to the hot spring might have conditioned the surrounding environment through sewage and farm effluents (Hill, 2003; Hooda et al., 2000; Pearce-Duvet, 2006), affecting eventually the microbial community composition of the hot spring itself. Species related to *Acinetobacter* were found only through sequence-based analysis and the phylogenetic investigations showed that most of them were closely related to human pathogens like *A. junii* and *A. baumannii*, two species well known in health care systems for their role in nosocomial infections (Hung et al., 2009; Visca et al., 2011). In the past, the role of *Acinebocater* spp. in geothermal settings has been associated with their capacity to use different organic carbon sources (Dixit et al., 2021) as well as their ability to degrade hydrocarbons (Freitas et al., 2023). Species related to *Pseudomonas*, instead, were found both in sequence-based and laboratory culture analysis. In particular, phylogenetic evaluations of DNA sequences from 16S rRNA libraries showed a similarity to species such as *P. fluorescens* and *P. fulva* related to human infections (Liu et al., 2014; Scales et al., 2014) while all laboratory isolates were closely associated to *Pseudomonas aeruginosa* strains that are well known for the high rate of acute and chronic infections in hospital settings (Crone et al., 2020; Huber et al., 2016; Obritsch et al., 2005),. The persistence of *P. aeruginosa* species in thriving in controlled settings like spas, hot springs, and swimming pools (Lutz and Lee, 2011; Moore et al., 2002) has been linked to its capacity to form biofilms (Hall-Stoodley and Stoodley, 2005) which can increase exponentially their survival in different niches (Berg et al., 1990, 2005; Guida et al., 2016; Mena and Gerba, 2009). The concentrations of *P. aeruginosa* colonies we found from recreational springs waters were orders of magnitude higher than the normal threshold accepted for natural spas (World Health Organization, 2003). This is a result in line with findings from

other contaminated environments exposed to anthropogenic pressure (Crone et al., 2020; Deredjian et al., 2014; Jardine et al., 2017; Mena and Gerba, 2009; Saini et al., 2023).

The ARGs detected from the investigated recreational hot springs indicated a low distribution of genes related to drug efflux functions, compared to the main occurrence of enzymatic degradation mechanisms by β-lactamases associated to *A. baumannii* and *P. aeruginosa* related genes (e.g., *OXA, ADC, and PDC*) which confer resistance to a broad range of antibiotics like carbapenems and cephalosporins (Evans and Amyes, 2014; Rao et al., 2020). Furthermore, these results were consistent with the potential resistance found in multiple *P. aeruginosa* type strains (see below), suggesting a correlation between the relative proportion of resistant bacteria with the anthropogenic activities associated to recreational hot springs, an observation already reported for other environments (Amos et al., 2014; Das et al., 2023; Di Cesare et al., 2015, 2017; Graham et al., 2011; Hatosy and Martiny, 2015; Luo et al., 2010; Popowska et al., 2012; Pruden et al., 2012).

The antibiotic resistance profiles, for the isolated *P. aeruginosa* type strains, showed varying results when different standard references (EUCAST vs. CLSI) were used. In accordance with the EUCAST guidelines, most of the *P. aeruginosa* strains displayed resistance against almost all the tested antibiotics, with a particular resistance rate against general antibiotics like Erythromycin (E, 30 μg), Cefsulodin (C, 30 μg), Kanamycin (K, 30 μg), and Ampicillin (A, 10 μg), already known for their mild efficacy against *P. aeruginosa* infections (Alam et al., 2019; Bălăşoiu and Bălăşoiu, 2014; Khan et al., 2015). Resistance was found also for more specific antibiotics like Ciprofloxacin (Cp, 5 μg), Cefepime (Cf, 30 μg), Gentamicin, and Meropenem (M, 10 μg), linked with *P. aeruginosa*'s *capacity* to acquire mutations on specific targets, as the case of the DNA gyrase and the topoisomerase IV enzymes in fluoroquinolones resistance (Drlica and Zhao, 1997; Jacoby, 2005) or through mechanisms for antibiotic cleavage, efflux, and reduced drug uptake in -Lactams resistance (Pfeifer et al., 2010; Poole, 2004, 2011). The detection of multidrug-resistant *P. aeruginosa* strains in over half of the investigated sites could be explained by the presence of a selective pressure able to escalate antibiotic resistance in the ecosystem (Bel Hadj Ahmed et al., 2020; Bravakos et al., 2021; Li et al., 2017). This was previously observed by Sharma et al. (2022) who investigated the spread and co-evolution of resistomes from pathogenic to non-pathogenic microorganisms in different Himalayan hot springs, attributing the occurrence of metal, drug, and biocide resistomes in these habitats to natural and anthropogenic activities. In this context, metals generally used for microbial metabolisms (Giovannelli, 2023; Hay Mele et al., 2023) as well as biocide contamination have been demonstrated to involve environmental multidrug resistance acquisition (Bengtsson-Palme et al., 2018; Catao et al., 2021; Farias et al., 2015; Lim et al., 2015; Mishra et al., 2023; Najar et al., 2022, 2020; Thomas et al., 2020), causing a hyperexpression of the drug efflux pumps in Gram-negative bacteria (Amsalu et al., 2020; Khan et al., 2018; Piddock, 2006). When the CLSI guidelines were used instead of the EUCAST ones, the number of *P. aeruginosa* strains resistant to the tested antibiotics decreased (Supplementary Fig. 1). The only resistance found was against gentamicin with the highest number of resistant isolates from the site S2, followed by S4 and S5 isolates. The latter was the only site where a low number *P. aeruginosa* type strains resistant to meropenem was found, suggesting a strong response to the antibiotic exposure since the CLSI has more stringent diameter breakpoints. The discrepancies between the two antimicrobial susceptibility testing systems (Supplementary Fig. 3) have been already observed, and the lack of agreement in the antibiotic breakpoints interpretation have critical implications on surveillance initiatives (Bork et al., 2017; Cusack et al., 2019; Hombach et al., 2013; Machuca et al., 2016; Rodríguez-Baño et al., 2012; Rodríguez-Martínez et al., 2011).

Overall our data suggest that hot springs represent an optimal reservoir of antimicrobial resistant opportunistic pathogens. The low abundance of putative pathogens and ARGs in natural occurring springs as well as the higher multidrug resistant pathogens observed only in the recreational hot springs suggests that the presence of pathogens is linked to anthropogenic activities. In this context, anthropogenic pressure together with environmental factors might play a key role in leading the differential occurrence of putative multidrug-resistant pathogens, making recreational hot springs a suitable reservoir for pathogen proliferation. The investigated recreational hot springs have a neutral pH and temperatures ranging between 40 °C and 60 °C that makes them more suitable for human bathing compared to naturally occurring hot springs. These conditions however also fall in the physiological range of common pathogens, supporting their higher abundances at recreational sites. In addition, a combined effect of anthropogenic pressure and natural events like the rainy season typical of the Central America regions may affect the ARG dynamics, facilitating the contamination of the soil and the consequent drainage to the hot spring fluids, contributing to the spreading of antibiotic resistance genes, as was previously reported for other pathogens (Di Cesare et al., 2017). However, the occurrence of putative pathogens in recreational hot springs might be supported by the lack of prevention strategies as well as insufficient management practices (Mavridou et al., 2018). The application of more stringent management protocols (Nichols, 2006), which include an in-depth investigation of the hot springs underground plumbing system to prevent potential contamination at the hydrothermal fluids source, more frequent drainage and cleaning operations, better water quality management, and regular microbiological testing might mitigate the risk. This is however a temporary solution to the rising threat of antimicrobial resistance in the environment, which will require direct and decisive legislative interventions to limit antibiotic use together with new investment in antimicrobial research (Majumder et al., 2020).

5. Conclusion

In conclusion, our study presents a combined sequence-based and laboratory culture survey to investigate the potential occurrence of putative pathogens and antibiotic resistance genes in several recreational and naturally occurring hot springs of Central America. The obtained results indicate that recreational hot springs harbor significantly higher abundances of multi drug resistant opportunistic pathogens, suggesting anthropogenic activities as the main factor in favoring the presence of putative pathogenic bacteria as well as the contamination and spreading of antibiotic resistance genes. This, together with the capacity of some opportunistic pathogens in persisting in high temperature conditions, highlights the need for a better understanding of the hot springs' role as reservoirs of potential multi resistant pathogens in the environment. Given the exponential rise in popularity of hot springs as tourist attractions globally, more effective management guidelines and prevention measures are necessary to ensure public safety and preserve the cultural and health legacy of this millenia-old leisure activity.

CRediT authorship contribution statement

Matteo Selci: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Monica Correggia:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Angelina Cordone:** Writing – review & editing, Methodology, Formal analysis. **Marco Guida:** Writing – review & editing, Methodology. **Grazia Marina Quero:** Writing – original draft, Validation, Methodology. **Roberta Piredda:** Writing – original draft, Methodology. **Costantino Vetriani:** Writing – review & editing, Writing – original draft, Validation, Conceptualization. **Karen G. Lloyd:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **J. Maarten de Moor:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Conceptualization. **Peter H. Barry:** Writing – review & editing, Resources, Funding acquisition, Conceptualization,

Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Matthew O. Schrenk:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Donato Giovannelli:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

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

https://github.com/giovannellilab/Selci_et_al_Hot_springs_pathogens permanently stored with DOI: https://doi.org/10.5281/zenodo.8274180.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.119841.

References

Abrahams-Sandí, E., Retana-Moreira, L., Castro-Castillo, A., Reyes-Batlle, M., Lorenzo-Morales, J., 2015. Fatal meningoencephalitis in child and isolation of Naegleria fowleri from hot springs in Costa Rica. Emerg. Infect. Dis. 21, 382.

Aburto-Medina, A., Shahsavari, E., Cohen, M., Mantri, N., Ball, A.S., 2020. Analysis of the microbiome (bathing Biome) in geothermal waters from an Australian Balneotherapy Centre. Water 12, 1705. https://doi.org/10.3390/w12061705.

Adjeroud, M., Escuder-Rodríguez, J.-J., González-Siso, M.-I., Kecha, M., 2020. Metagenomic investigation of bacterial and Archaeal diversity of Hammam Essalihine hot spring from Khenchela. Algeria. Geomicrobiology Journal 37, 804–817. https://doi.org/10.1080/01490451.2020.1783035.

Alam, S.T., Le, T.A.N., Park, J.-S., Kwon, H.C., Kang, K., 2019. Antimicrobial biophotonic treatment of ampicillin-resistant Pseudomonas aeruginosa with hypericin and ampicillin cotreatment followed by orange light. Pharmaceutics 11, 641.

Alcock, B.P., Huynh, W., Chalil, R., Smith, K.W., Raphenya, A.R., Wlodarski, M.A., Edalatmand, A., Petkau, A., Syed, S.A., Tsang, K.K., 2023. Card 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. Nucleic Acids Res. 51, D690–D699.

Amalfitano, S., Coci, M., Corno, G., Luna, G.M., 2015. A microbial perspective on biological invasions in aquatic ecosystems. Hydrobiologia 746, 13–22. https://doi.org/10.1007/s10750-014-2002-6.

Amos, G.C.A., Zhang, L., Hawkey, P.M., Gaze, W.H., Wellington, E.M., 2014. Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. Vet. Microbiol. 171, 441–447.

Amsalu, A., Sapula, S.A., De Barros Lopes, M., Hart, B.J., Nguyen, A.H., Drigo, B., Turnidge, J., Leong, L.E., Venter, H., 2020. Efflux Pump-Driven antibiotic and biocide cross-resistance in Pseudomonas aeruginosa isolated from different ecological niches: a case study in the development of multidrug resistance in environmental Hotspots. Microorganisms 8, 1647. https://doi.org/10.3390/microorganisms8111647.

Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. 55, 539–552.

APHA, 2017. Standard Methods for the Examination of Water and Wastewater, 23rd ed. American Public Health Association, Washington DC.

Arce-Rodríguez, A., Puente-Sánchez, F., Avendaño, R., Martínez-Cruz, M., de Moor, J.M., Pieper, D.H., Chavarría, M., 2019. Thermoplasmatales and sulfur-oxidizing bacteria dominate the microbial community at the surface water of a CO2-rich hydrothermal spring located in Tenorio Volcano National Park, Costa Rica. Extremophiles 23, 177–187. https://doi.org/10.1007/s00792-018-01072-6.

Bălăşoiu, M., Bălăşoiu, A.T., 2014. Pseudomonas aeruginosa resistance phenotypes and phenotypic highlighting methods. Current Health Sciences Journal 85–92. https://doi.org/10.12865/CHSJ.40.02.01.

Barben, J., Hafen, G., Schmid, J., Group, S.P.R.R., 2005. Pseudomonas aeruginosa in public swimming pools and bathroom water of patients with cystic fibrosis. J. Cyst. Fibros. 4, 227–231.

Barosa, B., Ferrillo, A., Selci, M., Giardina, M., Bastianoni, A., Correggia, M., di Iorio, L., Bernardi, G., Cascone, M., Capuozzo, R., 2023. Mapping the microbial diversity associated with different geochemical regimes in the shallow-water hydrothermal vents of the Aeolian archipelago, Italy. Front. Microbiol. 14, 1134114.

Barrantes, K., Chacón, L., Morales, E., Rivera-Montero, L., Pino, M., Jiménez, A.G., Mora, D.C., Jiménez, P.S., Silva, B., Romero-Esquivel, L.G., 2022. Occurrence of pathogenic microorganisms in small drinking-water systems in Costa Rica. J. Water Health 20, 344–355. https://doi.org/10.2166/wh.2022.230.

Barrett, J.R., 2012. Preventing antibiotic resistance in the wild: a new end point for environmental risk assessment. National Institute of Environmental Health Sciences 100, 110.

Barry, P.H., de Moor, J.M., Giovannelli, D., Schrenk, M., Hummer, D.R., Lopez, T., Pratt, C.A., Segura, Y.A., Battaglia, A., Beaudry, P., Bini, G., Cascante, M., d'Errico, G., di Carlo, M., Fattorini, D., Fullerton, K., Gazel, E., González, G., Halldórsson, S.A., Iacovino, K., Ilanko, T., Kulongoski, J.T., Manini, E., Martínez, M., Miller, H., Nakagawa, M., Ono, S., Patwardhan, S., Ramírez, C.J., Regoli, F., Smedile, F., Turner, S., Vetriani, C., Yücel, M., Ballentine, C.J., Fischer, T.P., Hilton, D.R., Lloyd, K.G., 2019. Forearc carbon sink reduces long-term volatile recycling into the mantle. Nature 568, 487–492. https://doi.org/10.1038/s41586-019-1131-5.

Bel Hadj Ahmed, A., Salah Abbassi, M., Rojo-Bezares, B., Ruiz-Roldán, L., Dhahri, R., Mehri, I., Sáenz, Y., Hassen, A., 2020. Characterization of Pseudomonas aeruginosa isolated from various environmental niches: new STs and occurrence of antibiotic susceptible "high-risk clones.". Int. J. Environ. Health Res. 30, 643–652. https://doi.org/10.1080/09603123.2019.1616080.

Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiology Reviews 42. https://doi.org/10.1093/femsre/fux053APHA (2017).

Berg, G., Seech, A.G., Lee, H., Trevors, J.T., 1990. Identification and characterization of a soil bacterium with extracellular emulsifying activity. Journal of Environmental Science & Health Part A 25, 753–764.

Berg, G., Eberl, L., Hartmann, A., 2005. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environ. Microbiol. 7, 1673–1685. https://doi.org/10.1111/j.1462-2920.2005.00891.x.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.

Bondarczuk, K., Markowicz, A., Piotrowska-Seget, Z., 2016. The urgent need for risk assessment on the antibiotic resistance spread via sewage sludge land application. Environ. Int. 87, 49–55. https://doi.org/10.1016/j.envint.2015.11.011.

Bork, J.T., Heil, E.L., Leekha, S., Fowler, R.C., Hanson, N.D., Majumdar, A., Johnson, J. K., 2017. Impact of CLSI and EUCAST Cefepime breakpoint changes on the susceptibility reporting for Enterobacteriaceae. Diagn. Microbiol. Infect. Dis. 89, 328–333.

Bowers, R.M., Kyrpides, N.C., Stepanauskas, R., Harmon-Smith, M., Doud, D., Reddy, T. B.K., Schulz, F., Jarett, J., Rivers, A.R., Eloe-Fadrosh, E.A., Tringe, S.G., Ivanova, N. N., Copeland, A., Clum, A., Becraft, E.D., Malmstrom, R.R., Birren, B., Podar, M., Bork, P., Weinstock, G.M., Garrity, G.M., Dodsworth, J.A., Yooseph, S., Sutton, G., Glöckner, F.O., Gilbert, J.A., Nelson, W.C., Hallam, S.J., Jungbluth, S.P., Ettema, T.J. G., Tighe, S., Konstantinidis, K.T., Liu, W.-T., Baker, B.J., Rattei, T., Eisen, J.A., Hedlund, B., McMahon, K.D., Fierer, N., Knight, R., Finn, R., Cochrane, G., Karsch-Mizrachi, I., Tyson, G.W., Rinke, C., Lapidus, A., Meyer, F., Yilmaz, P., Parks, D.H., Murat Eren, A., Schriml, L., Banfield, J.F., Hugenholtz, P., Woyke, T., 2017. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. Nat. Biotechnol. 35, 725–731. https://doi.org/10.1038/nbt.3893.

Bravakos, P., Mandalakis, M., Nomikou, P., Anastasiou, T.I., Kristoffersen, J.B., Stavroulaki, M., Kilias, S., Kotoulas, G., Magoulas, A., Polymenakou, P.N., 2021. Genomic adaptation of Pseudomonas strains to acidity and antibiotics in hydrothermal vents at Kolumbo submarine volcano, Greece. Sci. Rep. 11, 1336. https://doi.org/10.1038/s41598-020-79359-y.

Brenes-Guillén, L., Vidaurre-Barahona, D., Morales, S., Mora-López, M., Sittenfeld, A., Uribe-Lorío, L., 2021. Novel Cyanobacterial Diversity Found in Costa Rican Thermal Springs Associated with Rincon de la Vieja and Miravalles Volcanoes: A Polyphasic Approach. J. Phycol. 57, 183–198. https://doi.org/10.1111/jpy.13077.

Calero-Cáceres, W., Melgarejo, A., Colomer-Lluch, M., Stoll, C., Lucena, F., Jofre, J., Muniesa, M., 2014. Sludge as a potential important source of antibiotic resistance genes in both the bacterial and Bacteriophage Fractions. Environ. Sci. Technol. 48, 7602–7611. https://doi.org/10.1021/es501851s.

Catao, E.C., Gallois, N., Fay, F., Misson, B., Briand, J.-F., 2021. Metal resistance genes enrichment in marine biofilm communities selected by biocide-containing surfaces in temperate and tropical coastal environments. Environmental Pollution 268, 115835.

CDC, 2019. Antibiotic Resistance Threats in the United States, 2019. U.S. Department of Health and Human Services, CDC, Atlanta, GA.

Charnot-Katsikas, A., Dorafshar, A.H., Aycock, J.K., David, M.Z., Weber, S.G., Frank, K. M., 2009. Two cases of necrotizing fasciitis due to Acinetobacter baumannii. J. Clin. Microbiol. 47, 258–263.

Cordone, A., D'Errico, G., Magliulo, M., Bolinesi, F., Selci, M., Basili, M., De Marco, R., Saggiomo, M., Rivaro, P., Giovannelli, D., 2022. Bacterioplankton diversity and

distribution in relation to Phytoplankton community structure in the Ross Sea surface waters. Front. Microbiol. 13.
Cordone, A., Selci, M., Barosa, B., Bastianoni, A., Bastoni, D., Bolinesi, F., Capuozzo, R., Cascone, M., Correggia, M., Corso, D., Di Iorio, L., Misic, C., Montemagno, F., Ricciardelli, A., Saggiomo, M., Tonietti, L., Mangoni, O., Giovannelli, D., 2023. Surface Bacterioplankton community structure Crossing the Antarctic Circumpolar current Fronts. Microorganisms 11, 702. https://doi.org/10.3390/microorganisms11030702.
Core, R., 2021. Team. R: A Language and Environment for Statistical Computing, 2015.
Crespo-Medina, M., Twing, K.I., Sánchez-Murillo, R., Brazelton, W.J., McCollom, T.M., Schrenk, M.O., 2017. Methane dynamics in a tropical Serpentinizing environment: the Santa Elena Ophiolite, Costa Rica. Front. Microbiol. 8 https://doi.org/10.3389/fmicb.2017.00916.
Crone, S., Vives-Flórez, M., Kvich, L., Saunders, A.M., Malone, M., Nicolaisen, M.H., Martínez-García, E., Rojas-Acosta, C., Catalina Gomez-Puerto, M., Calum, H., Whiteley, M., Kolter, R., Bjarnsholt, T., 2020. The environmental occurrence of Pseudomonas aeruginosa. APMIS 128, 220–231. https://doi.org/10.1111/apm.13010.
Cui, Q., Huang, Y., Wang, H., Fang, T., 2019. Diversity and abundance of bacterial pathogens in urban rivers impacted by domestic sewage. Environmental Pollution 249, 24–35. https://doi.org/10.1016/j.envpol.2019.02.094.
Cusack, T.P., Ashley, E.A., Ling, C.L., Rattanavong, S., Roberts, T., Turner, P., Wangrangsimakul, T., Dance, D.A.B., 2019. Impact of CLSI and EUCAST breakpoint discrepancies on reporting of antimicrobial susceptibility and AMR surveillance. Clin. Microbiol. Infection 25, 910–911. https://doi.org/10.1016/j.cmi.2019.03.007.
Das, S., Najar, I.N., Sherpa, M.T., Kumar, S., Sharma, P., Mondal, K., Tamang, S., Thakur, N., 2023. Baseline metagenome-assembled genome (MAG) data of Sikkim hot springs from Indian Himalayan geothermal belt (IHGB) showcasing its potential CAZymes, and sulfur-nitrogen metabolic activity. World J. Microbiol. Biotechnol. 39, 179. https://doi.org/10.1007/s11274-023-03631-2.
De Jonckheere, J.F., 2005. The isolation of Naegleria italica from Peru indicates that this potentially pathogenic species occurs worldwide. Parasitol. Int. 54, 173–175. https://doi.org/10.1016/j.parint.2005.03.004.
De Sadeleer, N., Godfroid, J., 2020. The story behind COVID-19: animal diseases at the crossroads of wildlife, livestock and human health. European Journal of Risk Regulation 11, 210–227.
Deredjian, A., Colinon, C., Hien, E., Brothier, E., Youenou, B., Cournoyer, B., Dequiedt, S., Hartmann, A., Jolivet, C., Houot, S., Ranjard, L., Saby, N.P.A., Nazaret, S., 2014. Low occurrence of Pseudomonas aeruginosa in agricultural soils with and without organic amendment. Front. Cell. Infect. Microbiol. 4.
Des Marais, D.J., Walter, M.R., 2019. Terrestrial hot spring systems: Introduction. Astrobiology 19, 1419–1432. https://doi.org/10.1089/ast.2018.1976.
Destoumieux-Garzón, D., Mavingui, P., Boetsch, G., Boissier, J., Darriet, F., Duboz, P., Fritsch, C., Giraudoux, P., Le Roux, F., Morand, S., Paillard, C., Pontier, D., Sueur, C., Voituron, Y., 2018. The one health Concept: 10 Years old and a long Road Ahead. Front. Vet. Sci. 5 https://doi.org/10.3389/fvets.2018.00014.
Di Cesare, A., Eckert, E.M., Teruggi, A., Fontaneto, D., Bertoni, R., Callieri, C., Corno, G., 2015. Constitutive presence of antibiotic resistance genes within the bacterial community of a large subalpine lake. Mol. Ecol. 24, 3888–3900.
Di Cesare, A., Eckert, E.M., Rogora, M., Corno, G., 2017. Rainfall increases the abundance of antibiotic resistance genes within a riverine microbial community. Environmental Pollution 226, 473–478. https://doi.org/10.1016/j.envpol.2017.04.036.
Dixit, S., Behera, D.U., Gaur, M., Dey, S., Sahoo, R.K., Sahu, A., Das, A., Sahoo, S., Kumari, K.S., Subudhi, E., 2021. Evaluation of community Structures and their Physicochemical correlation with five hot springs in India. Geomicrobiol. J. 38, 655–671. https://doi.org/10.1080/01490451.2021.1917732.
Drlica, K., Zhao, X., 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiology and molecular biology reviews 61, 377–392.
Ebi, K.L., Vanos, J., Baldwin, J.W., Bell, J.E., Hondula, D.M., Errett, N.A., Hayes, K., Reid, C.E., Saha, S., Spector, J., 2021. Extreme weather and climate change: population health and health system implications. Annu. Rev. Publ. Health 42, 293–315.
Eckert, E.M., Di Cesare, A., Coci, M., Corno, G., 2018. Persistence of antibiotic resistance genes in large subalpine lakes: the role of anthropogenic pollution and ecological interactions. Hydrobiologia 824, 93–108.
Erfurt, P., 2021. Hot Springs and Their Cultural Heritage. In: Erfurt, P. (Ed.), The Geoheritage of Hot Springs. Springer International Publishing, Cham, pp. 183–214. https://doi.org/10.1007/978-3-030-60463-9_6.
EUCAST The European Committee on Antimicrobial Susceptibility Testing, n.d. Breakpoint tables for interpretation of MICs and zone diameters. version 8.1, http://www.eucast.org/clinical_breakpoints/.
Evans, B.A., Amyes, S.G.B., 2014. OXA β-Lactamases. Clin. Microbiol. Rev. 27, 241–263. https://doi.org/10.1128/CMR.00117-13.
Exner, M., Kramer, A., Lajoie, L., Gebel, J., Engelhart, S., Hartemann, P., 2005. Prevention and control of health care–associated waterborne infections in health care facilities. Am. J. Infect. Control 33, S26–S40. https://doi.org/10.1016/j.ajic.2005.04.002.
Fabros, M.R.L., Diesta, X.R.S., Oronan, J.A., Verdejo, K.S., Garcia, J.-A.S.M., Sophia, Romey, Ma, Milanez, G.D.J., 2021. Current report on the prevalence of free-living amoebae (FLA) in natural hot springs: a systematic review. J. Water Health 19, 563–574. https://doi.org/10.2166/wh.2021.101.
Falkinham, J.O., Hilborn, E.D., Arduino, M.J., Pruden, A., Edwards, M.A., 2015. Epidemiology and ecology of opportunistic Premise plumbing pathogens: *Legionella pneumophila* , *Mycobacterium avium* , and *Pseudomonas aeruginosa*. Environmental Health Perspectives 123, 749–758. https://doi.org/10.1289/ehp.1408692.
Farias, P., Espírito Santo, C., Branco, R., Francisco, R., Santos, S., Hansen, L., Sorensen, S., Morais, P.V., 2015. Natural hot Spots for Gain of multiple resistances: Arsenic and antibiotic resistances in heterotrophic, Aerobic bacteria from marine hydrothermal vent Fields. Appl. Environ. Microbiol. 81, 2534–2543. https://doi.org/10.1128/AEM.03240-14.
Finsinger, K., Scholz, I., Serrano, A., Morales, S., Uribe-Lorio, L., Mora, M., Sittenfeld, A., Weckesser, J., Hess, W.R., 2008. Characterization of true-branching cyanobacteria from geothermal sites and hot springs of Costa Rica. Environmental Microbiology 10, 460–473. https://doi.org/10.1111/j.1462-2920.2007.01467.x.
Fleming, I.D., Krezalek, M.A., Belogortseva, N., Zaborin, A., Defazio, J., Chandrasekar, L., Actis, L.A., Zaborina, O., Alverdy, J.C., 2017. Modeling Acinetobacter baumannii wound infections: the critical role of iron. J. Trauma Acute Care Surg. 82, 557.
Freitas, J.F., Silva, D.F.L., Silva, B.S., Castro, J.N.F., Felipe, M.B.M.C., Silva-Portela, R.C. B., Minnicelli, C.F., Agnez-Lima, L.F., 2023. Genomic and phenotypic features of Acinetobacter baumannii isolated from oil reservoirs reveal a novel subspecies specialized in degrading hazardous hydrocarbons. Microbiological Research 273, 127420. https://doi.org/10.1016/j.micres.2023.127420.
French, G.L., 2005. Clinical impact and relevance of antibiotic resistance. Adv. Drug Deliv. Rev. 57, 1514–1527.
Fullerton, K.M., Schrenk, M.O., Yücel, M., Manini, E., Basili, M., Rogers, T.J., Fattorini, D., Di Carlo, M., d'Errico, G., Regoli, F., Nakagawa, M., Vetriani, C., Smedile, F., Ramírez, C., Miller, H., Morrison, S.M., Buongiorno, J., Jessen, G.L., Steen, A.D., Martínez, M., de Moor, J.M., Barry, P.H., Giovannelli, D., Lloyd, K.G., 2021. Effect of tectonic processes on biosphere–geosphere feedbacks across a convergent margin. Nat. Geosci. 14, 301–306. https://doi.org/10.1038/s41561-021-00725-0.
Ghilamicael, A.M., Boga, H.I., Anami, S.E., Mehari, T., Budambula, N.L.M., 2018. Potential human pathogenic bacteria in five hot springs in Eritrea revealed by next generation sequencing. PLoS One 13, e0194554. https://doi.org/10.1371/journal.pone.0194554.
Gill, J.S., Arora, S., Khanna, S.P., Kumar, K.H., 2016. Prevalence of multidrug-resistant, extensively drug-resistant, and pandrug-resistant Pseudomonas aeruginosa from a tertiary level intensive care unit. J. Global Infect. Dis. 8, 155.
Giovannelli, D., 2023. Trace metal availability and the evolution of biogeochemistry. Nat. Rev. Earth Environ. 4, 597–598.
Giovannelli, D., d'Errico, G., Fiorentino, F., Fattorini, D., Regoli, F., Angeletti, L., Bakran-Petricioli, T., Vetriani, C., Yücel, M., Taviani, M., Manini, E., 2016. Diversity and distribution of Prokaryotes within a shallow-water Pockmark field. Front. Microbiol. 7 https://doi.org/10.3389/fmicb.2016.00941.
Giovannelli, D., Barry, P.H., de Moor, J.M., Jessen, G.L., Schrenk, M.O., Lloyd, K.G., 2022. Sampling across large-scale geological gradients to study geosphere–biosphere interactions. Front. Microbiol. 13 https://doi.org/10.3389/fmicb.2022.998133.
Goto, S., Enomoto, S., 1970. Nalidixic Acid Cetrimide agar A new selective plating medium for the selective isolation of Pseudomonas aeruginosa. Jpn. J. Microbiol. 14, 65–72.
Graciaa, D.S., Cope, J.R., Roberts, V.A., Cikesh, B.L., Kahler, A.M., Vigar, M., Hilborn, E. D., Wade, T.J., Backer, L.C., Montgomery, S.P., 2018. Outbreaks associated with untreated recreational water—United States, 2000–2014. MMWR Morb Mortal Wkly Rep, 67 (25), 701–706.
Graham, D.W., Olivares-Rieumont, S., Knapp, C.W., Lima, L., Werner, D., Bowen, E., 2011. Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba. Environmental science & technology 45, 418–424.
Grettenberger, C.L., Hamilton, T.L., 2021. Metagenome-Assembled Genomes of Novel Taxa from an Acid Mine Drainage Environment. Applied and Environmental Microbiology 87, e00772–21. https://doi.org/10.1128/AEM.00772-21.
Guida, M., Di Onofrio, V., Gallè, F., Gesuele, R., Valeriani, F., Liguori, R., Romano Spica, V., Liguori, G., 2016. Pseudomonas aeruginosa in swimming pool water: evidences and Perspectives for a new control Strategy. Int J Environ Res Public Health 13, 919. https://doi.org/10.3390/ijerph13090919.
Hall-Stoodley, L., Stoodley, P., 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol. 13, 7–10. https://doi.org/10.1016/j.tim.2004.11.004.
Harris, S.J., Cormican, M., Cummins, E., 2012. Antimicrobial Residues and antimicrobial-resistant bacteria: impact on the microbial environment and risk to human health—a review. Hum. Ecol. Risk Assess. 18, 767–809. https://doi.org/10.1080/10807039.2012.688702.
Hatosy, S.M., Martiny, A.C., 2015. The ocean as a global reservoir of antibiotic resistance genes. Appl. Environ. Microbiol. 81, 7593–7599.
Hay Mele, B., Monticelli, M., Leone, S., Bastoni, D., Barosa, B., Cascone, M., Migliaccio, F., Montemagno, F., Ricciardelli, A., Tonietti, L., 2023. Oxidoreductases and metal cofactors in the functioning of the earth. Essays Biochem. 67, 653–670.
Hayat, M.A., 2012. Fixation for Electron Microscopy. Elsevier (Eric).
Hill, V.R., 2003. Prospects for pathogen Reductions in livestock Wastewaters: a review. Crit. Rev. Environ. Sci. Technol. 33, 187–235. https://doi.org/10.1080/10643380390814532.
Hlavsa, M.C., Cikesh, B.L., Roberts, V.A., Kahler, A.M., Vigar, M., Hilborn, E.D., Wade, T. J., Roellig, D.M., Murphy, J.L., Xiao, L., 2018. Outbreaks associated with treated recreational water—United States, 2000–2014. MMWR Morb Mortal Wkly Rep 70 (20), 733–738.
Hombach, M., Wolfensberger, A., Kuster, S.P., Böttger, E.C., 2013. Influence of clinical breakpoint changes from CLSI 2009 to EUCAST 2011 antimicrobial susceptibility testing guidelines on multidrug resistance rates of Gram-negative Rods. J. Clin. Microbiol. 51, 2385–2387. https://doi.org/10.1128/JCM.00921-13.

Hooda, P.S., Edwards, A.C., Anderson, H.A., Miller, A., 2000. A review of water quality concerns in livestock farming areas. Sci. Total Environ. 250, 143–167. https://doi.org/10.1016/S0048-9697(00)00373-9.
Huber, P., Basso, P., Reboud, E., Attrée, I., 2016. Pseudomonas aeruginosa renews its virulence factors. Environmental microbiology reports 8, 564–571.
Hung, Y.-T., Lee, Y.-T., Huang, L.-J., Chen, T.-L., Yu, K.-W., Fung, C.-P., Cho, W.-L., Liu, C.-Y., 2009. Clinical characteristics of patients with Acinetobacter junii infection. J. Microbiol. Immunol. Infect. 42, 47–53.
Jack, S., Bell, D., Hewitt, J., 2013. Norovirus Contamination of a Drinking Water Supply at a Hotel Resort 126.
Jacoby, G.A., 2005. Mechanisms of resistance to quinolones. Clin. Infect. Dis. 41, S120–S126.
James, A.E., Kesteloot, K., Paul, J.T., McMullen, R.L., Louie, S., Waters, C., Dillaha, J., Tumlison, J., Haselow, D.T., Smith, J.C., Lee, S., Ritter, T., Lucas, C., Kunz, J., Miller, L.A., Said, M.A., 2022. Potential association of Legionnaires' disease with hot spring water, hot springs National Park and hot springs, Arkansas, USA, 2018–2019 - Volume 28, Number 1—January 2022 - Emerging Infectious Diseases journal - CDC. https://doi.org/10.3201/eid2801.211090.
Jardine, J.L., Abia, A.L.K., Mavumengwana, V., Ubomba-Jaswa, E., 2017. Phylogenetic analysis and antimicrobial profiles of cultured emerging opportunistic pathogens (phyla Actinobacteria and Proteobacteria) identified in hot springs. Int. J. Environ. Res. Publ. Health 14, 1070.
Jawad, A., Hawkey, P.M., Heritage, J., Snelling, A.M., 1994. Description of Leeds Acinetobacter Medium, a new selective and differential medium for isolation of clinically important Acinetobacter spp., and comparison with Herellea agar and Holton's agar. J. Clin. Microbiol. 32, 2353–2358.
Ji, W.-T., Hsu, B.-M., Chang, T.-Y., Hsu, T.-K., Kao, P.-M., Huang, K.-H., Tsai, S.-F., Huang, Y.-L., Fan, C.-W., 2014. Surveillance and evaluation of the infection risk of free-living amoebae and Legionella in different aquatic environments. Sci. Total Environ. 499, 212–219.
Katoh, K., Rozewicki, J., Yamada, K.D., 2019. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings Bioinf. 20, 1160–1166. https://doi.org/10.1093/bib/bbx108.
Khan, H.A., Ahmad, A., Mehboob, R., 2015. Nosocomial infections and their control strategies. Asian Pac. J. Trop. Biomed. 5, 509–514. https://doi.org/10.1016/j.apjtb.2015.05.001.
Khan, R., Roy, N., Choi, K., Lee, S.-W., 2018. Distribution of triclosan-resistant genes in major pathogenic microorganisms revealed by metagenome and genome-wide analysis. PLoS One 13, e0192277.
Kim, M., Christley, S., Khodarev, N.N., Fleming, I., Huang, Y., Chang, E., Zaborina, O., Alverdy, J.C., 2015. Pseudomonas aeruginosa wound infection involves activation of its iron acquisition system in response to fascial contact. J. Trauma Acute Care Surg. 78, 823–829.
Knapp, C., Lima, L., Olivares-Rieumont, S., Bowen, E., Werner, D., Graham, D.W., 2012. Seasonal Variations in antibiotic resistance gene transport in the Almendares river, Havana, Cuba. Front. Microbiol. 3.
Kraemer, S.A., Ramachandran, A., Perron, G.G., 2019. Antibiotic pollution in the environment: from microbial ecology to public policy. Microorganisms 7, 180.
Kresic, N., 2010. Chapter 2 - Types and classifications of springs, in: Kresic, N., Stevanovic, Z. (Eds.), Groundwater Hydrology of Springs. Butterworth-Heinemann, Boston, pp. 31–85. https://doi.org/10.1016/B978-1-85617-502-9.00002-5.
Kumar, S., Najar, I.N., Sharma, P., Tamang, S., Mondal, K., Das, S., Sherpa, M.T., Thakur, N., 2023. Temperature – a critical abiotic paradigm that governs bacterial heterogeneity in natural ecological system. Environ. Res. 234, 116547 https://doi.org/10.1016/j.envres.2023.116547.
Lamoreaux, P.E., 2005. History and Classification of Springs. Geological Society of America Abstract with Programs, p. 324.
Leclerc, H., Schwartzbrod, L., Dei-Cas, E., 2002. Microbial agents associated with waterborne diseases. Crit. Rev. Microbiol. 28, 371–409.
Li, L.-G., Xia, Y., Zhang, T., 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. The ISME journal 11, 651–662.
Lim, J.C., Goh, K.M., Shamsir, M.S., Ibrahim, Z., Chong, C.S., 2015. Characterization of aluminum resistant Anoxybacillus sp. SK 3–4 isolated from a hot spring. J. Basic Microbiol. 55, 514–519. https://doi.org/10.1002/jobm.201400621.
Liu, Y., Liu, K., Yu, X., Li, B., Cao, B., 2014. Identification and control of a Pseudomonas spp (P. fulva and P. putida) bloodstream infection outbreak in a teaching hospital in Beijing, China. Int. J. Infect. Dis. 23, 105–108.
Luo, Y.I., Mao, D., Rysz, M., Zhou, Q., Zhang, H., Xu, L., Jj Alvarez, P., 2010. Trends in antibiotic resistance genes occurrence in the Haihe River, China. Environmental science & technology 44, 7220–7225.
Lutz, J.K., Lee, J., 2011. Prevalence and antimicrobial-resistance of Pseudomonas aeruginosa in swimming pools and hot tubs. Int. J. Environ. Res. Publ. Health 8, 554–564.
Machuca, J., Briales, A., Díaz-de-Alba, P., Martínez-Martínez, L., Rodríguez-Martínez, J.-M., Pascual, Á., 2016. Comparison of clinical categories for Escherichia coli harboring specific qnr and chromosomal-mediated fluoroquinolone resistance determinants according to CLSI and EUCAST. Enferm. Infecc. Microbiol. Clín. 34, 188–190.
Majumder, M.A.A., Rahman, S., Cohall, D., Bharatha, A., Singh, K., Haque, M., Gittens-St Hilaire, M., 2020. Antimicrobial stewardship: Fighting antimicrobial resistance and protecting global public health. Infect. Drug Resist. 4713–4738.
Manini, E., Baldrighi, E., Ricci, F., Grilli, F., Giovannelli, D., Intoccia, M., Casabianca, S., Capellacci, S., Marinchel, N., Penna, P., Moro, F., Campanelli, A., Cordone, A., Correggia, M., Bastoni, D., Bolognini, L., Marini, M., Penna, A., 2022. Assessment of Spatio-temporal Variability of Faecal pollution along coastal waters during and after Rainfall events. Water 14, 502. https://doi.org/10.3390/w14030502.
Martins, M.T., Sato, M.I.Z., Alves, M.N., Stoppe, N.C., Prado, V.M., Sanchez, P.S., 1995. Assessment of microbiological quality for swimming pools in South America. Water Res. 29, 2417–2420.
Mavridou, A., Pappa, O., Papatzitze, O., Dioli, C., Kefala, A.M., Drossos, P., Beloukas, A., 2018. Exotic tourist destinations and transmission of infections by swimming pools and hot springs—a literature review. Int. J. Environ. Res. Publ. Health 15, 2730.
McCausland, W.J., Cox, P.J., 1975. Pseudomonas infection traced to motel whirlpool. J. Environ. Health 37, 455–459.
McConnell, M.J., Pérez-Romero, P., Lepe, J.A., Pérez-Ordóñez, A., Valencia, R., Vázquez-Barba, I., Pachón, J., 2011. Positive Predictive Value of Leeds Acinetobacter Medium for Environmental Surveillance of Acinetobacter baumannii. Journal of Clinical Microbiology 49, 4416. https://doi.org/10.1128/jcm.05412-11, 4416.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome Census data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217.
Mena, K.D., Gerba, C.P., 2009. Risk assessment of Pseudomonas aeruginosa in water. In: Whitacre, D.M. (Ed.), Reviews of Environmental Contamination and Toxicology Vol 201, Reviews of Environmental Contamination and Toxicology. Springer, US, Boston, MA, pp. 71–115. https://doi.org/10.1007/978-1-4419-0032-6_3.
Menzel, P., Ng, K.L., Krogh, A., 2016. Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nat. Commun. 7, 1–9.
Mills, M.C., Lee, J., 2019. The threat of carbapenem-resistant bacteria in the environment: Evidence of widespread contamination of reservoirs at a global scale. Environmental Pollution 255, 113143. https://doi.org/10.1016/j.envpol.2019.113143.
Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A., Lanfear, R., 2020. IQ-TREE 2: new models and Efficient methods for phylogenetic inference in the genomic Era. Mol. Biol. Evol. 37, 1530–1534. https://doi.org/10.1093/molbev/msaa015.
Mishra, A., Kesarwani, S., Jaiswal, T.P., Bhattacharjee, S., Chakraborty, S., Mishra, A.K., Singh, S.S., 2023. Decoding whole genome of Anoxybacillus rupiensis TPH1 isolated from tatapani hot spring, India and giving insight into bioremediation ability of TPH1 via heavy metals and azo dyes. Res. Microbiol. 174, 104027 https://doi.org/10.1016/j.resmic.2023.104027.
Montalbano Di Filippo, M., Novelletto, A., Di Cave, D., Berrilli, F., 2017. Identification and phylogenetic position of Naegleria spp. from geothermal springs in Italy. Exp. Parasitol. 183, 143–149. https://doi.org/10.1016/j.exppara.2017.08.008.
Moore, J.E., Heaney, N., Millar, B.C., Crowe, M., Elborn, J.S., 2002. Incidence of Pseudomonas aeruginosa in recreational and hydrotherapy pools. Comm. Dis. Publ. Health 5, 23–26.
Motbainor, H., Bereded, F., Mulu, W., 2020. Multi-drug resistance of blood stream, urinary tract and surgical site nosocomial infections of Acinetobacter baumannii and Pseudomonas aeruginosa among patients hospitalized at Felegehiwot referral hospital, Northwest Ethiopia: a cross-sectional study. BMC Infect. Dis. 20 https://doi.org/10.1186/s12879-020-4811-8.
Moussard, H., L'Haridon, S., Tindall, B.J., Banta, A., Schumann, P., Stackebrandt, E., Reysenbach, A.-L., Jeanthon, C., 2004. Thermodesulfatator indicus gen. nov., sp. nov., a novel thermophilic chemolithoautotrophic sulfate-reducing bacterium isolated from the Central Indian Ridge. Int. J. Syst. Evol. Microbiol. 54, 227–233. https://doi.org/10.1099/ijs.0.02669-0.
Mukherjee, S., ArunimaSaha, A.K.R., Chowdhury, A.R., Mitra, A.K., 2012. Identification and characterization of a green pigment producing bacteria isolated from Bakreshwar Hot Spring, West Bengal, India. International Journal of Environmental Sciences and Research 2, 126–129.
Myers, S.S., Patz, J.A., 2009. Emerging threats to human health from global environmental change. Annu. Rev. Environ. Resour. 34, 223–252.
Najar, I.N., Sherpa, M.T., Das, Sayak, Das, Saurav, Thakur, N., 2018. Microbial ecology of two hot springs of Sikkim: Predominate population and geochemistry. Sci. Total Environ. 637–638, 730–745. https://doi.org/10.1016/j.scitotenv.2018.05.037.
Najar, I.N., Sherpa, M.T., Das, Sayak, Das, Saurav, Thakur, N., 2020. Diversity analysis and metagenomic insights into antibiotic and metal resistance among Himalayan hot spring bacteriobiome insinuating inherent environmental baseline levels of antibiotic and metal tolerance. Journal of Global Antimicrobial Resistance 21, 342–352. https://doi.org/10.1016/j.jgar.2020.03.026.
Najar, I.N., Das, S., Kumar, S., Sharma, P., Mondal, K., Sherpa, M.T., Thakur, N., 2022. Coexistence of heavy metal tolerance and antibiotic resistance in thermophilic bacteria belonging to genus Geobacillus. Front. Microbiol. 13 https://doi.org/10.3389/fmicb.2022.914037.
Nicastri, E., Petrosillo, N., Martini, L., Larosa, M., Gesu, G.P., Ippolito, G., 2003. Prevalence of nosocomial infections in 15 Italian hospitals: first point prevalance study for the INF-NOS project. Infection 31, 10–15.
Nichols, G., 2006. Infection risks from water in natural and man-made environments. Euro Surveill. 11, 1–2. https://doi.org/10.2807/esm.11.04.00611-en.
Niewolak, S., Opieka, A., 2000. Potentially pathogenic microorganisms in water and bottom sediments in the Czarna Hancza River. Pol. J. Environ. Stud. 9, 183–194.
Nurk, S., Meleshko, D., Korobeynikov, A., Pevzner, P.A., 2017. metaSPAdes: a new versatile metagenomic assembler. Genome Res. 27, 824–834.
Obeidat, M., Al-Shomali, B., 2023. Moderately thermophilic bacteria from Jordanian hot springs as Possible sources of Thermostable enzymes and Leukemia Cytotoxic agents. Jordan J. Biol. Sci. 16.
Obritsch, M.D., Fish, D.N., MacLaren, R., Jung, R., 2005. Nosocomial infections due to multidrug-resistant Pseudomonas aeruginosa: epidemiology and treatment options. Pharmacotherapy 25, 1353–1364.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2018. Vegan: Community Ecology Package.

Overbey, K.N., Hatcher, S.M., Stewart, J.R., 2015. Water quality and antibiotic resistance at beaches of the Galápagos Islands. Front. Environ. Sci. 3.
Page, S.J., Essex, S., Causevic, S., 2014. Tourist attitudes towards water use in the developing world: a comparative analysis. Tourism Manag. Perspect. 10, 57–67. https://doi.org/10.1016/j.tmp.2014.01.004.
Pandey, A., Dhakar, K., Sharma, A., Priti, P., Sati, P., Kumar, B., 2015. Thermophilic bacteria that tolerate a wide temperature and pH range colonize the Soldhar (95 C) and Ringigad (80 C) hot springs of Uttarakhand, India. Ann. Microbiol. 65, 809–816.
Parada, A.E., Needham, D.M., Fuhrman, J.A., 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples: primers for marine microbiome studies. Environ. Microbiol. 18, 1403–1414. https://doi.org/10.1111/1462-2920.13023.
Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25, 1043–1055. https://doi.org/10.1101/gr.186072.114.
Paul, R., Rogers, T.J., Fullerton, K.M., Selci, M., Cascone, M., Stokes, M.H., Steen, A.D., Moor, J.M. de, Chiodi, A., Stefánsson, A., Halldórsson, S.A., Ramirez, C.J., Jessen, G. L., Barry, P.H., Cordone, A., Giovannelli, D., Lloyd, K.G., 2023. Complex organic matter degradation by secondary consumers in chemolithoautotrophy-based subsurface geothermal ecosystems. PLoS One 18, e0281277. https://doi.org/10.1371/journal.pone.0281277.
Pearce-Duvet, J.M.C., 2006. The origin of human pathogens: evaluating the role of agriculture and domestic animals in the evolution of human disease. Biol. Rev. 81, 369–382. https://doi.org/10.1017/S1464793106007020.
Pérez-Cataluña, A., Tapiol, J., Benavent, C., Sarvisé, C., Gómez, F., Martínez, B., Terron-Puig, M., Recio, G., Vilanova, A., Pujol, I., Ballester, F., Rezusta, A., Figueras, M.J., 2017. Antimicrobial susceptibility, virulence potential and sequence types associated with Arcobacter strains recovered from human faeces. J. Med. Microbiol. 66, 1736–1743. https://doi.org/10.1099/jmm.0.000638.
Performance, CLSI., 2018. Standards for Antimicrobial DiskSusceptibility Tests. In: CLSI guideline M02, 13th ed. Clinical and Laboratory Standards Institute.
Pfeifer, Y., Cullik, A., Witte, W., 2010. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. International Journal of medical microbiology 300, 371–379.
Piddock, L.J., 2006. Multidrug-resistance efflux pumps? not just for resistance. Nat. Rev. Microbiol. 4, 629–636.
Poole, K., 2004. Resistance to β-lactam antibiotics. Cellular and Molecular Life Sciences CMLS 61, 2200–2223.
Poole, K., 2011. Pseudomonas aeruginosa: resistance to the Max. Front. Microbiol. 2.
Popowska, M., Rzeczycka, M., Miernik, A., Krawczyk-Balska, A., Walsh, F., Duffy, B., 2012. Influence of soil use on prevalence of tetracycline, streptomycin, and erythromycin resistance and associated resistance genes. Antimicrobial agents and chemotherapy 56, 1434–1443.
Prouzet-Mauléon, V., Labadi, L., Bouges, N., Ménard, A., Mégraud, F., 2006. *Arcobacter butzleri* : Underestimated Enteropathogen. Emerg. Infect. Dis. 12, 307–309. https://doi.org/10.3201/eid1202.050570.
Pruden, A., Arabi, M., Storteboom, H.N., 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environmental science & technology 46, 11541–11549.
Purnell, S., Halliday, A., Newman, F., Sinclair, C., Ebdon, J., 2020. Pathogen infection risk to recreational water users, associated with surface waters impacted by de facto and indirect potable reuse activities. Sci. Total Environ. 722, 137799 https://doi.org/10.1016/j.scitotenv.2020.137799.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.
Rahel, C., Adriyani, R., Nurfitria, H.A., 2021. Health risk in hot springs: a literature review. Jour. Health Sci Prev 5, 88–99. https://doi.org/10.29080/jhsp.v5i2.524.
Rao, M., Rashid, F.A., Shukor, S., Hashim, R., Ahmad, N., 2020. Detection of antimicrobial resistance genes associated with carbapenem resistance from the whole-genome sequence of Acinetobacter baumannii isolates from Malaysia. Canadian Journal of Infectious Diseases and Medical Microbiology, 2020, 5021064.
Rawat, N., Joshi, G.K., 2019. Bacterial community structure analysis of a hot spring soil by next generation sequencing of ribosomal RNA. Genomics 111, 1053–1058. https://doi.org/10.1016/j.ygeno.2018.06.008.
Reisman, J.S., Weinberg, A., Ponte, C., Kradin, R., 2012. Monomicrobial Pseudomonas necrotizing fasciitis: a case of infection by two strains and a review of 37 cases in the literature. Scand. J. Infect. Dis. 44, 216–221.
Rodrigues, C., Cunha, M.Â., 2017. Assessment of the microbiological quality of recreational waters: indicators and methods. Euro-Mediterranean Journal for Environmental Integration 2, 1–18.
Rodríguez-Baño, J., Picón, E., Navarro, M.D., López-Cerero, L., Pascual, A., Group, E.-R., 2012. Impact of changes in CLSI and EUCAST breakpoints for susceptibility in bloodstream infections due to extended-spectrum β-lactamase-producing Escherichia coli. Clin. Microbiol. Infection 18, 894–900.
Rodríguez-Martínez, J.M., Briales, A., Velasco, C., Díaz de Alba, P., Martínez-Martínez, L., Pascual, A., 2011. Discrepancies in fluoroquinolone clinical categories between the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI for Escherichia coli harbouring qnr genes and mutations in gyrA and parC. Journal of antimicrobial chemotherapy 66, 1405–1407.
Rogers, T.J., Buongiorno, J., Jessen, G.L., Schrenk, M.O., Fordyce, J.A., de Moor, J.M., Ramírez, C.J., Barry, P.H., Yücel, M., Selci, M., Cordone, A., Giovannelli, D., Lloyd, K.G., 2022. Chemolithoautotroph distributions across the subsurface of a convergent margin. ISME J. https://doi.org/10.1038/s41396-022-01331-7.
Rosen, B.H., Croft, R., Atwill, E.R., Wade, S., Stehman, S., 2000. Waterborne pathogens in agricultural watersheds. US Department of Agriculture, Natural Resources Conservation Service.
Rupasinghe, R., Amarasena, S., Wickramarathna, S., Biggs, P.J., Chandrajith, R., Wickramasinghe, S., 2022. Microbial diversity and ecology of geothermal springs in the high-grade metamorphic terrain of Sri Lanka. Environmental Advances 7, 100166. https://doi.org/10.1016/j.envadv.2022.100166.
Saini, N., Aamir, M., Singh, V.K., Deepak, B., Mona, S., 2023. Unveiling the microbial diversity and functional dynamics of Shiv Kund, Sohna hot spring, India through a shotgun metagenomics approach. Arch. Microbiol. 205, 323. https://doi.org/10.1007/s00203-023-03664-z.
Scales, B.S., Dickson, R.P., LiPuma, J.J., Huffnagle, G.B., 2014. Microbiology, genomics, and clinical significance of the Pseudomonas fluorescens species complex, an unappreciated colonizer of humans. Clin. Microbiol. Rev. 27, 927–948.
Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541.
Segawa, T., Takeuchi, N., Rivera, A., Yamada, A., Yoshimura, Y., Barcaza, G., Shinbori, K., Motoyama, H., Kohshima, S., Ushida, K., 2013. Distribution of antibiotic resistance genes in glacier environments. Environmental microbiology reports 5, 127–134.
Sharma, N., Kumari, R., Thakur, M., Rai, A.K., Singh, S.P., 2022. Molecular dissemination of emerging antibiotic, biocide, and metal co-resistomes in the Himalayan hot springs. J. Environ. Manag. 307, 114569 https://doi.org/10.1016/j.jenvman.2022.114569.
Sheehan, K.B., Henson, J.M., Ferris, M.J., 2005. *Legionella* species diversity in an Acidic biofilm community in Yellowstone National Park. Appl. Environ. Microbiol. 71, 507–511. https://doi.org/10.1128/AEM.71.1.507-511.2005.
Sheik, C.S., Reese, B.K., Twing, K.I., Sylvan, J.B., Grim, S.L., Schrenk, M.O., Sogin, M.L., Colwell, F.S., 2018. Identification and Removal of contaminant sequences from ribosomal gene Databases: Lessons from the Census of deep Life. Front. Microbiol. 9 https://doi.org/10.3389/fmicb.2018.00840.
Spinks, A.T., Dunstan, R.H., Harrison, T., Coombes, P., Kuczera, G., 2006. Thermal inactivation of water-borne pathogenic and indicator bacteria at sub-boiling temperatures. Water Research 40, 1326–1332. https://doi.org/10.1016/j.watres.2006.01.032.
Steenwyk, J.L., Iii, T.J.B., Li, Y., Shen, X.-X., Rokas, A., 2020. ClipKIT: a multiple sequence alignment trimming software for accurate phylogenomic inference. PLoS Biol. 18, e3001007 https://doi.org/10.1371/journal.pbio.3001007.
Tanwar, J., Das, S., Fatima, Z., Hameed, S., 2014. Multidrug resistance: an emerging crisis. Interdisciplinary Perspectives on Infectious Diseases 2014.
Tate, D., Mawer, S., Newton, A., 2003. Outbreak of Pseudomonas aeruginosa folliculitis associated with a swimming pool inflatable. Epidemiol. Infect. 130, 187–192.
Tello, A., Austin, B., Telfer, T.C., 2012. Selective pressure of antibiotic pollution on bacteria of importance to public health. Environmental health perspectives 120, 1100–1106.
Thomas IV, J.C., Oladeinde, A., Kieran, T.J., Finger Jr, J.W., Bayona-Vásquez, N.J., Cartee, J.C., Beasley, J.C., Seaman, J.C., McArthur, J.V., Rhodes Jr, O.E., 2020. Co-occurrence of antibiotic, biocide, and heavy metal resistance genes in bacteria from metal and radionuclide contaminated soils at the Savannah River Site. Microbial biotechnology 13, 1179–1200.
Thorolfsdottir, B., Marteinsson, V., 2013. Microbiological analysis in three diverse natural geothermal bathing pools in Iceland. IJERPH 10, 1085–1099. https://doi.org/10.3390/ijerph10031085.
Uribe-Lorío, L., Brenes-Guillén, L., Hernández-Ascencio, W., Mora-Amador, R., González, G., Ramírez-Umaña, C.J., Díez, B., Pedrós-Alió, C., 2019. The influence of temperature and pH on bacterial community composition of microbial mats in hot springs from Costa Rica. MicrobiologyOpen 8, e893. https://doi.org/10.1002/mbo3.893.
Uritskiy, G.V., DiRuggiero, J., Taylor, J., 2018. MetaWRAP—a flexible pipeline for genome-resolved metagenomic data analysis. Microbiome 6, 158. https://doi.org/10.1186/s40168-018-0541-1.
van Tubergen, A., van der Linden, S., 2002. A brief history of spa therapy. Annals of the rheumatic diseases 61, 273.
Visca, P., Seifert, H., Towner, K.J., 2011. Acinetobacter infection – an emerging threat to human health. IUBMB Life 63, 1048–1054. https://doi.org/10.1002/iub.534.
Vugia, D.J., Richardson, J., Tarro, T., Vareechon, C., Pannaraj, P.S., Traub, E., Cope, J.R., Balter, S., 2019. Notes from the field: fatal Naegleria fowleri meningoencephalitis after swimming in hot spring water—California, 2018. MMWR (Morb. Mortal. Wkly. Rep.) 68, 793.
Walters, S.P., Thebo, A.L., Boehm, A.B., 2011. Impact of urbanization and agriculture on the occurrence of bacterial pathogens and stx genes in coastal waterbodies of central California. Water Res. 45, 1752–1762. https://doi.org/10.1016/j.watres.2010.11.032.
Wang, Y., Chen, Y., Zheng, X., Gui, C., Wei, Y., 2017. Spatio-temporal distribution of fecal indicators in three rivers of the Haihe River Basin, China. Environ. Sci. Pollut. Res. 24, 9036–9047. https://doi.org/10.1007/s11356-015-5907-3.
Wang, Z., Gao, J., Zhao, Y., Dai, H., Jia, J., Zhang, D., 2021. Plastisphere enrich antibiotic resistance genes and potential pathogenic bacteria in sewage with pharmaceuticals. Sci. Total Environ. 768, 144663 https://doi.org/10.1016/j.scitotenv.2020.144663.
Weiser, R., Donoghue, D., Weightman, A., Mahenthiralingam, E., 2014. Evaluation of five selective media for the detection of Pseudomonas aeruginosa using a strain panel from clinical, environmental and industrial sources. J. Microbiol. Methods 99, 8–14. https://doi.org/10.1016/j.mimet.2014.01.010.

Wickham, H., 2011. ggplot2. Wiley interdisciplinary reviews: Comput. Stat. 3, 180–185.
World Health Organization, 2003. In: Guidelines for Safe Recreational Water Environments. World Health Organization, Geneva.
World Health Organization, 2006. Guidelines for safe recreational water environments. 2: Swimming pools and similar environments. 1-118.
Wright, G.D., 2010. Antibiotic resistance in the environment: a link to the clinic? Curr. Opin. Microbiol. 13, 589–594.
Zhang, C., Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z., Li, P., Wang, L., Cui, J., Wang, P., Huang, L., Klena, J.D., Song, H., 2013. Higher isolation of NDM-1 producing Acinetobacter baumannii from the sewage of the hospitals in Beijing. PLoS One 8, e64857. https://doi.org/10.1371/journal.pone.0064857.
Wu, L., Long, H., Huang, S., Niu, X., Li, S., Yu, X., You, L., Ran, X., Wang, J., 2023. Bacterial diversity in water from Xifeng Hot Spring in China. Braz J Microbiol 54, 1943–1954. https://doi.org/10.1007/s42770-023-01070-7.
Zhang, T., Ji, Z., Li, J., Yu, L., 2022. Metagenomic insights into the antibiotic resistome in freshwater and seawater from an Antarctic ice-free area. Environmental Pollution 309, 119738.