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Artículo

Assessing the microbial contamination and antibiotic resistance genes of two Amazon Bolivian watercourses

Evaluación de la contaminación microbiana y genes de resistencia a antibióticos en dos cursos de agua de la Amazonía boliviana

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Abstract
Arroyo Maige and the Beni River are subjected to different types of contamination. This study aimed to evaluate microbial pollution using metagenomic analysis and the physicochemical properties of the rivers. The high conductivity of Arroyo Maige, the identification of heterotrophic microorganisms, and the likely anaerobic conditions favored the presence of prokaryotes that thrive in such an environment. Some bacterial species previously obtained from wastewater, sewer pipes, human feces, and intestinal tracts were identified in the metagenomes of the Bolivian rivers. Additionally, the disease symptoms experienced by locals who consumed water from Arroyo Maige were similar to those caused by *Arcobacter butzleri*, which was identified in our study; however, a clinical evaluation is required. Multidrug antibiotic resistance genes in efflux pumps, currently considered high-risk for global health, were also found in the metagenomes. The expression of these genes in pathogens and the occurrence of integrons, known to mobilize such genes among bacteria, are potential threats to the population living near the rivers. Overall, our work showed that the non-existent sanitation treatment of wastewater in the Bolivian Amazon adds to the detrimental environmental conditions in the zone.
Key words: Beni River, Arroyo Maige, Metagenomic analysis, Bacterial pollution.

Resumen
El arroyo Maige y el río Beni están sujetos a diferentes tipos de contaminación. Este estudio tuvo como objetivo evaluar la contaminación microbiana mediante análisis metagenómicos y las propiedades fisicoquímicas de los ríos. La alta conductividad del Arroyo Maige, la identificación de microorganismos heterótrofos y las probables condiciones anaeróbicas favorecieron la presencia de procariotas que prosperan en tal ambiente. Algunas especies bacterianas obtenidas previamente de aguas residuales, tuberías de alcantarillado, heces humanas y tracto intestinal fueron identificadas en los metagenomas de los ríos bolivianos. Además, los síntomas de la enfermedad que experimentaron los pobladores que consumieron agua del Arroyo Maige fueron similares a los causados por *Arcobacter butzleri*, que fue identificada en nuestro estudio; sin embargo, se requiere una evaluación clínica. En los metagenomas también se encontraron genes de resistencia a múltiples fármacos antibióticos incluidos en las bombas de eflujo, actualmente consideradas de alto riesgo para la salud mundial. La expresión de estos genes en patógenos y la presencia de integrones, conocidos por movilizar dichos genes entre las bacterias, son amenazas potenciales para la población que vive cerca de los ríos. En general, nuestro trabajo demostró que el inexistente tratamiento sanitario de las aguas residuales en la Amazonia boliviana se suma a las condiciones ambientales perjudiciales en la zona.
Palabras clave : Análisis metagenómico, Arroyo Magne, Polución bacteriana, Río Beni.

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Introduction

Freshwater ecosystems provide essential ecosystem services, including recreation, tourism, water supply, water quality control, food supply, and climate regulation (Vári *et al.* 2022). However, these ecosystems are among the most endangered worldwide because they are exposed to

agricultural and land use effects throughout the watershed and accumulate pollutants, such as phosphorus, organic-rich waste, pesticides, and pharmaceuticals (Vári *et al.* 2022). In this respect, legislations such as the US Clean Water Act (1972) and the EU Water Framework Directive (2000) are intended to improve water quality and restore freshwater habitats, including better wastewater treatment and control of air pollution (Haase *et al.* 2023). These actions have led to the development of biodiversity gains in freshwater resources in Europe during the 1990s and the 2000s; nevertheless, progress has decelerated and plateaued since 2010, requiring further mitigation efforts (Haase *et al.* 2023). In China, studies on 186 black-odorous rivers polluted with organic matter have shown that their prokaryotic communities resemble those of sewage and differ from those of eutrophic or oligotrophic waters (Liang *et al.* 2023). Research on sewage-polluted streams in Brazil revealed that conductivity increased in those with high dissolved organic carbon. There were also reduced oxygen concentrations when the water flow in the rivers was low. In contrast, water conductivity and organic matter had low levels when the streams had high flow, implying the dissolution of the contaminants (Daniel *et al.* 2002).

Environmental metagenomics has been used to analyze the DNA sequences of organisms present in different natural habitats. This method generates reliable phylogenetic data and enables comprehensive studies of microbial communities (Ruppert *et al.* 2019). Therefore, metagenomics has been used to evaluate anthropogenic contamination of lakes and rivers. The distinctive microbiome of darkened contaminated rivers in China revealed that organic matter triggered the overgrowth of prokaryotic and eukaryotic microorganisms. Aerobic and anaerobic heterotrophic bacteria and fungi compete for nutrients and electron donors (Liang *et al.* 2023). On the other hand, antimicrobial resistance genes (AMRGs) have also been identified in microbiomes of polluted waters (Zhang *et al.* 2022). Antibiotic residues and microbial co-selection prompted by heavy metals or nutrients may also enable the spread of antibiotic resistance genes (ARGs) (Zhang *et al.* 2018). Moreover, sewage pipes also contribute to mobilizing pathogens because they contain bacteria dominated by a few genera, among which *Acinetobacter* and *Arcobacter* are known to host several ARGs (McLellan & Roguet 2019). Horizontal gene transfer by transposons and plasmids facilitates the dissemination of ARGs among bacteria (Zhang *et al.* 2018). These mobile genetic elements usually include integrons, whose presence in metagenomes has been correlated with human impact on water bodies (Corno *et al.* 2023). In Bolivia, many multidrug ARGs have been identified in an urban lake contaminated with sewage and industrial wastewater (Quillaguamán *et al.* 2021). The hazards of various ARGs have recently been evaluated, and multidrug genes have been classified into the group with the highest risk (Zhang *et al.* 2022).

The Amazon is the biodiversity-richest ecosystem on a subcontinental scale, including more than 10 % of all named species of plants and vertebrates (Albert *et al.* 2023). However, the Amazon ecosystem is rapidly deteriorating due to industrial human activities. The Bolivian Amazon contains intermittent and perennial rivers, among which the Beni River is a large perennial river, is the principal migration route for fish, and is close to indigenous communities (Miranda-Chumacero *et al.* 2020). Currently, many areas around the river are negatively affected by overfishing, gold mining, and deforestation due to the placement of sugar plantations (Miranda-Chumacero *et al.* 2020). Arroyo Maige is a small intermittent stream and a tributary to the Beni River. Local people have used this river to fish, but recently, a drainage channel, presumably from a sugarcane company, has contaminated the water. Many people suffer from diarrhea or stomach pain, nausea, vomiting, or fever when consuming water from the river. Gradually, the river darkened and developed a foul odor. To the best of our knowledge, there have been no metagenomic analyses of the Bolivian Amazon Rivers.

This study aimed to evaluate microbial pollution using metagenomic analysis and the physicochemical properties of the Beni River and Arroyo Maige. Our metagenomic studies revealed the overall diversity of the samples, including prokaryotic orders and eukaryotic groups, with the highest relative abundances. Statistical analysis aided in recognizing the organisms whose abundances differed significantly between Arroyo Maige and the Beni River. Potential bacterial pathogens, ARGs, and integrons carrying AMRGs were identified in the metagenomes of the rivers.

Methods

Sample location, collection, and treatment. Individual water samples were collected from the surface of Arroyo Maige and the Beni River on October 25th, 2021 (the geographical locations and descriptions of the sampling sites are presented in Table 1 and Fig. 1). In the study area, October is characterized as a transition month between the dry and wet seasons with low cloudiness. For the analysis of metal ions, the water samples were filtered by passing each sample through a 0.45 μm filter (Econofilter nylon, Agilent Technologies).

Water samples (approximately 800 ml) were collected separately in sterile glass jars for metagenomic analysis. The samples were immediately filtered for metagenomic analysis upon reaching the laboratory. The remaining water samples were used to determine conductivity, pH, ammonium, orthophosphate, sulfate, sulfide, nitrate, nitrite, metals, and sugars. All samples were refrigerated and transported to the

laboratory within 40 hours and stored at 4 °C until analytical measurements were made.

Chemical and physical analyses of the samples: Conductivity and pH were determined using a portable device (Orion Star A329, Thermo Scientific). A photometer (Spectroquant Move100, Merck) and appropriate kits were used to determine ammonium (kit 1.14558.0001), orthophosphate (kit 1.00673.0001), sulfate (kit 1.01812.0001), sulfide (kit 1.14779.0001), nitrate (kit 1.01842.0001), and nitrite (1.00609.0001).

Table 1. Description of the sampling points selected on Arroyo Maige and the Beni River.

Sampling point	Geographic location	Description
P1	14° 20' 44.88"S 67° 34' 13.70"W	A sample was obtained at the conjunction of a drainage channel and Arroyo Maige.
P2	14° 20' 56.85"S 67° 33' 27.25"W	A sample was taken from Arroyo Maige before joining the Beni River.
P3	14° 20' 57.34"S 67° 33' 32.39"W	A water sample was taken from the Beni River upstream from its junction with Arroyo Maige.

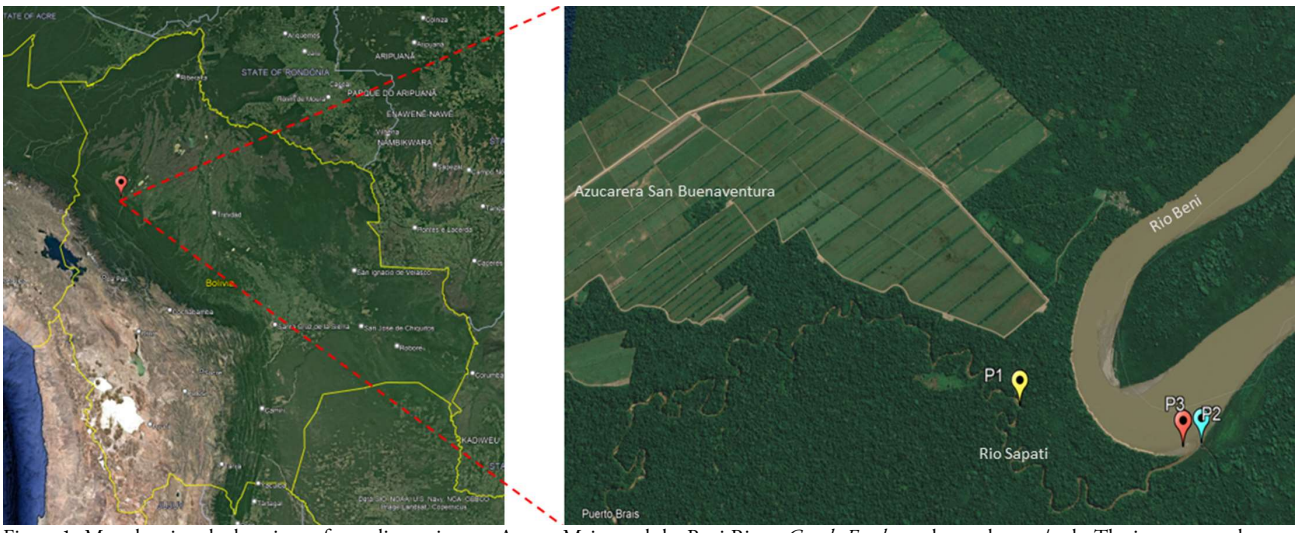


Figure 1. Map showing the locations of sampling points on Arroyo Maige and the Beni River. *Google Earth*, earth.google.com/web. The image was taken on July 2, 2023.

Open reading frames (ORFs) were obtained from the assembled sequences using FragGeneScan v1.31 with default software options provided by the authors (Rho *et al.* 2010).

Furthermore, the taxonomic composition of the communities was assessed by extracting and classifying small subunit (SSU) rRNA fragments for prokaryotes and eukaryotes from the paired-end reads of each metagenome with Metaxa2 v2.2 using default options (Bengtsson-Palme *et al.* 2015). Metaxa2 taxonomic transversal tool was used to identify microorganisms at different taxonomic levels. The percent identity cutoff was 99% for a sequence to be derived from a reference entry. The relative abundance of the microbial groups was obtained by normalizing the number of their sequence counts that, for this purpose, were divided by the number of SSU rRNA sequences assigned by the second classification of the software and multiplied by 10^6, that is, the number of reads corresponding to the order per million reads. The second Metaxa2 classification provides statistics about the number of SSU rRNA sequences assigned to Archaea, Bacteria, and Eukaryota, which were used to normalize the sequence counts corresponding to each microbial kingdom.

To identify potentially harmful bacterial species, trimmed paired-end metagenomes at sampling points P1 and P3 were taxonomically analyzed using MetaPhlAn v4.0.3 (Blanco-Míguez *et al.* 2023) with the default settings provided by the authors. The relative abundances of species

found in the orders Firmicutes and the Gamma-Proteobacteria class were analyzed in circular representations of taxonomic and phylogenetic trees with the help of the software GraPhlAn (Asnicar *et al.* 2015).

Detection of antimicrobial resistance genes and gene cassettes, including known antibiotic resistance genes. As described previously (Daga-Quisbert *et al.* 2023), abundances of ARGs were quantified by mapping the ORFs attained with FragGeneScan against genes from the CARD database v3.1.4 (Alcock *et al.* 2023) using Vsearch v2.23.0 (Rognes *et al.* 2016), requiring at least 65% identity over 60 nucleotides (options "--usearch_global --id 0.65 --mincols 60"). The number of matches to each resistance gene was counted and normalized to the length of the respective gene, in kilobases (kb), to avoid bias due to the differential length of the genes. The relative abundance of each gene was further normalized to the number of SSU rRNA sequences assigned by the second Metaxa2 classification and multiplied by 10^4. To target ARGs with the highest abundance in the metagenomes, genes with fewer than 13 matching DNA fragments were removed.

Potential gene transfer of mobile resistance integrons among microorganisms was assessed by identifying gene cassettes that harbor antibiotic resistance genes. For this purpose, a feature database of previously compiled cassettes (Partridge *et al.* 2009) was used as a reference. Blastn from the software BLAST v2.14.0 (Camacho *et al.* 2009) was used, requiring an expected value of 10^{-20} and a nucleotide sequence identity of at least 97% (options "-max_target_seqs 1 -evalue 1e-20 -perc_identity 97"); moreover, sequences covering no less than 95% of the reference were selected to ensure the presence of genuine gene cassettes. Because almost complete sequences were achieved, the relative abundance was obtained by dividing cassette counts by the number of SSU rRNA sequences assigned by the second Metaxa2 classification and multiplying by 10^4.

Statistical analyses. All statistical analyses were performed using the program R v4.2.2. The diversity of microbial orders was estimated using the Shannon-Weaver method based on relative abundances using Vegan v2.5-7 (Oksanen *et al.* 2022). Relative abundances of microbial orders with statistical difference $p < 0.01$ were obtained with edgeR v.3.41.9 (Robinson *et al.* 2010) after comparing the abundances found in the samples in all possible combinations. Principal component analysis with the Hellinger method was used to evaluate physicochemical data on the same scale; correspondence analysis was utilized to relate relative abundances of microbial orders with sample location, while canonical correspondence analysis and the PERMANOVA test were used to find the relationships between the abundances of microbial orders with the physicochemical data in Vegan v2.5-7.

Results

The microbiomes reveal different types of organisms between Arroyo Maige and the sample point in the Beni River. Shannon diversity was estimated for the polluted Arroyo Maige (sampling points P1 and P2) and the Beni River sample point (P3) (Fig. 2a). There were no significant differences ($p > 0.05$) between the sampling points. Most identified sequences (over 97%) corresponded to the Bacteria domain, followed by the Eukaryota domain (Fig. 2b). Therefore, the relative abundances of the microbial orders were quantified, and the means of the most abundant prokaryotic orders and eukaryotic groups are shown in Fig. 2c. The organisms with the highest relative abundances were those in the Craniata group (Fig. 2c). Different groups of eukaryotes, Stramenopiles, Chrysophyceae, Hexapoda, and Chlorophyta, were among those with the highest abundance. Furthermore, the methane-producing archaea of the Methanobacteriales were more abundant in Arroyo Maige than in the Beni River sample point, whereas other methanogens of the Methanosarcinales had somewhat higher abundances in Arroyo Maige than in the Beni River sample point. Bacterial orders Burkholderiales, Pseudomonadales, and Lactobacillales, which contain heterotrophic members, were also identified.

Microaerophilic and anaerobic bacteria are likely to be found in Arroyo Maige. Physicochemical analysis of the Amazon rivers showed an almost neutral pH; no sugars were found in the samples (Table 2). However, conductivity in Arroyo Maige was approximately double that observed in the Beni River sample point. Congruently, the principal component analysis revealed that sodium, potassium, and manganese were more likely found in P1 and P2 than in P3 (Fig. 3a). Magnesium, lead, and nutrients, such as ammonium, orthophosphate, and sulfate, were common at all sampling points.

After comparing the abundances found in the samples in all possible combinations, we sought the orders of the organisms whose relative abundances differed ($p < 0.01$) using edgeR v.3.34.0. We found that six eukaryotic groups (Fig. 3b) and 32 bacterial orders (Fig. 3c) differed between Arroyo Maige and the Beni River sample point. The sample point in the Beni River was more habitable for various eukaryotes than Arroyo Maige (Fig. 3b). Interestingly, Arroyo Maige was an ecosystem where microaerophilic or anaerobic microorganisms, including Bacteroidales, Clostridiales, Desulfobacterales, Methanobacteriales, and Desulfovibrionales, thrive. In contrast, the Beni River sample point was a more favorable habitat for bacterial groups belonging to various phyla with distinctive

metabolisms (Fig. 3c). Additionally, canonical correspondence analysis showed that none of the physicochemical parameters could explain a significant difference ($p > 0.05$) in the relative abundance of the organisms at the sampling points.

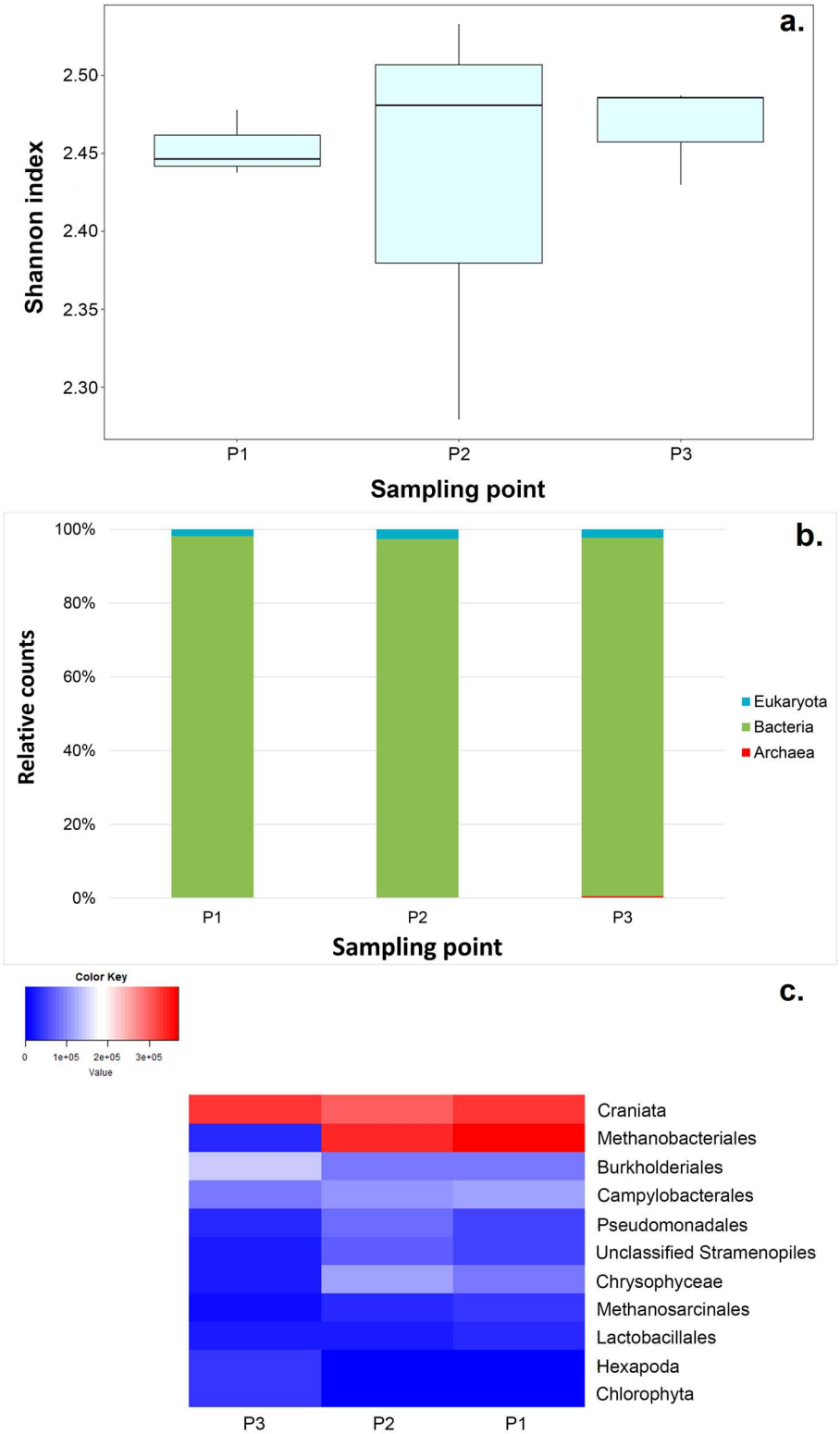
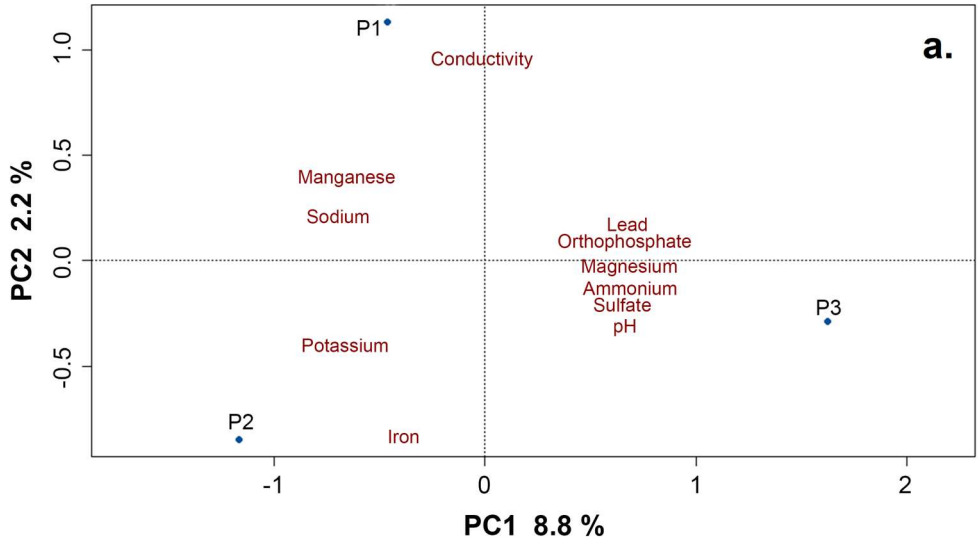
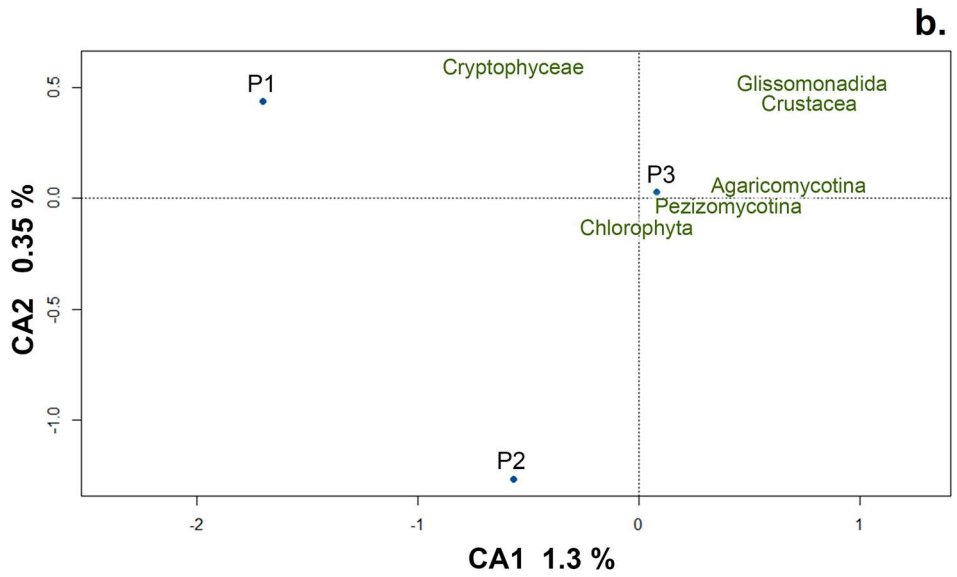


Figure 2. Description of the biological diversity encountered at Arroyo Maige and the Beni River sampling points. The figures show **a.** The biological diversity estimated by the Shannon-Weaver method, **b.** Relative counts of the domains identified in the rivers; **c.** Microbial groups with the highest relative abundances. The sampling points are detailed in Table 1.

Table 2. Physical and chemical characteristics of Arroyo Maige and the Beni River. The results show average values of samples taken at two locations from the Arroyo Maige (points P1 and P2) and one from the River Beni (point P3). The concentrations of sucrose, glucose, xylose, arabinose, fructose, zinc, selenium, cobalt, cadmium, nickel, nitrate, nitrite, and sulfide at all sampling points, as well as cadmium for samples P1 and P2, were below the detection limit of the analytical procedure used. The numbers in parentheses show the standard deviation of the mean values.

	Sampling points		
Parameter	P1	P2	P3
pH	6.84	6.89	6.95
Conductivity (µS/cm)	275.7	271.4	104.0
Ammonium (mg/l)	0.39 (±0.07)	0.34 (±0.03)	0.36 (±0.02)
Orthophosphate (mg/l)	0.13 (±0.02)	0.09 (±0.06)	0.09 (±0.02)
Sulfate (mg/l)	3.3 (±0.50)	2.6 (±0.4)	4.53 (±0.23)
Sodium (mg/l)	20.59 (±0.51)	21.59 (±0.19)	1.03 (±0.00)
Potassium (mg/l)	11.15 (±0.00)	16.79 (±0.44)	3.20 (±0.44)
Magnesium (mg/l)	5.84 (± 0.12)	4.57 (±0.04)	4.28 (±0.08)
Manganese (mg/l)	0.44 (± 0.03)	0.36 (±0.02)	0.007 (±0.0001)
Iron (mg/l)	0.012(±0.002)	2.94 (±0.07)	0.08 (±0.01)
Lead (ng/ml)	2.00 (±0.00)	1.26 (±0.32)	1.44 (±0.00)





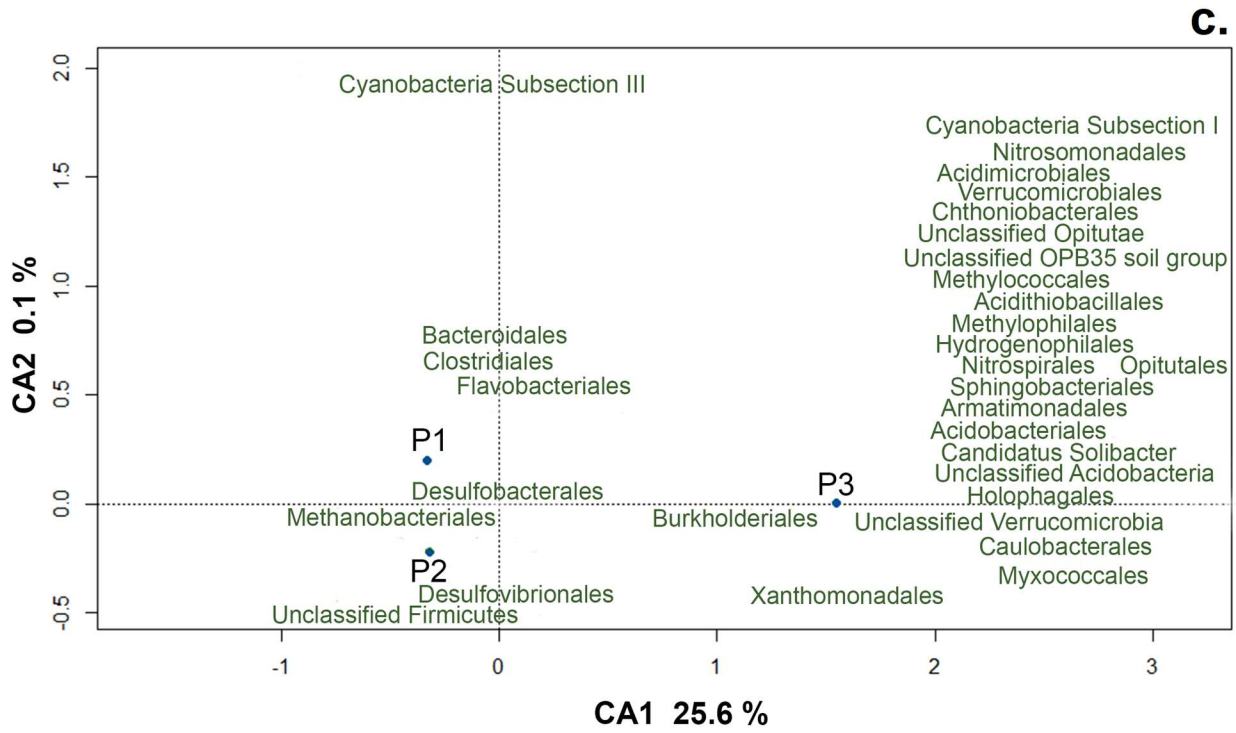


Figure 3. Statistical analysis of microbial groups and physicochemical features of Arroyo Maige and the Beni River sampling points. The statistics show **a.** Principal component analysis regarding physicochemical features, **b.** Correspondence analysis focused on eukaryotic groups, and **c.** Correspondence analysis focused on prokaryotic orders.

Identifying bacterial species in distinctive bacterial orders of the Amazon rivers

Regarding the symptoms experienced by locals who consumed water from Arroyo Maige, we identified bacteria from DNA in the metagenomes using MetaPhlAn software. The species with the highest relative abundance were *Sulfurospirillum cavolei* (abundance = 21.7), followed by *Arcobacter butzleri* (abundance = 10.5) at sampling point P1. In the Beni River sample point, *S. cavolei* and *A. butzleri* had similar abundances, c.a. 18 and 7.2, respectively, to those found at P1, although *Limnohabitans* sp. 103DPR2 showed the highest abundance, c.a. 20. We also considered Firmicutes, phylum frequently found in large amounts in the human and animal intestines (Casals-Pascual *et al.* 2018), because some of their orders were common in Arroyo Maige (Fig. 3c). In addition, we analyzed the Gamma-Proteobacteria class, which includes various opportunistic human pathogens (Rizzatti *et al.* 2017).

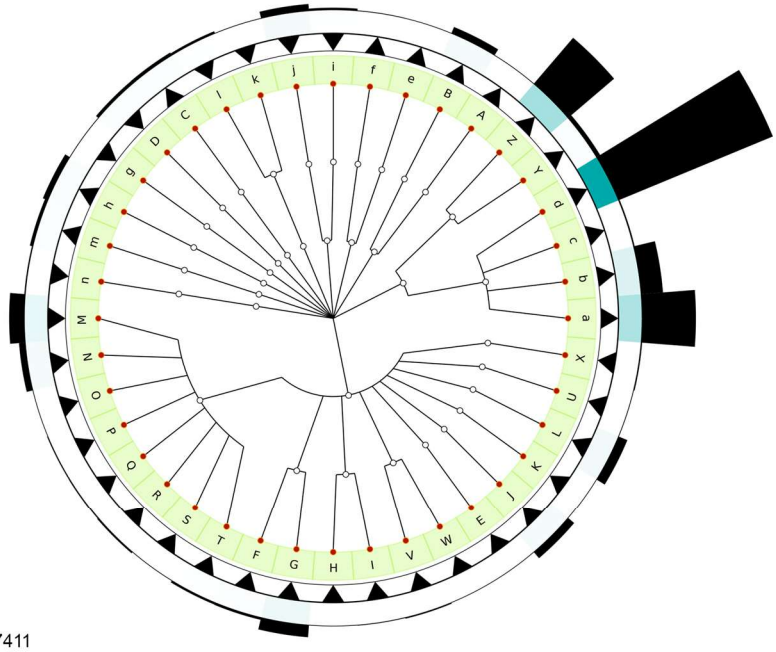
The Firmicutes with the highest relative abundance in both rivers were *Megasphaera* sp. BL7, *Dialister hominis*, *Megasphaera paucivorans*, *Megasphaera hominis*, and *Lacticaseibacillus manihotivorans* (Figs. 4a-b). However, the relative abundances of bacteria in Arroyo Maige were between 3.3 and 9.8 times higher than those in the sample point on the Beni River, and the occurrence of *Lactobacillus* spp. and anaerobic bacteria was noteworthy in Arroyo Maige (Figs. 4a y 4b). Gamma-Proteobacteria in the rivers were mainly represented by *Acinetobacter brisouii* and *Pseudomonas* sp. A1 (Figs. 4c-d), whose abundances in Arroyo Maige were 2.4 and 3 times those in the sample point in the Beni, respectively. Moreover, many bacteria, including the potential pathogen *Acinetobacter baumannii* (abundance = 0.048) (Carvalheira *et al.* 2021), were found in Arroyo Maige and undetected in the Beni River sample point (Figs 4c-d).

Antibiotic resistance genes and gene cassettes with AMRGs were detected in the Bolivian Amazon rivers

The same types of ARGs were found in the rivers, although the relative abundance of these genes was higher in the sample point on the Beni River in most cases (Fig. 5a). These genes are involved in resistance to rifamycin (*rpoB2*, *rpoB*), peptide antibiotics (*ugd*, *arnA*), mupirocin (*mupA*, *mupB*), which use an alternative isoleucyl-tRNA synthase (*ileS*) to confer resistance, and tetracycline (*otr(A)*). All other genes were related to efflux pumps.

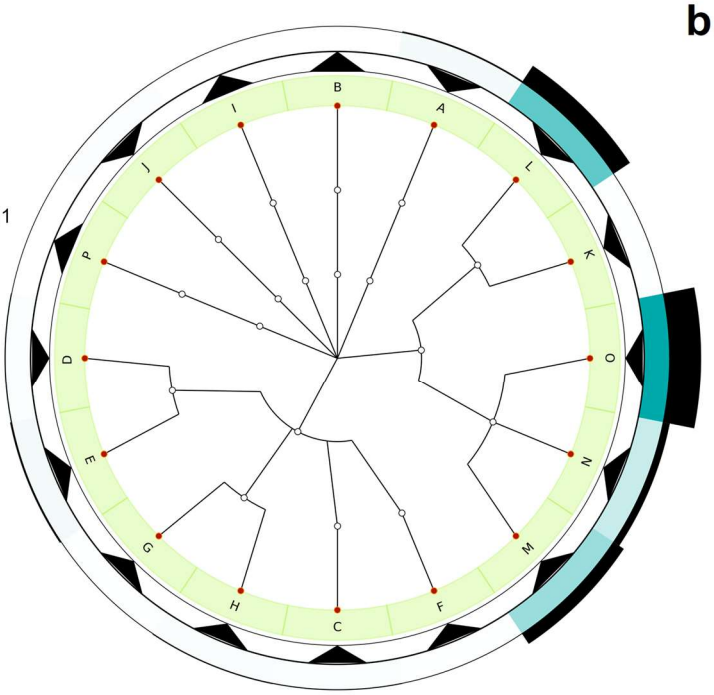
a.

A: *Pectinatus frisingensis*
B: *Selenomonas* SGB5910
C: *Erysipelatoclostridium ramosum*
D: Ruminococcaceae bacterium BL-4
E: *Liquorilactobacillus vini*
F: *Lactobacillus delbrueckii*
G: *Lactobacillus amylovorus*
H: *Lactobacillus mucosae*
I: *Lactobacillus panis*
J: *Lactiplantibacillus pentosus*
K: *Lactobacillus perolens*
L: *Lactobacillus coryniformis*
M: *Lacticaseibacillus manihotivorans*
N: *Lacticaseibacillus pantheris*
O: *Lacticaseibacillus rhamnosus*
P: *Lacticaseibacillus suibinensis*
Q: *Lacticaseibacillus suibinensis*
R: *Lacticaseibacillus paracasei*
S: *Lacticaseibacillus porcinae*
T: *Lacticaseibacillus camelliae*
U: *Lactobacillus vaccinostercus*
V: *Lactobacillus similis*
W: *Lactobacillus silagincola*
X: *Companilactobacillus farciminis*
Y: *Dialister* SGB5817
Z: *Dialister hominis*
a: *Megasphaera paucivorans*
b: *Megasphaera hominis*
c: *Megasphaera cereviciae*
d: *Megasphaera* sp. BL-7
e: *Anaerobium acetethylicum*
f: *Parasporobacterium paucivorans*
g: *Exiguobacterium alkaliphilum*
h: *Trichococcus ilyis*
i: *Eubacterium aggregans*
j: Eubacteriaceae unclassified SGB37411
k: *Clostridium* sp. DMHC-10
l: *Clostridium* sp. C2-6-12
m: Oscillospiraceae bacterium LBM18003
n: *Anaeroarcus burkinensis*



b.

A: *Selenomonas* SGB5910
B: Ruminococcaceae bacterium BL-4
C: *Lactobacillus coryniformis*
D: *Lacticaseibacillus pantheris*
E: *Lacticaseibacillus manihotivorans*
F: *Lactiplantibacillus pentosus*
G: *Lactobacillus delbrueckii*
H: *Lactobacillus amylovorus*
I: Eubacteriaceae unclassified SGB37411
J: *Clostridium* sp. C2-6-12
K: *Dialister* SGB5817
L: *Dialister hominis*
M: *Megasphaera paucivorans*
N: *Megasphaera hominis*
O: *Megasphaera* sp. BL-7
P: *Anaeroarcus burkinensis*



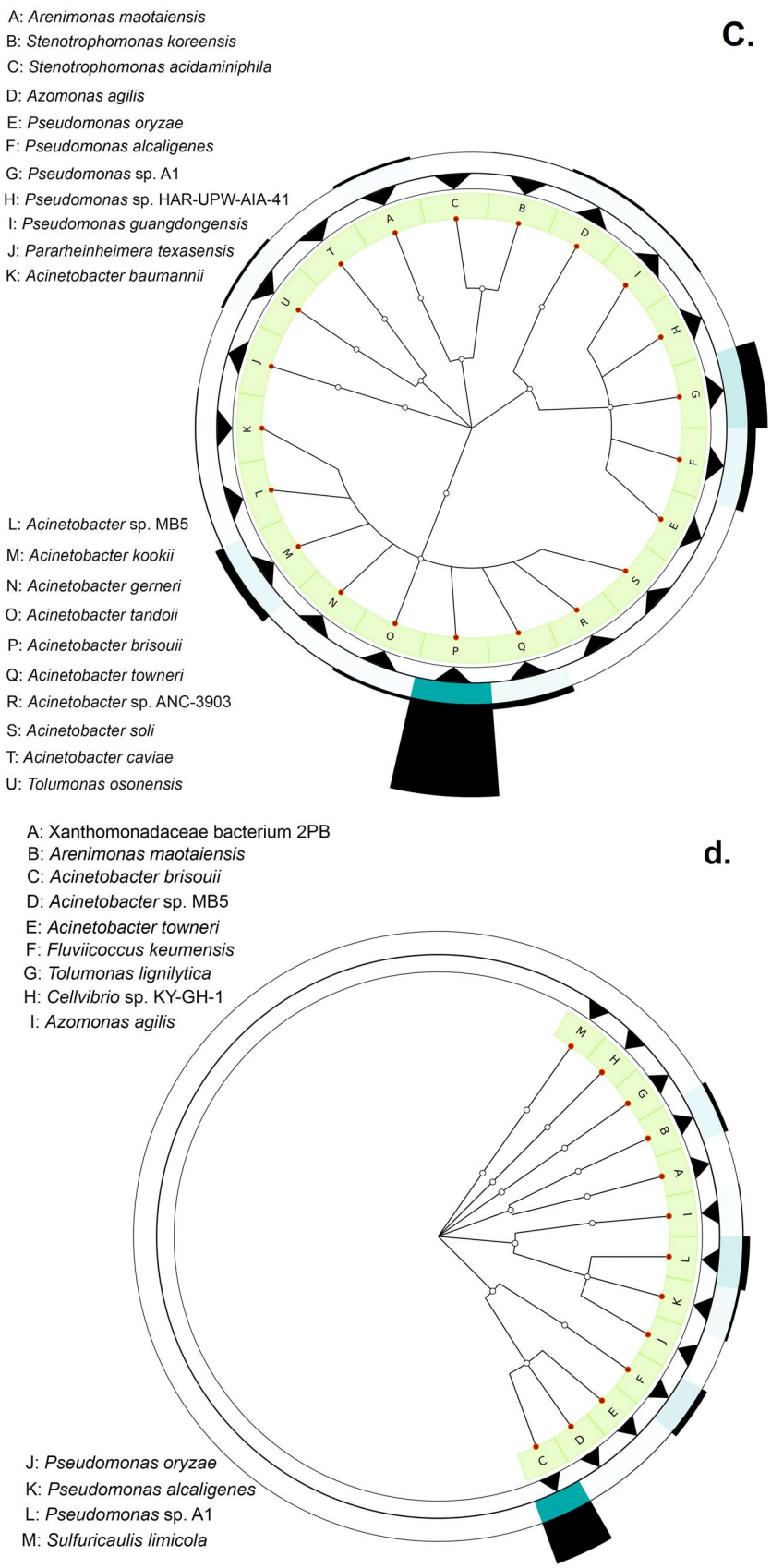


Figure 4. Identified bacterial species in Arroyo Maige (P1) and the Beni River (P3) metagenomes. The figures show a. Species of the phylum Firmicutes at P1, b. Species of the order Firmicutes at P3, c. Species of the class Gamma-Proteobacteria at P1, d. Species of the class Gamma-Proteobacteria at P3. Taxonomy and relative abundances of the identified species were obtained using MetaPhlAn (Blanco-Míguez *et al.* 2023) and plotted with the aid of GraPhlAn (Asnicar *et al.* 2015).

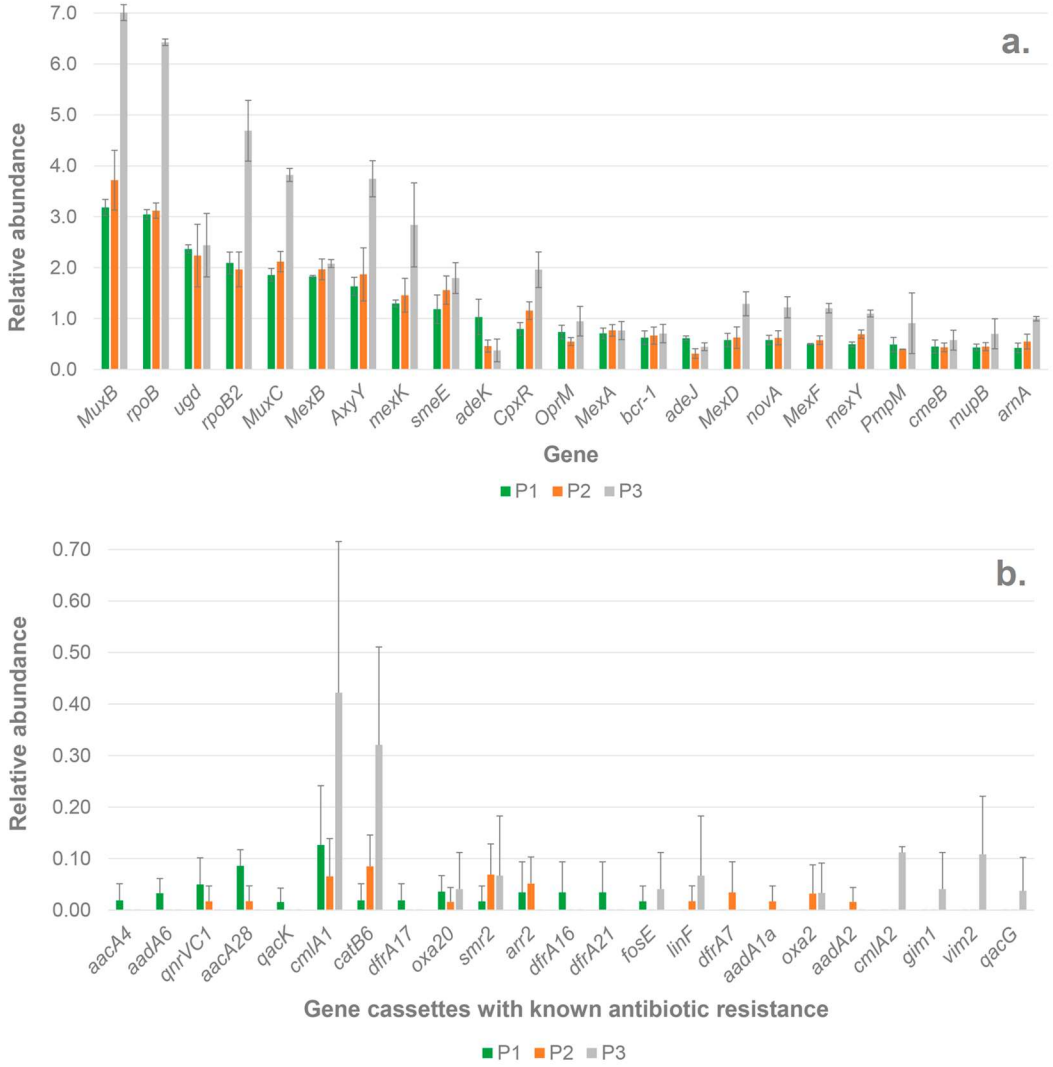


Figure 5. Identification of antibiotic resistance genes and gene cassettes with known AMRGs in Arroyo Maige and the Beni River metagenomes. Figures show a. ARGs with the highest relative abundances; the genes were identified in the metagenomes using the software Vsearch (Rognes *et al.* 2016) and the CARD database (Alcock *et al.* 2023), b. Gene cassettes were identified taken as reference a compiled feature database (Partridge *et al.* 2009); the genes were identified in the metagenomes using the software BLAST (Camacho *et al.* 2009). Standard deviations of the average values are shown as error bars.

To evaluate the mobile genetic elements that carry AMRGs, complete or almost complete DNA gene cassette sequences included in integrons were sought in the metagenomes of Amazon rivers (Fig. 5b). Some integrons were detected only in Arroyo Maige (that is, *aacA4*, *aadA6*, *qnrVC1*, *aacA28*, *qacK, dfrA16, dfrA21*) or in the Beni River sample point (*cmlA2*, *gim1*, *vim2*, *qacG*). These integrons combine genes known to confer resistance to more than one antibiotic. However, according to the characteristics of the gene cassettes, the integrons found in the rivers can be classified as (Partridge *et al.* 2009) aminoglycoside (6') acetyltransferases (*aacA4*, *aacA28*), streptomycin/spectinomycin resistance (*aadA1a*, *aadA2*, *aadA6*), quinolone resistance (*qnrVC1*), quaternary ammonium compound efflux (*qacG*, *qacK*), chloramphenicol exporters (*cmlA1, cmlA2*), chloramphenicol acetyltransferases (*catB6*), trimethoprim resistance (*dfrA7, dfrA16*, *dfrA17*, *dfrA21*), class D β-lactamases (*oxa2*, *oxa20*), small multidrug resistance proteins (*smr2*), rifampicin resistance (*arr2*), fosfomycin resistance (*fosE*), lincomycin nucleotidyltransferases (*linF*), and class B metallo-β-lactamases (*gim1, vim2*).

Discussion

Locals use Arroyo Maige to fish; however, drainage discharges intermittently pollute the river, where the water becomes dark and smells foul. People also witnessed fish dying in the river. Moreover, people become ill when they consume the river water. We performed the first metagenomic analysis on the Bolivian rivers. Both rivers are located in the Bolivian Amazon region, which is regarded as a biodiversity epicenter (Mariac *et al.* 2022). Therefore, different eukaryotic groups with high relative abundances

should be expected in the rivers. Moreover, as observed in three rivers in the Brazilian Amazon (Santos-Júnior *et al.* 2017), most DNA sequences of the microbiomes were related to bacteria. Nonetheless, the severe conditions in the Arroyo Maige did not affect its diversity compared to the sample point in the Beni River, suggesting a rearrangement in the abundance of species.

Heterotrophs were prevalent among the bacteria in the Bolivian rivers, indicating the availability of dissolved organic carbon (DOC), which did not account for sugars in our samples. A study of 186 black-odorous polluted rivers in China demonstrated that DOC was crucial in the darkening and foul smell of the water, and the microbial communities were similar to those found in sewage (Liang *et al.* 2023). In these rivers, ammonium was the primary nitrogen source, likely derived from DOC. The ammonium concentration ranged from 0.16 to 127.36 mg/l, while the total phosphorus concentration was between 0.01 and 8.36 mg/l (Liang *et al.* 2023). The concentrations of ammonium and orthophosphate in Arroyo Maige and sample point in the Beni Rivers are within these ranges and should maintain the active growth of bacteria. Furthermore, ammonium in the Chinese rivers was significantly correlated with the number and abundance of heterotrophic microorganisms, while organic matter triggered the overgrowth of prokaryotic and eukaryotic microorganisms (Liang *et al.* 2023). Excessive growth of aerobic organisms is usually accompanied by rapid oxygen consumption. In fact, studies of sewage-polluted streams in Brazil have shown that when water flow decreases, and a stream is heavily contaminated, an increase in DOC leads to a reduction in dissolved oxygen and an increase in water conductivity, whereas in less contaminated streams, dissolved oxygen and conductivity remain approximately constant (Daniel *et al.* 2002). The high water conductivity and the favored presence of microbial orders, which include microaerophilic and anaerobic bacteria, differentiated Arroyo Maige from the sample point in the Beni River and were consistent with observations in highly polluted streams in Brazil. The conductivity of Arroyo Maige was mainly related to the concentrations of sodium and potassium in the samples. These metals are present at high titers in gray water, which includes wastewater from laundries, washbasins, washing machines, dishwashing, bathrooms, and kitchen sinks (Shaikh & Ahammed 2020). Gray water may contaminate the Arroyo Maige because there are no wastewater treatment plants in the Bolivian Amazon region, which is the subject of our study. Furthermore, Desulfobacterales and Desulfovibrionales, which encompass sulfate-reducing representatives (Kuever *et al.* 2015) and were likely to be found in Arroyo Maige, can generate hydrogen sulfide, a noxious compound with a strong smell of rotten eggs (Locey 2005). The combination of these biological and chemical features hindered the abundance of some eukaryotes; in particular, the limitation or absence of oxygen may have had a detrimental effect on fish.

The presence of *Arcobacter butzleri* among the bacteria identified with a high relative abundance in the Bolivian rivers is noteworthy because it has been regarded as a foodborne pathogen (Ferreira *et al.* 2019). Moreover, *A. butzleri* and *A. cryaerophilus* are the predominant species isolated from sewage, and *A. butzleri* is a common pathogen detected in human diarrheal feces (Ferreira *et al.* 2014). This bacterium is also associated with non-diarrheal gastrointestinal illness, characterized by abdominal pain, nausea, vomiting, or fever (Van Den Abeele *et al.* 2014), which has been experienced by the population in the study area. Bacteria of the genera *Arcobacter* and *Acinetobacter*, whose number of species was higher in Arroyo Maige than in the sample point in the Beni River, are widespread in lakes, rivers, and coastal waters in or near urban areas (Lee *et al.* 2012). Some studies have described the occurrence of *Arcobacter* spp. in water with high levels of fecal pollution (Lee *et al.* 2012). Another species in the Bolivian rivers, with high abundance, shared DNA similarities with *Megasphaera* sp. BL7 (NCBI:txid1285585), *Dialister hominis*, and *Megasphaera hominis*, which were previously obtained from human feces or the intestinal tract (Sakamoto *et al.* 2020, Liu *et al.* 2021,). Our study suggests that the lack of adequate wastewater sanitation in the Bolivian Amazon region negatively affects its rivers, with a more significant impact on those with low water flow, such as Arroyo Maige.

ARGs are widespread worldwide, although regions with high population density have abundant ARGs (Zhang *et al.* 2022). This study used the same bioinformatics and normalization procedures described for the Bolivian lakes Alalay and Pastos Grandes (Daga-Quisbert *et al.* 2023). The former is an urban lake severely polluted with municipal and industrial wastewater (Quillaguamán *et al.* 2021), whereas the latter is a high-altitude lake with little anthropogenic influence (Daga-Quisbert *et al.* 2023). The types of ARGs in the Bolivian Amazon rivers were similar to those found in the Bolivian lakes. Nevertheless, the relative abundance of rifamycin-resistant genes *rpoB* and *rpoB2* found in the Beni River sample point was about the same as that of Lake Pastos Grandes, twice that of Arroyo Maige, and approximately four times lower than that found in Lake Alalay. The *rpoB* gene fragments detected in the Amazon rivers and Lake Alalay were related to *Bifidobacterium adolescentis* (Quillaguamán *et al.* 2021), a commensal bacterium in the digestive tract of the human population. In contrast, the abundance of these genes appeared to be a consequence of high exposure to UV radiation in Lake Pastos Grandes (Daga-Quisbert *et al.*

2023). Being at a low altitude (c.a., 270 m), Arroyo Maige and the sample point in the Beni River should not be subjected to elevated UV; instead, pollution originating from wastewater may have a more significant effect on the presence of the gene. On the other hand, genes associated with efflux pumps are not generally considered a hazard when they are found in metagenomes because antibiotic resistance is related to the mutation of a repressor gene that upregulates the expression of the efflux pump rather than the presence of the gene (Fitzpatrick & Walsh 2016). They are also ubiquitous genes likely to be involved in fundamental processes of bacterial physiology (Martínez *et al.* 2015). However, a recent study assessed the global health risks of ARGs based on their transmission from the environment to bacteria in humans, their presence in pathogens, and their relationship to the current use of antibiotics (Zhang *et al.* 2022). The evaluation considered four categories, Q1 to Q4, with Q1 being high-risk and found in large numbers in the genomes of the pathogenic bacteria. Many multidrug ARGs are bacterial efflux pumps (Zhang *et al.* 2022). Based on this classification, several efflux pump genes from the Bolivian rivers (*ugd, MexB, AxyY, MexK, smeE, OprM, MexA, adeJ, MexD, MexF, mexY, PmpM, mexI, smeB, OpmB, mexW, acrB, ceoB,* and *mexQ*) belonged to category Q1. Consequently, these genes are potential threats in these rivers, and their harmful effects depend on their expression in bacterial pathogens. Additionally, the mobility of AMRGs involved in resistance to various types of antibiotics is relevant to Arroyo Maige owing to the detection of potential pathogens, such as *Arcobacter* species and *Acinetobacter baumannii*, known to be capable of acquiring ARGs from different microorganisms (Carvalheira *et al.* 2021).

Conclusions

The Arroyo Maige is located in the Bolivian Amazon region, is small, and has been intermittently contaminated with a drainage discharge. Contrastingly, the Beni River is large. The elevated conductivity of Arroyo Maige, the identification of heterotrophic microorganisms, and the likely anaerobic conditions that favored the presence of prokaryotes that thrive in such an environment are consistent with observations in darkened-odorous rivers and streams, which have been polluted with wastewater and can be found elsewhere. In fact, some bacterial species previously obtained from wastewater, sewer pipes, human feces, or intestinal tracts were identified in the metagenomes of the Bolivian rivers. The smaller size of Arroyo Maige probably enhanced the distressing sensorial effects on people compared with the Beni River. Additionally, the disease symptoms experienced by locals who consumed water from Arroyo Maige were similar to those caused by *Arcobacter butzleri*; however, a clinical evaluation is required. The potential pathogen *Acinetobacter baumannii* was further detected in Arroyo Maige. Multidrug ARGs included in efflux pumps, currently considered high-risk for global health, were also detected in the metagenomes. The expression of these genes in pathogens and the occurrence of integrons, known to mobilize ARGs among bacteria, are potential threats to the population living near the rivers. Overall, our work showed that the nonexistent sanitation treatment of wastewater in the Bolivian Amazon adds to the detrimental environmental conditions in the zone and should call for adequate political and technological actions.

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