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A case study on pharmaceutical residues and antimicrobial resistance genes in Costa Rican rivers: A possible route of contamination for feline and other species

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ABSTRACT

In this investigation, the presence of antibiotics and pharmaceuticals in Costa Rican surface waters, specifically in regions near feline habitats, was examined. The study revealed that 47% of the water samples contained detectable traces of at least one antibiotic. Ciprofloxacin and norfloxacin were the most frequently detected compounds, each with a detection rate of 27%. Other antibiotics, such as erythromycin, roxithromycin, and trimethoprim, were also found but at lower frequencies, around 14%. Notably, all antibiotic concentrations remained below 10 ng/L, with ciprofloxacin, norfloxacin, and erythromycin showing the highest concentrations. Furthermore, the investigation revealed the presence of non-antibiotic pharmaceutical residues in the water samples, typically at concentrations below 64 ng/L. Tramadol was the most frequently detected compound, present in 18% of the samples. The highest concentrations were observed for acetaminophen and tramadol, measuring 64 and 10 ng/L, respectively. Comparing these findings with studies conducted in treated wastewater and urban rivers, it became evident that the concentrations of antibiotics and pharmaceuticals were notably lower in this study. While previous research reported higher values, the limited number of studies conducted in protected areas raises concerns about the potential environmental impact on biodiversity.

In summary, these results emphasize the importance of monitoring pharmaceutical residues and antimicrobial resistance genes ARGs in vulnerable ecosystems, especially those in close proximity to feline habitats in Costa Rica. Additionally, the study delved into the detection of (ARGs). All tested water samples were positive for at least one ARG, with the bla_{TEM} gene being the most prevalent at 82%, followed by *tet*S at 64% and *qnr*B at 23%. Moreover, this research shed light on the complexity of evaluating ARGs in environmental samples, as their presence does not necessarily indicate their expression. It also highlighted the potential for co-selection and co-regulation of ARGs, showcasing the intricate behaviors of these genes in aquatic environments.

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[1] Rest in peace.

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1. Introduction

Antimicrobial resistance (AMR) has emerged as a pressing global concern, presenting an escalating threat to human health (Qiao et al., 2018; Ventola, 2015). Alarming estimates project that antibiotic-resistant bacterial infections could claim the lives of 10 million individuals annually worldwide by 2050 (Amarasiri et al., 2020). The origins of antimicrobial resistance genes (ARGs) are multifaceted, encompassing environments such as healthcare and veterinary facilities where bacteria encounter high antibiotic concentrations (Ng and Gin, 2019). Furthermore, sources of environmental contamination by antimicrobial substances, antibiotic-resistant bacteria, and ARGs have been identified in households, wastewater treatment plants, livestock, and agricultural activities (Amarasiri et al., 2020; de la Cruz et al., 2014; Ng and Gin, 2019). ARGs, constitute a pivotal element within antimicrobial resistance. These genes empower bacteria, viruses, and various microorganisms with the capability to withstand antimicrobial agents. ARGs have the capacity to transfer between distinct microorganisms via mechanisms like conjugation, transduction, and transformation, thereby promoting the dissemination of antimicrobial resistance throughout diverse bacterial species. Comprehending the intricate interplay between AMR and ARGs is paramount for the efficient mitigation of the antimicrobial resistance challenge.

Recent reports have highlighted the presence of antimicrobial contamination in aquatic environments, promoting the proliferation of AMR. Even at low concentrations, these substances have been shown to sustain resistant bacterial populations, emphasizing the urgency of this issue (Karkman et al., 2019; Ng and Gin, 2019; Wellington et al., 2013; Amarasiri et al., 2020; Gullberg et al., 2011). Costa Rica provides an exceptional setting for AMR research due to its unique combination of climatic conditions, intricate river networks, and extensive antibiotic usage in veterinary and agricultural contexts. Remarkably, by 2009, the National Animal Health Service (SENASA) had registered an astonishing 473 antibiotics (Rojas et al., 2017). This extensive antibiotic usage raises concerns about pharmaceutical waste management, potentially leading to the detection of (ARGs) within feline fecal samples (Angulo et al., 2023). Our research builds upon prior work that examined 16 ARGs in the fecal samples of jaguars (*Panthera onca*) and pumas (Puma concolor) within Costa Rican conservation areas. This work revealed the presence of ARGs, particularly those associated with tetracycline, sulfonamides, phenicols, and quinolones. This discovery prompted our focus on understanding the extent of contamination affecting felines and other wildlife species (Angulo et al., 2023). In this paper, we present compelling evidence of the presence of antibiotics, other pharmaceuticals, and ARGs in small rivers in Costa Rica, this innovative perspective diverges from the typical focus on human health to highlight the significance of wildlife health. We concentrated on smaller river flows, known for their accessibility to wildlife, particularly wild felids that rely on them for drinking water. This situation introduces the potential risk of contamination from pollutants carried by these waters, emphasizing broader environmental consequences. We selected sampling sites near sentinel and umbrella species, such as wildcats with easy access to these rivers, and considered potential human impact on these areas. Our analysis also includes environmental metrics like the Remaining Vegetation Index (RVI), reflecting forest cover percentages at each site (Table S3), and an index of human activity based on land use. The selection of compounds and ARGs builds upon previous studies conducted in Costa Rica (Angulo et al., 2023; Causanilles Llanes, 2018; Ramírez Morales et al., 2021a; Rodríguez et al., 2006; Spongberg et al., 2011; Wilkinson et al., 2022). To safeguard the privacy of the numerous subsistence activities taking place near these rivers and mitigate their potential vulnerability to our findings, we have opted to anonymize the river names.

2. Materials and methods

2.1. Sampling

In 2021, 22 rivers in Costa Rica were sampled once for analysis of antibiotics, drugs, and ARGs. The sites were chosen due to the possibility that wild animals were drinking water from these rivers. The study area includes the Braulio Carrillo (Fig. 1) and Los Quetzales National Parks and their surrounding area. Previous data on feline faecal samples, confirm the presence of wildcats at selected sites. Anthropogenic influences such as agriculture, fishing, livestock, and human settlements were delimited. Each sampling site has been assigned a side of the code to keep the name of the rivers anonymous, and in all cases, the geographic coordinates were taken.

2.2. Chemicals

Analytical reference standards and their isotopically-labeled internal standards (ILIS) were purchased from LGC (Teddington, UK) and Merck (Darmstadt, Germany). Methanol, acetonitrile, formic acid (LC-MS grade), and ammonium acetate (>98%) were acquired from Scharlab (Scharlab, Barcelona, Spain). LC-MS grade water was obtained using an Ultramatic Plus GR from Wasserlab (Navarra, Spain). Literature can provide more details (Botero-Coy et al., 2018; Fabregat-Safont et al., 2023).

In total, 46 target compounds were selected: 20 antibiotics (amoxicillin, ampicillin, cloxacillin, cefuroxime, cefditoren, azithromycin, clarithromycin, clindamycin, erythromycin, roxithromycin, ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin, doxycycline, sulfamethoxazole, sulfadiazine, tetracycline, metronidazole, and trimethoprim), and 25 additional pharmaceuticals (acetaminophen, phenazone, levamisole, primidone, nalidixic acid, carbamazepine, salbutamol, oxonolic acid, flumequine, tramadol, metropopol, venlafaxine, diclofenac, alprazolam, lorazepam, omeprazole, furaltadone, enalapril, pantoprazole, losartan, irbersartan, valsartan, atorvastatin, ioprimide, gabapentin).

2.2.1. Analytical methodology

Pharmaceutical residue analysis was performed by direct injection followed by LC-MS/MS measurement. Briefly, 2 mL of each sample was centrifuged at 12,000 rpm for 10 min 950 μL of supernatant was transferred to a glass vial, and 50 μL of a mixture of ILIS at 5 μg/L was added (final ILIS concentration in samples 0,25 μg/L). A high number of analyte-ILIS were used to correct for matrix effects and other instrumental analysis variations, improving accurate quantification. Two injections were made into the LC-MS/MS system: one for the determination of 20 antibiotics, and the other for the determination of 25 additional pharmaceuticals. The absence of sample pre-treatment -Safont et al., 2023.

LC-MS/MS analysis were made using an ACQUITY H-CLASS UPLC chromatographic system (Waters Corp, Milford, MA, USA) coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp., Wilmslow, Manchester, UKK), equipped with an electrospray ionization interface (ESI), operated in positive mode. Cone voltage was optimized for each compound. More details on the analytical methodology applied on and instrumental conditions can be found in Botero-Coy et al. (2018); Fabregat-Safont et al. (2023).

The samples were quantified with a 6-level calibration curve in a concentration rangefrom 0.5 ng/L to 100 ng/L. Together with the samples, quality control (QC) samples were also prepared by spiking real-world river water samples at two concentration levels (5 and 50 ng/L). QC recoveries between 60 and 140% were considered as acceptable SANTE/12682/2019. (s. f.). Table S1 shows the QCs recoveries for the compounds that were quantified in the samples. Recoveries were acceptable at all spiked concentrations, except for roxithromycin at the low spiked level used for antibiotics (0.5 ng/L) and tramadol at the lowest level used for the rest of pharmaceuticals (100 ng/L).

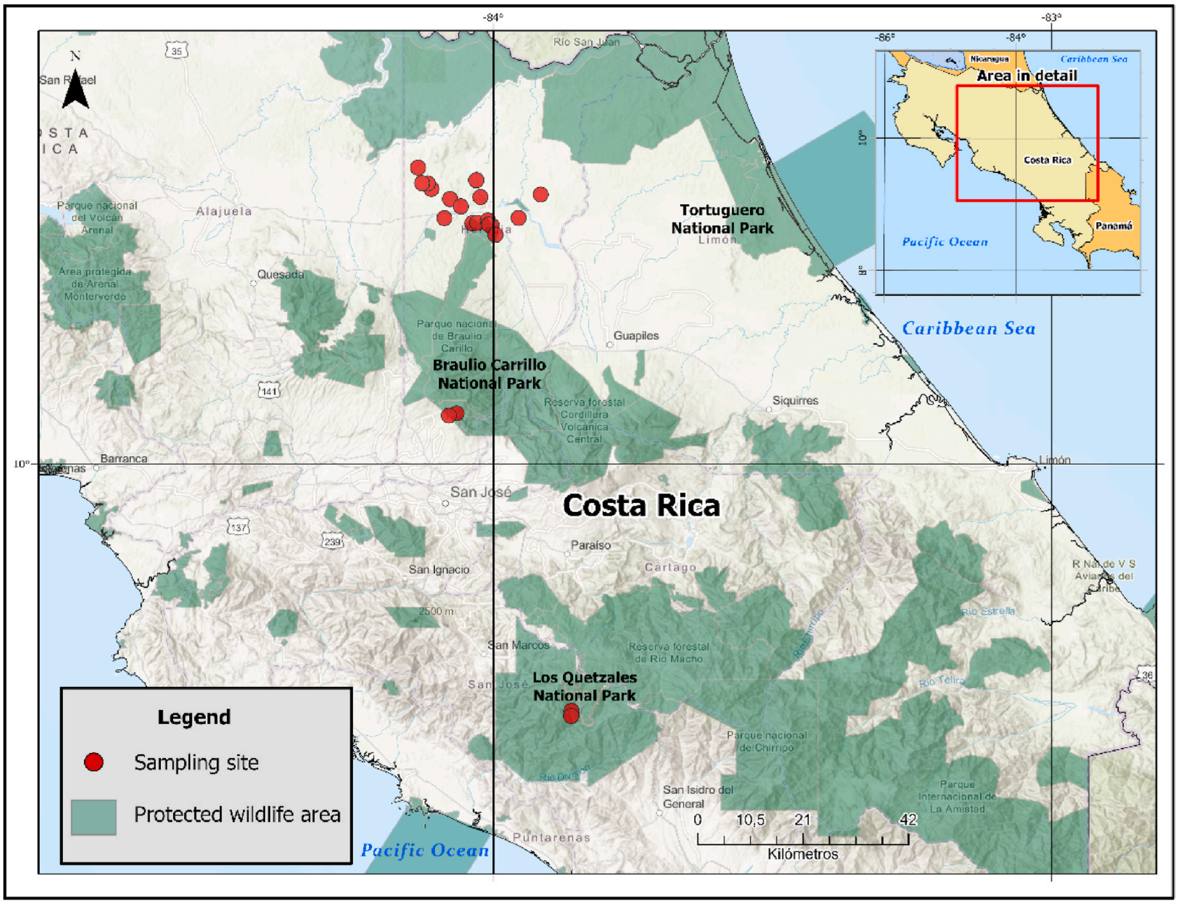

Fig. 1. Location of the sampling sites (n = 22) in rivers near feline habitats.

For compound identification, q/Q ion ratios were calculated from the peak areas of the quantification ion (Q) and two confirmation ions (q1 and q2). The identity of the substance in the samples was confirmed if at least one experimental ion ratio (q1/Q and/or q2/Q) did not deviate more than ±30 %, and retention time more than ± 0.1 min compared to the average value of reference standards injected in the calibration and/or QC samples analysed in the sample batch SANTE/12682/2019. (s. f.). The analyses were performed at the Research Institute for Pesticides and Water, University Jaume I, Castelló, Spain.

2.3. Antimicrobial resistance genes (ARGs)

Water samples were filtered using 0.2 mm filters (Thermo Scientific Nalgene, USA). The genomic DNA from the water samples was extracted with the PowerDNA kit (Mo Bio). With the 16 S rRNA gene, bacterial genetic material was identified in each sample, following the methodology previously proposed (Doi and Arakawa, 2007). A valid one was one whose 10-fold dilution showed a cycle threshold (ct) less than 30.

The 21 ARGs analysed were selected as representatives of eight antibiotic groups: tetracyclines (*tet*A, *tet*B, *tet*K, *tet*M, *tet*Q, *tet*S, *tet*W, *tet*Y), chloramphenicols (*cat*I, *cat*II), sulfonamides (*sul*I, *sul*II), aminoglycosides (*str*, *aad*A), macrolides (*erm*B, *erm*F), beta-lactams (bla_{TEM}, $bla_{\mathrm{CTX\text{-}M}}$), quinolones (*qnr*S, *qnr*S), and polymyxins (*mcr*-1) ((Table S2).). All genes were analysed by real-time PCRs, using SYBR Green. The protocols were described previously (Nieto-Claudin et al., 2019) and quantified using the following formula:

$$log\ 10(percentage\ of\ an\ ARG) = 2 + 0.33 * (ct16SrRNA - ctARG)$$

where $ct_{16SrRNA}$ is the cycle threshold for the bacterial determination, ct_{ARG} is the cycle threshold for each gene, and the value 0.33 is the mean slope for all the genes tested. The results were expressed in $\log_{10}$ of the hypothetical percentage of bacteria each gene presented for the ARGs percentage load, with 10^{-7} as the minimum detectable value. This formula also allows the results to be expressed by percentage, highly correlated with those previously published ($R^2 = 0.9097$; data not shown). A value of 10^{-8} was assumed when a result had not been determined. This value represents the PCR's minimum detectable load. A negative PCR result indicates concentrations equal to or below 10^{-8}, as values lower than this threshold cannot be detected. All DNA extractions were conducted at the Regional Institute for Studies on Toxic Substances, Universidad Nacional, Costa Rica. The gene determination and quantification were performed at the European University, Madrid, Spain.

2.4. Remaining vegetation index (RVI)

The study area was first delimited using Geographic Information Systems (GIS). First, the watershed boundary limits were identified. This curved line is defined by the altitudes, which have a close point to the location of the sampling sites and includes the distinction of the drainages. Next, the remaining vegetation index to each watershed (**RVI**) expresses the forest cover (remaining vegetation) as its total percentage. With its calculi, it is possible to characterize regions using the following formula (Marquez, 2000; Quijano Pinzón, 2016):

$$RVI = \left(\frac{AVR}{At}\right) * 100$$

where **ARV** represents the remaining vegetation area, and **At** is the total area of the unit in Km^2.

The forest cover was obtained as a product of the restitution carried out with orthoimages produced between 2015 and 2018 by the Costa Rican National Geographic Institute. They are available through the National Territorial Information System (SNIT, 2021). The categories identified with this index are shown in Table S3.

In addition, from the SNIT, the layers of rivers, roads, urban use, pastures, and crops used for the different micro-watersheds were obtained, and the distances concerning the sampling points were determined. For this, the ArcGIS Pro version 3.0.3 software and the select by location tool were used, which takes the features of the input layer (sampling points) and selects the elements within a specified distance (using the distance Euclidean) of the selected entity.

2.5. Data analysis

Descriptive statistics were used to show the results of antibiotics and other pharmaceuticals. The cumulative antibiotic and other pharmaceutical concentrations were calculated as the sum of all those quantified at each specific site.

For ARGs detection, it was possible to elaborate a heatmap to visualize the most common ARGs in the rivers using the packages heatmap (Kolde, 2019) and ggplot2 (Wickham, 2009) from R. This visualization technique allowed the measurement of the magnitude of a phenomenon in two-dimensional colors and the identification of the most representative variable. For a better graphical interpretation, the highest values (>1) were scaled to visualize those lower than 0.1 since they are representative. Subsequently, a Cluster was performed to classify the rivers based on the ARGs burden and the Bray Curtis dissimilarity index to consider the presence and concentration of interest variables. In this line, the Vegan package (Oksanen et al., 2022) was employed for the clustering.

To determine if there is a difference in ARGs concentrations according to the RVI, a one-way PERMANOVA test (Vicente-Gonzalez & Vicente-Villardon, s. f.) with 99909 permutations was applied. So, we wanted to corroborate whether the more forest cover, the lower the ARG concentration. Subsequently, Kruskall-Wallis and posthoc tests were performed to determine whether remnant vegetation treatments differed (Lê et al., 2008; Oksanen et al., 2022; Wickham, 2009).

Finally, Redundancy Analysis (RDA) was performed to determine a relationship between ARGs and registered pharmaceutical residues in the sampled rivers. Vegan package (Oksanen et al., 2022) was used to carry out the analysis.

3. Results and discussion

3.1. Antibiotic and other pharmaceuticals detection

At least one antibiotic residue was detected in 47% of the samples. Ciprofloxacin and norfloxacin were the most frequently observed compounds, each detected in 27% of the samples. Erythromycin, roxithromycin, and trimethoprim had detection frequencies of 14%. All antibiotic concentrations were below 10 ng/L. The highest concentrations were recorded for ciprofloxacin (8.2 ng/L), norfloxacin (7.5 ng/L), and erythromycin (6.3 ng/L) (Tables S4 and S6).

Pharmaceutical residues other than antibiotics were also detected at low concentrations (mostly below 10 ng/L) in several samples. The most frequently observed compound was tramadol (18%), while the highest concentrations were recorded for acetaminophen (paracetamol) (64 ng/L) and tramadol (10 ng/L) (Tables S4 and S6, Fig. S1).

Comparing the frequencies and concentrations of pharmaceuticals with those reported in other publications, particularly in treated wastewater and urban rivers, it is observed that data reported in other papers are much higher than the values obtained in our campaigns (Beattie et al., 2018; Bijlsma et al., 2021; Causanilles Llanes, 2018; Fonseca et al., 2020; Grenni, 2022; Guan et al., 2018; Lopez et al., 2022; Ramírez-Morales et al., 2021a, 2021b; Spongberg et al., 2011; Wilkinson et al., 2022). Unfortunately, studies in protected areas are scarce (Pereira et al., 2020), which makes troublesome the comparison of data. More studies are needed to get a better knowledge on anthropogenic pollution effects on biodiversity.

Despite the lack of data from protected areas, we did find some similarities between the substances listed in Table 1 and compounds previously reported in Costa Rica and other regions worldwide for urban rivers. Some examples are: Ramírez Morales et al. (2021a) confirmed the presence of pharmaceuticals such as acetaminophen (18.5%), as well as antibiotics such as ciprofloxacin and trimethoprim (18.5% and 3.7%, respectively) in the San José rivers, Costa Rica. Another study in the surrounding surface waters of an animal production area in the highlands of San José, Costa Rica, reported the presence of sulfamethoxazole (12%), tetracycline (22%), and doxycycline (77%), with norfloxacin (28%), and ciprofloxacin (15%), frequencies similar to our results (Ramírez-Morales et al., 2021b). In our study erythromycin, roxithromycin, and trimethoprim were the most frequent compounds. These same pharmaceuticals were also reported in the Ba River in Xi'an, China (Guan et al., 2018). In another study, Botero-Coy et al. (2018) reported levels above 1 μg/L for ciprofloxacin and norfloxacin in urban wastewater from Colombia, even higher than those found in Costa Rican urban areas.

The cumulative concentration of pharmaceuticals in the rivers ranged from 3 to 70 ng/L (Fig. S2). Thanks to the excellent sensitivity of the LC-MS/MS technique, even by direct injection of the samples, we achieved quantification limits in the range of 0.5 and 1.0 ng/L. Although these reported concentrations are very low, recent scientific evidence indicates that resistant bacteria are positively selected even at low concentrations (Amarasiri et al., 2020; Beattie et al., 2018; Gullberg et al., 2011).

3.2. Antibiotic Resistance Genes detection

All samples were positive for at least one ARG. The encoding one to *bla*$_{\mathrm{TEM}}$ was the most frequent in all samples (82%), followed by *tet*S (64%) and *qnr*B (23%). ARGs encoding *bla*$_{\mathrm{TEM}}$, *qnr*B, and *mec*A showed the highest concentrations. No gene encoding phenicols was detected (Table S5).

Table 1 shows the presence of ARGs in the study area. It is worth mentioning that the detection of ARGs by PCR is not precisely equivalent to its presence orabsence. This is because an ARG may be present below the detection limit, which is influenced mainly by the nature of the matrix, especially the presence of PCR inhibitors and nontarget DNA (Storteboom et al., 2010).

The ARG detected most frequently and in high concentrations was *bla*$_{\mathrm{TEM}}$, although no β-lactam residues were identified in the water samples. β-Lactam-resistant bacteria have been reported in several Latin American countries (Domínguez et al., 2021), and other studies have shown high *bla*$_{\mathrm{TEM}}$ gene copy numbers in urban rivers worldwide (Guan et al., 2018). Penicillins of the β-lactam class are one of the most widely used families of antibiotics in human and veterinary medicine, but when monitored in rivers, their concentrations are usually very low due to their rapid degradation in environmental media by photolysis and hydrolysis of β-lactams (Grenni, 2022; Timm et al., 2019). For this reason, it is necessary to strengthen the monitoring of ARGs, especially considering that the TEM-β-lactamases encoded by the *bla*$_{TEM}$ gene represent one of the most clinically important β-lactamase families (Guan et al., 2018).

Other publications have mentioned, as do our results, that antibiotics (e.g., aminoglycosides, macrolides, quinolones and fluoroquinolones, sulfonamides, and tetracyclines) have also been reported in low concentrations in rivers (Grenni, 2022; Krzeminski et al., 2019). This can be explained by the degradation/biodegradation of the antimicrobials in water, as their low molecular weight (<1000 g/mol) and relatively low octanol/water partition coefficients (K_{OW}), which favor their rapid dissolution in water bodies (Grenni, 2022; Krzeminski et al., 2019).

Tetracycline-resistant genes were the second most frequent group of

Table 1
Characterization of sampling areas according to their Remaining Vegetation Index (**RVI**) and findings of antibiotics, other pharmaceuticals, and antimicrobial resistance genes (ARGs) in surface waters located close to Costa Rican wild feline habitats in two national parks and surroundings.

Side Code	Location	Characteristics	RVI %	Category	Antibiotic	Other pharmaceuticals	ARGs
EGR[a]	Sarapiquí	ZAI	62	Partially transformed	Ciprofloxacin, norfloxacin, furaltadone	n.d.	*tet*S, *str, aad*A, bla_{TEM}
CRC	Sarapiquí	ZAI	68	Partially transformed	Ciprofloxacin, norfloxacin, furaltadone	Acetaminophen	*tet*S, *qnr*B, bla_{TEM}
TC	Sarapiquí	ZAI	68	Partially transformed	Ciprofloxacin, norfloxacin	n.d.	*tet*S, *sul* II, *aad*A, *qnr*B, bla_{TEM}
CNC	Sarapiquí	ZAI	46	Moderally transformed	n.d.	n.d.	bla_{TEM}, *mec*A
PR	Sarapiquí	Biological Station	94	Not transformed or scarcely transformed	Roxithromycin, trimethoprim	n.d.	*erm*
SR	Sarapiquí	Biological Station	100	Not transformed or scarcely transformed	Erythromycin, trimethoprim	n.d.	*qnr*B, *mec*A
SuC	Sarapiquí	Biological Station	98	Not transformed or scarcely transformed	n.d.	n.d.	*tet*S, bla_{TEM}
ESC	Sarapiquí	Biological Station	100	Not transformed or scarcely transformed	Ciprofloxacin, norfloxacin	n.d.	bla_{TEM}
S3C	Sarapiquí	Biological Station	98	Not transformed or scarcely transformed	n.d.	n.d.	*str, qnr*B, bla_{TEM}
PrR	Dota	LQNP	86	Not transformed or scarcely transformed	n.d.	n.d.	*tet*S
PoR	Dota	LQNP	85	Not transformed or scarcely transformed	Ciprofloxacin, norfloxacin, erythromycin, sulfamethoxazole, roxithromycin	Acetaminophen, Tramadol	bla_{TEM}, *mec*A
PLVR	Sarapiquí	Wildlife Refuge	61	Partially transformed	n.d.	n.d.	*str, mec*A, bla_{TEM}
SaR	Sarapiquí	ZAI	91	Not transformed or scarcely transformed	Roxithromycin	n.d.	*tet*S, bla_{TEM}
SMC	Sarapiquí	ZAI	50	Partially transformed	n.d.	n.d.	bla_{TEM}
EC	Sarapiquí	ZAI	82	Not transformed or scarcely transformed	n.d.	n.d.	*tet*S, bla_{TEM}
GC	Sarapiquí	ZAI	67	Partially transformed	Roxithromycin, trimethoprim	n.d.	*tet*A, *tet*M, *tet*S, bla_{TEM}
So2C	Sarapiquí	ZAI	54	Partially transformed	n.d.	n.d.	*tet*S, bla_{TEM}
SaR	Sarapiquí	ZAI	66	Partially transformed	n.d.	n.d.	*tet*S, bla_{TEM}
TiR	Sarapiquí	ZAI	55	Partially transformed	Norfloxacin	Tramadol	*tet*S, bla_{TEM}, *mcr*-1
CDC	Sarapiquí	BCNP	99	Not transformed or scarcely transformed	Ciprofloxacin, norfloxacin	n.d.	*tet*S, *qnr*B, bla_{TEM}
CD1C	Sarapiquí	BCNP	99	Not transformed or scarcely transformed	Erythromycin, sulfamethoxazole	Acetaminophen	*tet*S, *sul* II, *str*, *aad*A, bla_{TEM}
GR	Sarapiquí	ZAI	97	Not transformed or scarcely transformed	n.d.	n.d.	*tet*S, *tet*K

[a] ZAI: Zone of anthropogenic influence, LQNP: Los Quetzales National Park, BCNP: Braulio Carrillo National, nd: no detected.

ARGs *tet*S (64%). This antibiotic is widely used in both veterinary and human medicine, primarily as an additive in animal feed. Granados-Chinchilla et al. (2014) evidenced concentrations to this product and abundant Gram-positive bacteria resistant in farm animal feeds marketed in Costa Rica. For example, fish feeds contained the highest amounts of tetracyclines (119–8365 mg kg^{-1}) and the highest proportion of bacteria resistant to 10 μg mL^{-1} (1.8–92.4%). So, this can be considered as a possible contamination way.

Guan et al. (2018) analysed antimicrobials in both water and sediment samples, finding that tetracyclines mainly accumulated in sediment compared to water samples, while the concentration of trimethoprim in water was higher than in sediment. They justified these differences in antibiotic distribution depending on antibiotics' physicochemical stability and geographical area characteristics, confirming that tetracyclines were highly absorbed in organic matter, sediments, and soil (Christou et al., 2017; Guan et al., 2018). Besides, they are usually detected in aqueous matrices at deficient concentrations (<10 ng/L) because they form a stable complex with suspended matter (Grenni, 2022). These characteristics could explain the contamination with *tet*A, *tet*K, *tet*M, and *tet*S, although no tetracycline residues were identified in the water samples.

Although the ARGs are naturally present in the environment, very few studies have focused on "less impacted" rivers to assess their background levels (Grenni, 2022). This is worrying considering that different human activities surround these areas, including crops and livestock production, are potential sources of contamination (Doi and Arakawa, 2007). E.g., the lack of wastewater treatment and the use of manure as fertilizer on farmland promote the spread of resistant bacteria and ARGs (Pereira et al., 2020). Moreover, runoff can facilitate the transport of manure pollutants to surface water systems (Beattie et al., 2018), especially considering that between 25% and 75% of antibiotics can be excreted unchanged in urine or feces, providing direct environmental screening pressure (Beattie et al., 2018).

3.3. Remaining vegetation index (RVI)

As shown in Table 1, most sites (54%) have an untransformed or scarcely transformed forest cover (RVI ≥70%). The forest cover was partially transformed in 41% of the sampling sites (50% ≤ RVI<70%). The number of sites with a moderately transformed forest cover is low (4.5%), with an RVI between ≥30%, but <49%.

The diverse human activities in the monitoring areas could explain why that conserved riparian forests exhibited similar concentrations of chemicals and ARGs as areas with partial and moderate disturbances. This can be explained by the Costa Rican water network complexity. In our case, some small rivers can be used to discharge wastewater from towns or human activities, and their water flows into the main channel of the rivers studied. This discharge generally occurs upstream of the watershed, where no degree of conservation exists. One example corresponds to the PoR River (Fig. 2), with 84% vegetation cover; Despite being located within the Los Quetzales National Park, ciprofloxacin, norfloxacin, erythromycin, sulfamethoxazole, roxithromycin, acetaminophen, and tramadol were determined, as along with bla_{TEM} and *mec*A (see Table 1). Upstream from PoR, there is a small village where

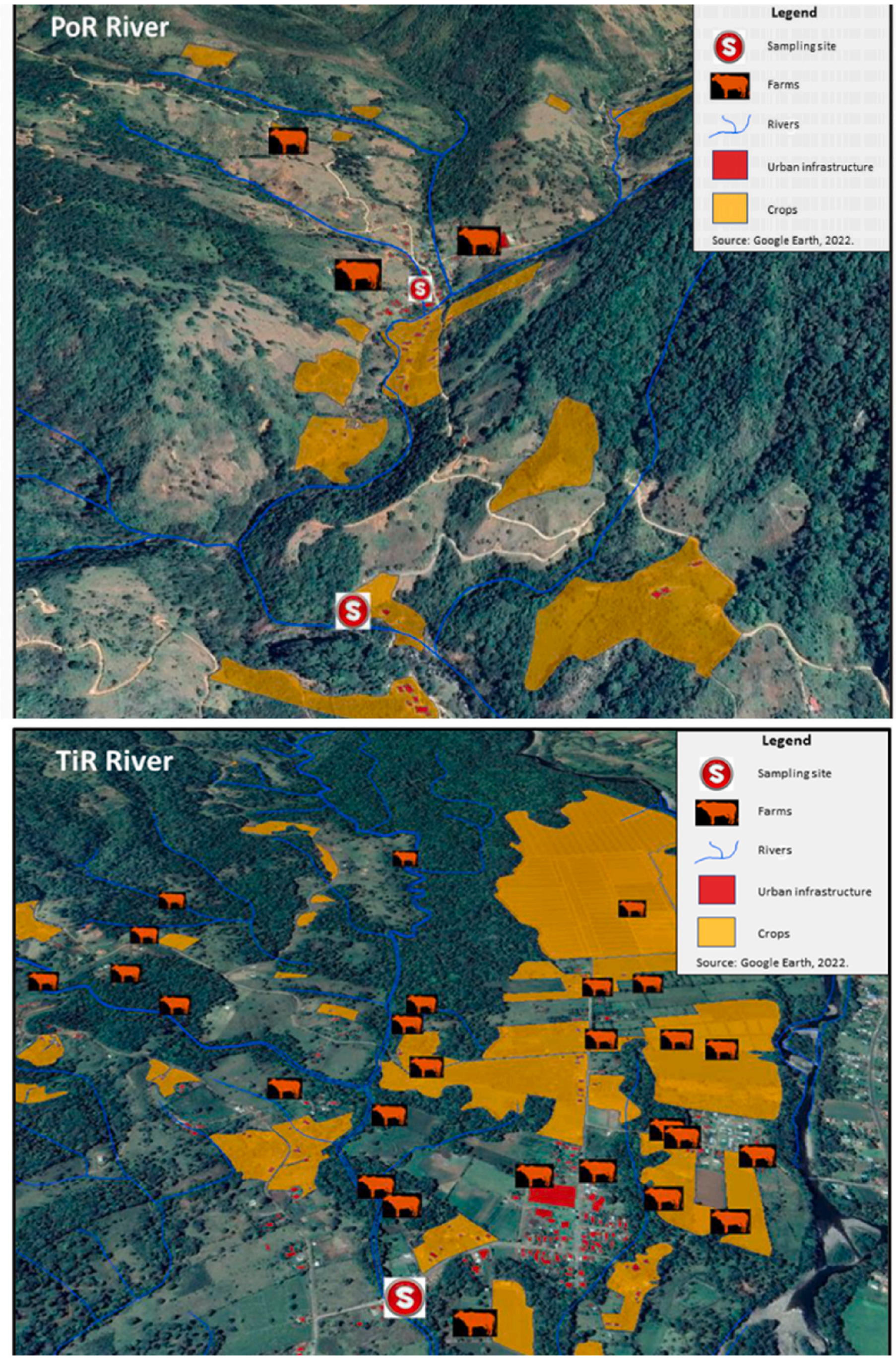

Fig. 2. Geographic distribution, farms, crops, urban infrastructure, surrounding. samplings in PoR and TiR river.

the inhabitants are dedicated to farming, cattle raising, and trout farming (see Fig. 2). Although a medical prescription is required in the country to obtain antibiotics, these drugs are commonly used in productive activities, such as livestock and agriculture, without adequate technical supervision and, in some cases, without full consideration of the environmental and health consequences (Angulo et al., 2023; de la Cruz et al., 2014).

The CDC, one of the entrances to Braulio Carrillo National Park, with an RVI of 99%, can be considered a potential source of contamination due to the proximity of a pet shelter. The proximity raises concerns about potential veterinary treatments occurring at the shelter and could lead to contamination. These activities will likely require additional wastewater treatment infrastructure, especially in the light of the finding of ciprofloxacin, norfloxacin, *tet*S, *qnr*B, and $\textit{bla}_{\text{TEM}}$ (Table 1).

The presence of ARGs has already been approved in wildlife species in Costa Rica, such as monkeys, jaguars, free-living pumas from nature reserves, and pigeons from urban areas (Angulo et al., 2023; Arias-Andrés et al., 2014; Blanco-Peña et al., 2017; Granados-Chinchilla et al., 2014; Palacios, 2007; Rodríguez et al., 2006). In some of these studies, the possibility of wildlife contamination through superficial waters was proposed. This hypothesis can be supported by the data reported here, which can even imply the potential for modification of the microbial communities' homeostasis, impacting biodiversity and both animal and human health by generating resistant agents (Arias-Andrés et al., 2014, p.; Granados-Chinchilla et al., 2014). Furthermore, this study supports the idea that only focusing on the issue of protected areas is not enough for assessing a "healthy" environment.

3.4. Relation between laboratory results and the remaining vegetation index (RVI)

There was no differentiation in the ARGs presence according to RVI (Permanova. f: 0.865; p = 0.734). This indicates that sites with a conserved riparian forest exhibited similar concentrations to areas with partial and moderate disturbances. The highest ARGs numbers were detected in CTC (5) and CDC (5), followed by EGR (4) and CGC (4). CTC and CDC were also the sites with the highest cumulative pharmaceutical concentrations (15 and 21 ng/L, respectively), as well as EGR and CGC (16 and 15 ng/L, respectively), see Fig. S2 and Table S4. Thus, they could be considered the most affected by pharmaceuticals, despite having total concentrations below 25 ng/L. Interesting, according to the RVI index, these rivers had high percentages of vegetation cover (86–100 %), showing that a "healthy environment" is not always related to forest cover.

No pharmaceutical residues were detected in PrR (RVI = 86), and SMC (RVI = 50). In each one, only one ARG was identified. Therefore, they could be considered low-contaminated rivers. What is most striking is the difference in the RVI index: PrR (not transformed, 86%) but SMC (Partially transformed, 50%), with the latter being one of the lowest (Table 1).

TC, SaR, ESC, TiR, and CRC are rivers near the Braulio Carrillo National Park. Their surroundings also encompass small towns, roads, animal production, and both banana and pineapple plantations (Fig. 2). The cumulative concentration of pharmaceuticals (Fig. S2) in these rivers ranged from 3 to 17 ng/L, and several ARGs were also quantified in them (*tet*S, *sul* II, *aad*A, *qnr*B, *bla*$_{\text{TEM}}$, and *mcr*-1).

ESC is located close to a biological station within a protected area (RVI = 100%), attracting scientists and students from all over the world to study one of the world's most diverse and intricate ecosystems. It also experiences "scientific tourism" (personal communication). Residues of antimicrobials, such as ciprofloxacin and norfloxacin, were detected with concentrations ranging from 3 to 17 ng/L. Only the ARG *bla*$_{\text{TEM}}$ was confirmed.

3.5. Data analysis

The heatmap shown in Fig. 3 presents the most contaminated rivers for *bla*$_{\text{TEM}}$ (SC and S2C, followed by TiR, PQR, and PoR). High relative concentrations for *mec*A and *qnr*B were observed at one biological station SR and PoR. Medium relative concentrations of *tet*S were also found in SC, GR, and PrR, and medium relative concentrations of *tet*K was also reported only in GR. In CDC, the genes encoding *qnr*B and *bla*$_{\text{TEM}}$ were found in low relative concentrations. Samples with only one ARGs were: EC, ESC, PrR, and SMC.

The RDA (Fig. 4) did not demonstrate significant correlations between ARGs and their corresponding antibiotics. This issue can be attributed to several factors. Firstly, ARGs, like other genes, are heritable and transferable, enabling them to be acquired from progenitor or nearby bacteria even without antibiotic contamination in the environment (Chen et al., 2018; Matviichuk et al., 2022).

The presence of ARGs does not necessarily indicate their current expression; some may be silenced genes that provide future advantages to the bacterium. Secondly, the most common mechanisms involve mutations in gyrase and topoisomerase genes, which are not epidemiologically significant as they are not transmitted through plasmids. Less frequent plasmid-transmitted genes, such as *qnr*D or *qnr*A, have also been identified (Chen et al., 2018; Guan et al., 2018). Thirdly, ancient genes encoding resistance to tetracycline are frequently found in mobile genetic elements alongside other currently employed genes, including other ARGs or genes conferring resistance to heavy metals or organic disinfectants. This complexity highlights the diverse behaviors of ARGs, with some being eliminated upon the removal of selective pressure while

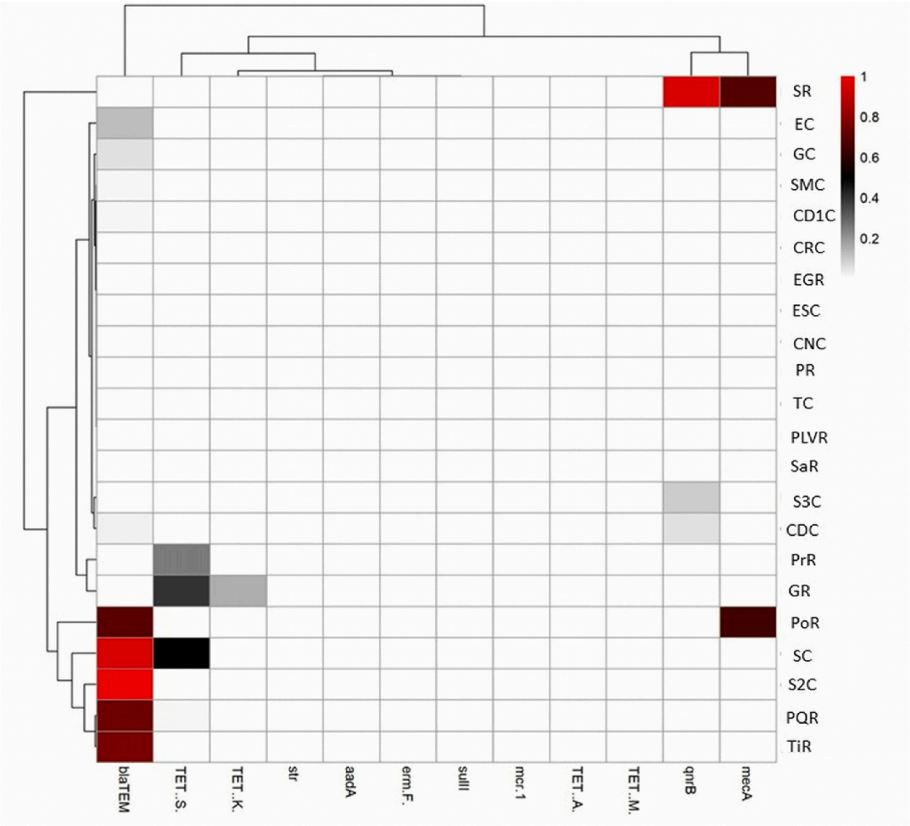

Fig. 3. Heat map representing the percentage load of (ARGs) in water surface waters close to Costa Rican wild feline habitats in two national parks and surroundings. The scale indicates the relative concentration of each gene. The outer lines show the degree of clustering between all the variables.

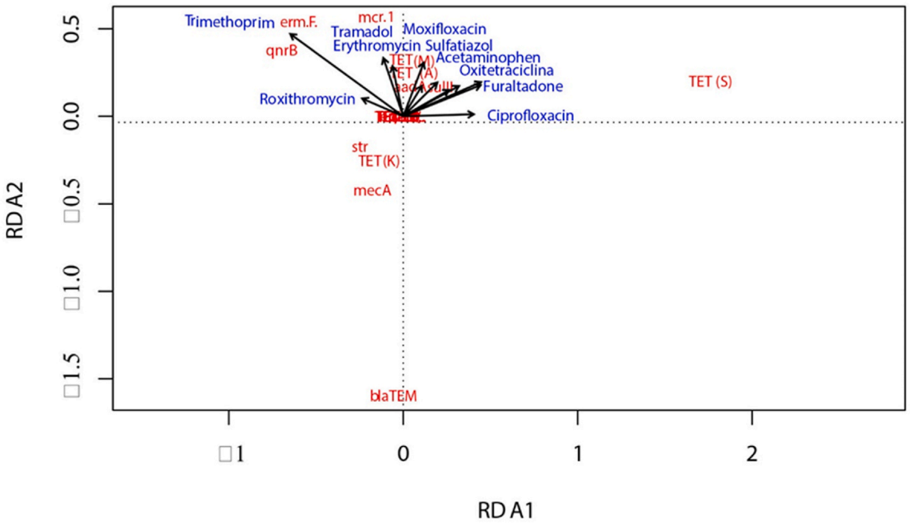

Fig. 4. Redundancy analysis (RDA); where the statistical association between resistance genes and drugs recorded in different water bodies is shown. The analysis accounts for 75% of the total variance; R2 = 0.5318.

others persist over time when they are no longer exposed to a source, while others persist over time (Dodd, 2012; Zhou et al., 2022). Fourthly, pharmaceutical residue loads are more evident in wastewater or urban rivers than in samples from protected areas. This case study reports only a few substances present in low concentrations. Antimicrobials degrade rapidly in the environment through photolysis, hydrolysis, or biodegradation (Grenni, 2022; Krzeminski et al., 2019; Timm et al., 2019). It is possible that they were present in the river at some point, but when the samples were taken, they could no longer be quantified due to being below the limits of detection or degradation, but bacteria can develop resistance in aquatic environments even at low antibiotic concentrations (Amarasiri et al., 2020; Gullberg et al., 2011). Some ARGs were also found to correlate resistance to certain antibiotics against which they should not confer resistance. E.g., between *erm*F and trimethoprim, and *mcr*1 and erythromycin, indicating a potential for co-selection, which results from co-localization of ARGs on the same genetic element, such as a plasmid, transposon, or integron; cross-resistance, which occurs when a single cellular response can combat multiple chemicals; or co-regulation, when two resistance regulatory systems are transcriptionally linked (Garner et al., 2016). A relationship between *qnr*B and trimethoprim is seen in Fig. 4, although *qnr*B encodes resistance to quinolones. One study evaluated whether different antimicrobial agents induce the expression of *qnr*B. Indicator gene assays showed that *qnr*B genes were induced by ciprofloxacin, as expected, but also by trimethoprim (Briales et al., 2012).

It is concerning that the most available information pertains to wastewater treatments and urban rivers, while there is limited data for rivers where wildlife may be contaminated through drinking. In this regard, our study is pioneering and offers valuable insights that can be of significance to stakeholders, despite being a case study. Given that we have demonstrated the impact on these rivers, a more comprehensive campaign is needed in the near future to determine whether these contaminants are present during specific time periods or if their levels are increasing.

4. Conclusion

1. This study revealed that antibiotic residues were detected in 47% of the water samples, with ciprofloxacin and norfloxacin being the most frequently found compounds. Notably, all antibiotic concentrations were below 10 ng/L, indicating low levels of antibiotic contamination. Additionally, pharmaceutical residues, such as acetaminophen and tramadol, were also present in the samples.
2. Despite the relatively low concentrations detected, it is noteworthy that every water sample in our study tested positive for at least one ARG. Among these genes, the most prevalent was *bla*$_{\text{TEM}}$, followed by *tet*S and *qnr*B. Intriguingly, the abundance of the *bla*$_{\text{TEM}}$ gene was notable, even in the absence of detectable β-lactam antibiotic residues.
3. This study brought to light the scarcity of research dedicated to “less impacted” rivers concerning (ARGs). This knowledge gap is a cause for concern, considering the diverse contamination sources originating from human activities in the surrounding areas. Our findings underscore the question of whether protected areas alone can effectively ensure the maintenance of a “healthy” environment. The presence of ARGs and pharmaceutical residues in these areas raises critical implications for wildlife and their potential vulnerability to these contaminants.

Our study represents a pioneering effort in understanding the presence of antibiotic residues and ARGs in protected areas. To build upon these findings, future work should include.

4. Conducting a more comprehensive and prolonged campaign to monitor contaminant levels over specific time periods, allowing for the assessment of trends and fluctuations. Expanding the study to include a broader range of protected areas and their surrounding environments to explore variations in contamination levels.
5. Investigating the potential impact of contaminants on wildlife in protected areas and their role in the transmission of antibiotic resistance. Assessing the long-term persistence of antibiotic residues and ARGs in the environment and their potential consequences.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.117665.

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