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Exploring Pathogens and Antibiotic Resistance Genes (ARGs) on Environmental Biofilms in Machángara and San Pedro Rivers: A Spatio-Temporal Study in Quito, Ecuador

Tesis en torno a una hipótesis o problema de investigación y su contrastación

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DEDICATORIA

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RESUMEN

Las biopelículas ambientales cumplen un papel importante en el monitoreo de la contaminación ambiental y de los patógenos clínicamente relevantes para la salud pública, funcionando como ecosistemas dinámicos que reflejan las interacciones entre los microorganismos y sus entornos. El presente estudio evaluó la dinámica microbiana y los niveles de contaminación química en biopelículas ambientales colectadas en distintos puntos del río Machángara (M0, M1 y M2) y San Pedro (SP0, SP1 y SP2) durante las estaciones seca, y lluviosas. La cuantificación de *E. coli* y coliformes totales se realizó mediante ensayos de unidades formadoras de colonia por peso húmedo de biopelícula (UFC/g). Además, se realizó identificación molecular mediante PCR convencional y secuenciación para detectar patógenos potenciales y genes codificantes de β-lactamasas de tipo espectro extendido (BLEE). Por último, se caracterizó los elementos mayores y traza en muestras de biopelículas. Nuestros resultados revelaron variaciones estacionales en las concentraciones microbianas con niveles más elevados observados durante las estaciones lluviosa 2 y seca en comparación a la lluviosa 1. Durante la estación seca, el río Machángara exhibió las mayores concentraciones de *E. coli* ($1.5x10^5$ UFC/g) y coliformes totales ($1.5x10^6$ UFC/g). Por otra parte, el análisis molecular detectó diversos patógenos potenciales como especies de *Campylobacter*, especies de *Mycobacterium tuberculosis*, *H. pylori* y parásitos como *Giardia intestinalis* y *Cryptosporidium parvum*. Las concentraciones de elementos mayores y traza variaron según los puntos de muestreo y temporalmente. Por ejemplo, durante la temporada de transición el punto M2 en el río Machángara presentó niveles elevados de cobre (Cu, 64.41), zinc (Zn, 250.84 ppm) y titanio (Ti, 401.16 ppm), mientras que el punto SP0 en el río San Pedro mostró mayores concentraciones de calcio (Ca, 3747.69 ppm), sodio (Na, 619.92 ppm) y potasio (K, 796.13 ppm). Finalmente, los genes codificantes de β-lactamasas fueron prevalentes en aislados

de *E. coli* y coliformes; siendo el gen *bla*$_{CTX\text{-}M}$ fue el más común, detectado en el 98% de los aislados de *E. coli* de ambos ríos. Estos hallazgos resaltan la compleja interacción que existe entre los factores ambientales, la dinámica microbiana y los niveles de contaminación en las biopelículas ambientales. Estos resultados destacan la necesidad de tener estrategias integrales de monitoreo y gestión para mitigar los riesgos potenciales para la salud humana y el ambiente.

Palabras clave: Ríos urbanos, biopelículas ambientales, recursos naturales de agua dulce, calidad microbiana, elementos mayores y traza, genes de resistencia a antibióticos, enfoque una sola salud.

ABSTRACT

Environmental biofilms play a crucial role in monitoring environmental pollution and clinically relevant pathogens for public health, serving as dynamic ecosystems that reflect the interactions between microorganisms and their surrounding environments. This study evaluated microbial and chemical contamination levels in environmental biofilms from different collection sampling points of the Machángara (M0, M1, and M2) and San Pedro (SP0, SP1, and SP2) Rivers across dry and rainy seasons. Quantification of *E. coli*, and total coliforms, was conducted using colony-forming unit assays per biofilm humid weight (CFU/g). In addition, molecular identification via PCR and Sanger sequencing was employed to detect potential pathogens and β-lactamase encoding genes. Lastly, trace metals and major elements in biofilm samples were also analyzed. Our results revealed seasonal variations in microbial concentrations with higher levels observed during rainy season 2 and dry seasons when compared to the rainy season 1. During the dry season, the Machángara River exhibited the highest concentrations of *E. coli* ($1.5x10^5$ CFU/g), and total coliforms ($1.5x10^6$ CFU/g). Molecular analysis detected diverse potential pathogens such as *Campylobacter* species, *Mycobacterium tuberculosis*, *H. pylori*, and parasites such as *Giardia intestinalis* and *Cryptosporidium parvum*. Trace metal and major element concentrations varied spatially and temporally. For instance, during the rainy 2 season, point M2 in the Machángara River had elevated levels of copper (Cu, 64.41 ppm), zinc (Zn, 250.84 ppm), and titanium (Ti, 401.16 ppm), while point SP0 in the San Pedro River showed higher concentrations of calcium (Ca, 3747.69 ppm), sodium (Na, 619.92 ppm), and potassium (K, 796.13 ppm). Finally, β-lactamase encoding genes were prevalent in *E. coli* and coliform isolates with $bla_{\text{CTX-M}}$ being the most common gene detected in 98% of *E. coli* isolates from both rivers. These findings underscore the complex interplay between environmental factors, microbial dynamics, and contamination levels in environmental biofilms, highlighting the need for comprehensive monitoring and management strategies to mitigate potential risks to human and environmental health.

Keywords: Urban rivers; Environmental biofilms; Natural freshwater resources; Microbial quality; Physicochemical parameters; Major and trace elements; Antibiotic Resistance Genes (ARGs); One Health approach.

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PART 1: LITERATURE REVIEW

Introduction

The continuous release of wastewater and chemical pollutants into freshwater reservoirs poses a significant environmental threat, particularly in developing countries where inadequate infrastructure and resources for treating domestic and industrial wastewaters are common (da Silva et al., 2020; UN-Water et al., 2023). This ongoing discharge leads to the accumulation of pollutants in water bodies, such as rivers, which represents a severe threat to public health (Valdés et al., 2021). The effects of this environmental contamination go beyond its degradation, affecting various economic sectors such as agriculture, livestock, manufacturing, and even recreational activities (Cely-Ramírez et al., 2021; Puspitasari & Hadi, 2022). Additionally, the proliferation of microorganisms and anthropogenic contaminants increases the risk of pathogens outbreaks, bacterial antibiotic resistance, and associated public health costs (Fradette et al., 2022; Kneis et al., 2022).

On a global scale, the inadequate treatment of wastewater persists as a prevalent issue and it is estimated that more than 80% of residual water is released into the environment without proper treatment or reuse (UN-Water et al., 2023; Vinueza et al., 2021). Consequently, billions of individuals are exposed to contaminated water sources annually, leading to waterborne illnesses that contribute significantly to morbidity and mortality rates worldwide (Borja-Serrano et al., 2020; UN-Water et al., 2023). In this context, understanding the dynamics of contamination, the presence of clinically relevant pathogens, and antibiotic resistance in river ecosystems becomes imperative. Several studies have demonstrated that pollutants in rivers can alter the composition, activity, and resistance profiles of microorganisms, especially those within biofilm communities (Chonova et al., 2018; Kneis et al., 2022; Matviichuk et al., 2023). Environmental biofilms, composed of diverse microbial communities, serve as a crucial biomarker for assessing contamination levels and pathogen dissemination in freshwater resources due to their capacity to accumulate contaminants and harbor pathogens derived from untreated wastewater (Guerrieri et al., 2022; Masangkay et al., 2020). Therefore, this short-review aims to gather relevant information related to contamination in rivers to shed light on the current environmental challenges worldwide.

Microbial contamination in rivers

Microbial contamination in rivers is a critical problem with various environmental microorganisms inhabiting these aquatic ecosystems (Borja-Serrano et al., 2020; Vinueza et al., 2021; Zhang et al., 2015). The principal sources of contamination in aquatic ecosystems are urban discharges, industrial wastewaters, and agricultural runoffs (Ahmed et al., 2019; da Silva et al., 2020; Noorhosseini et al., 2017; Proia et al., 2016). To understand the dynamics of contaminations, most of the studies seek to analyze the microorganisms that have been characterized as indicators of contamination, which most of them are commonly abundant in the intestines of warm-blooded animals being indicative of fecal contamination in water and being related to the presence of other pathogenic microorganisms (Duarte et al., 2021; Wen et al., 2020).

Water quality monitoring protocols vary among countries, often based on international guidelines. Despite this, some microorganisms are standardly analyzed such as *Escherichia coli* and *Enterococcus* spp., which are commonly quantified due to their resilience in the environment and their consistent presence in animal and human intestines, making them primary indicators of fecal contamination. Additionally, total coliforms, which include several bacterial genera such as *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Escherichia* spp., are assessed for their potential association with environmental contamination (Boni et al., 2021; Gionchetta et al., 2023; Wen et al., 2020).

On the other hand, parasites, such as *Cryptosporidium* and *Giardia* spp., are also monitored and known to cause outbreaks when consumed through contaminated water. Their infective forms (cysts) persist in fecal-contaminated water for extended periods, rendering them valuable indicators of water quality (Fradette et al., 2022; Hamilton et al., 2018; Jellison et al., 2020; Lefebvre et al., 2020; Sammarro Silva & Sabogal-Paz, 2021). Furthermore, the analysis of potential pathogens, such as *Campylobacter* species (*C. jejuni*, *C. coli,* and *C. upsaliensis*), helps to identify potential contamination sources being usually linked to certain animal hosts, urban pollution, or agricultural runoff (Denis et al., 2011; Maal-Bared et al., 2012; Mughini-Gras et al., 2016). The aforementioned species of *Campylobacter* have been linked to specific natural reservoirs according to their species. For instance, *C. jejuni* has been associated with chickens, pigs, and ruminants (Bronowski et al., 2014; Epps et al., 2013; Mughini-Gras et al., 2016; Whiley et al., 2013). Conversely, *C. upsaliensis* has been mainly associated with cats and dogs as hosts, while *C. coli* has been linked to pigs, swine, and wild birds (Elmonir et al., 2022; Epps et al., 2013; Mughini-Gras et al., 2016; Whiley et al., 2013; Workman et al., 2005). Other potential pathogen menace englobes mycobacterial species, such as *M. tuberculosis* and *M. leprae* (Arraes et al., 2017; Kesarwani et al., 2022; Mtetwa et al., 2022). However, the detection of these and many other potential pathogens is hard by using traditional cultivation methods, so this type of analysis is often performed using molecular techniques (Arraes et al., 2017; Chavarro-Portillo et al., 2019; Mtetwa et al., 2022). These clinically relevant pathogens may enter water sources via untreated hospital discharges or merely urban wastewater of contaminated individuals among the population. Additionally, *Helicobacter pylori* is the main cause of gastrointestinal complications in many developed and developing countries, but its route of transmission remains poorly understood (Duarte et al., 2021; Horiuchi et al., 2021; Mezmale et al., 2020). Analyzing its presence in water sources can offer insights into whether contaminated water contributes to its global prevalence.

Monitoring various microbial indicators and potential pathogens in rivers is necessary to safeguard public health and understand the main contamination sources in order to develop policies to reduce this problematic.

Major and minor elements

Major and minor element analysis is also important for assessing water quality in aquatic environments (Kumar et al., 2023; Li et al., 2022). These elements originate from various sources, including natural processes like rock weathering, soil erosion, and the dissolution of salts (Kumar et al., 2023). However, anthropogenic activities, such as agriculture, mining, and industrial operations, contribute to their presence in water bodies leading to elevated concentrations that can be harmful (Alqahtani et al., 2020). Contaminants like heavy metals or increased concentrations of major and minor elements pose significant risks to both human health and the environment in short and/or long exposure time. It is well-known that these elements have the potential to bioaccumulate in the food chain, posing long-term health hazards to organisms and ecosystems (Kumar et al., 2023; Paul, 2017). Given that humans rely on these water sources for various activities, including drinking, livestock production, food manufacturing, and agriculture, it is important to monitoring the permissible limits for major and minor elements to safeguard public health and ecosystem integrity (Borja-Serrano et al., 2020; Lenart-Boroń et al., 2017; Li et al., 2022; Ministry of Environment of Ecuador (MAE), 2015).

Exceeding these limits can result in severe health issues such as cancer, diabetes, and various neurological disorders, underlining the importance of rigorous monitoring and evaluation of water quality parameters (Kumar et al., 2023; Paul, 2017). Moreover, the geological characteristics of a study area play a crucial role in determining the normal or expected levels of these elements in rivers. Studies have shown that rivers located near volcanic zones may exhibit higher levels of certain elements like aluminum, which can significantly impact water quality assessment (Borja-Serrano et al., 2020; Vargas-Solano et al., 2019; Vinueza et al., 2021). Therefore, understanding the geological context of a region is essential for accurately interpreting water quality data and implementing effective management strategies to mitigate contamination risk.

Environmental Biofilms as biomarkers

Biofilms are defined as a community of microorganisms embedded within a matrix of extracellular polymeric substances (EPS) that underwent a phenotypic shift being composed of metabolic active and latent cells with different functions and interactions within biofilm allowing an increase of resistance against numerous external stresses (Machado et al., 2023; Matviichuk et al., 2023; Yang et al., 2021). These communities can be attached to different biotic and abiotic surfaces in aquatic ecosystems including sediments, woods, glass, animals, fishes, plants, cement structures, rocks, and plastics (Chonova et al., 2018; Gionchetta et al., 2023; Kneis et al., 2022). The majority of environmental biofilms are composed of different microorganisms including non-pathogenic and pathogenic multispecies of bacteria, fungi, algae, and protozoa (Mao et al., 2021; Masangkay et al., 2020; Reichert et al., 2021). Their significance as environmental biomarkers lies in serving as reservoirs for chemical compounds (metals, drugs, among others), as well as resistant bacteria, antibiotic resistance genes (ARGs), and mobile genetic elements (MGE). Regardless of their pathogenicity nature, microorganisms within biofilms can acquire different genes, including ARGs or resistance to heavy metals, through several mechanisms such as horizontal gene transfer (Haenelt et al., 2023; Matviichuk et al., 2023). Studies have shown that biofilms facilitate horizontal gene transfer (HGT) mechanisms like conjugation, transduction, and transformation, with conjugation being the most dominant HGT mechanism due to the proximity of bacterial cells within the biofilm

(Machado et al., 2023; Michaelis & Grohmann, 2023). These factors collectively increase the risk of antibiotic resistance propagation into the different ecosystems. Environmental biofilms also provide advantages to inhabiting microorganisms, serving as sources of protection against natural predators or competitors, food, and other resources such as public or private goods and siderophores among other essential compounds to their metabolism or through synergetic interactions among cells (Lefebvre et al., 2020; Michaelis & Grohmann, 2023). Additionally, biofilms can accumulate a wide range of contaminants, including antibiotics, metals, and other chemicals (such as caffeine, pesticides, and biological drugs). This accumulation allows for the detection of compounds present in low concentrations in water samples (Aubertheau et al., 2017; Balcázar et al., 2015; Matviichuk et al., 2022; Tien & Chen, 2013; Yadav, 2018).

The use of environmental biofilms as biomarkers for different types of contamination has been proposed, particularly in water analysis as a complementary tool for the existing analysis (Bastos et al., 2023; Guerrieri et al., 2022; Yadav, 2018). This suggestion arises from the ability of microorganisms to survive for longer periods within biofilms compared to the water itself (Carafa et al., 2021; Guerrieri et al., 2022; Jellison et al., 2020; Serra et al., 2010). Water is subjected to various environmental changes, including fluctuations in temperature, flow rate, movement, precipitation, and other factors that can kill bacteria or dilute the concentration of other contaminants. In contrast, biofilms maintain more stable communities over extended periods due to their previously described intrinsic characteristics (Haenelt et al., 2023). Moreover, biofilms offer a favorable environment for analyzing the dynamics of antibiotic resistance among bacteria within them as primary or secondary colonizers (Machado & Cerca, 2015; Matviichuk et al., 2023; Winkworth, 2013).

Antimicrobial resistance

Over the past few years, antimicrobial resistance (AMR) has emerged as a critical global public health issue. The World Health Organization (WHO) identifies AMR as a significant threat to worldwide health, food safety, and the environment, substantially reducing the effectiveness of common antibiotics in combating widespread bacterial infections (Velazquez-Meza et al., 2022; World Health Organization (WHO), 2023). The rapid rise and spread of AMR are primarily linked to the extensive use of antibiotics in different fields, such as human and veterinary medicine, as well as in food manufacturing and animal production, where they are mainly used as growth promoters (Velazquez-Meza et al., 2022). Furthermore, the ease with which bacteria exchange genetic material among themselves, particularly when they are within biofilms, accelerates the dissemination of ARGs (Haenelt et al., 2023; Machado et al., 2023).

It has been known that certain ARGs were present in natural environments before the widespread use of antibiotics. Anthropogenic activities and exposure to synthetic antimicrobial agents have altered bacterial ecology, leading to the prevalence of certain genes over others (Cantón et al., 2012; Jiang et al., 2021). A prime example of this phenomenon is the emergence of resistance to third-generation cephalosporins, which are broad-spectrum antibiotics commonly employed in human and veterinary medicine to combat infections, particularly those caused by Gram-negative bacteria (Matviichuk et al., 2023; Tacão et al., 2022). Many of the genes conferring resistance to these antibiotics encode various enzymes, with extended spectrum β-lactamases (ESBLs) being a well-studied group capable of hydrolyzing third-generation cephalosporins (Salinas et al., 2021; Siddiqui et al., 2018). Numerous studies have highlighted the global dissemination of ESBLs over the past decade with these enzymes being detected in diverse natural environments and linked to outbreaks of resistance (Lenart-Boroń, 2017; Montero et al., 2021; Salinas et al., 2021; Siddiqui et al., 2018). Some of the most important genes studied in environmental contexts are *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{OXA}$. While *bla*$_{TEM}$ and *bla*$_{SHV}$ were predominant genes associated with hospital outbreaks during the 1980s and 1990s, the *bla*$_{CTX-M}$ gene has rapidly proliferated since the 2000s, often facilitated by its association with mobile genetic elements such as plasmids (Cangui-Panchi et al., 2023; Cantón et al., 2012; Girlich et al., 2020; Seyedjavadi et al., 2016). Contrary, during the 2000s, *blaOXA* genes were considered unique ESBLs mainly because they were often found in *Pseudomonas* species rather than in *Enterobacteriaceae* species. However, in the last decade, bacteria have generated variations of this gene which can disperse to other enterobacterial species and also have been reported to coexist with other β-lactamase genes such as *bla*$_{CTX-M}$ (Cantón et al., 2012; Fang et al., 2008)

Moreover, the contamination of freshwater reservoirs by wastewater and chemical pollutants poses significant environmental and public health challenges on a global scale. The release of untreated wastewater, particularly in developing countries, contributes to the accumulation of pollutants in water bodies, endangering ecosystems and humans. Efforts to address these challenges require a multidisciplinary approach that takes into consideration water quality monitoring, microbial contamination analysis, and assessment of major and minor elements in rivers. In the same way, adopting a One Health approach that integrates human, animal, and environmental health, can be directed to develop comprehensive strategies for mitigating contamination, preserving water resources, and safeguarding public health.

PART 2: SCIENTIFIC ARTICLE

Exploring Pathogens and Antibiotic Resistance Genes (ARGs) on Environmental Biofilms in Machángara and San Pedro Rivers: A Spatio-Temporal Study in Quito, Ecuador

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Introduction

The contamination of environmental water resources, such as rivers, is one of the most important global problems, posing a challenge in safeguarding water quality and public health worldwide (Borja-Serrano et al., 2020; Tacão et al., 2022). As highlighted by the United Nations (UN-Water et al., 2023), water quality degradation is primarily due to inadequate water treatment practices, particularly in low-income countries, while in higher-income nations, agricultural runoff emerges as a primary source of pollutants. Furthermore, industrial activities exert pressure on freshwater reservoirs through the discharge of hazardous chemicals, contributing to the proliferation of emerging pollutants, including microplastics and pharmaceuticals (Mokarram et al., 2020; Ranjan et al., 2022). The cumulative impact of these factors emphasizes the urgent need for strategies to mitigate environmental degradation and ensure the sustainability of water resources on a global scale.

The continuous discharge of effluents into freshwater resources due to anthropogenic activities exposes inhabiting microorganisms to low but constant concentrations of various contaminants, including heavy metals, fertilizers, pesticides, and pharmaceuticals such as antibiotics (Yadav, 2018). Several studies conducted in rivers have shown that pollutants significantly influence the composition, activity, and resistance of biofilm communities. These environmental communities are commonly attached to various substrates, such as rocks, sediments, cobbles, cement structures, wood, glass, and even plastics (Chonova et al., 2018; Gionchetta et al., 2023; Kneis et al., 2022; Matviichuk et al., 2022).

Environmental biofilms in rivers can be composed of a diverse collection of microorganisms, for instance, multispecies bacteria, fungi, and algae. Moreover, these environmental biofilms can harbor pathogenic organisms within their community, primarily derived from sewer discharges (Mao et al., 2021; Masangkay et al., 2020; Reichert et al., 2021;

Wu et al., 2019). Similarly, biofilms serve as significant reservoirs of resistance to various substances such as antibiotics. Owing to the proximity in which microbial communities grow inside biofilms, the accumulation of different mobile elements increases and thereby favors the environmental dissemination of antibiotic resistance genes (ARGs) (Haenelt et al., 2023). Nowadays, one of the main problems is the resistance to third-generation cephalosporins, antibiotics commonly used due to their broad spectrum, particularly against Gram-negative bacteria. Biofilm communities play an important role in spreading genes responsible for this resistance, encoding extended-spectrum β-lactamase enzymes that are now disseminated in natural environments (Matviichuk et al., 2023; Tacão et al., 2022).

The pollution of the Machángara and San Pedro Rivers, located in the capital city of Ecuador, Quito, poses a significant challenge due to discharges from various sources, mainly agricultural, industrial, and urban areas. The presence of biofilms in these rivers could be a key biomarker to understand the dynamics of contamination, clinically relevant pathogens, and resistance to antibiotics. The environmental biofilms possess the ability to accumulate contaminants and harbor pathogens derived from untreated wastewater in natural freshwater resources. According to literature, in Quito, Ecuador, less than 3.5% of the effluents are adequately treated, while the rest remain contaminating freshwater sources such as the rivers previously mentioned (Borja-Serrano et al., 2020; EPMAPS, 2023; Vinueza et al., 2021). The main goal of the present study was to assess the microbiological quality and metal concentration of environmental biofilms in the Machángara and San Pedro Rivers, focusing on parameters such as microbial load (*Escherichia coli* and total coliforms counts), the presence of potential pathogenic microorganism using PCR detection and further Sanger Sequencing methods, and chemical parameters (including major and trace metallic elements) according to US Standard Methods from the American Public Health Association (Benítez et al., 2018; Grube et al., 2020). Additionally, an analysis of microbial and physicochemical parameters was simultaneously conducted in surface water samples in another study (Cabrera-Ontaneda et al., 2024), complementing the evaluation of environmental biofilms as environmental biomarkers in the present work. Furthermore, colonies of *E. coli* and coliforms resistant to the antibiotic ceftriaxone (a third-generation cephalosporin) were also studied to identify the presence of ARGs within biofilm samples. This comprehensive analysis covered three longitudinal sampling points along both rivers and was conducted over three distinct seasons (rainy season 1, rainy season 2, and dry season) from November 2022 to July 2023.

Materials and Methods

Sample site and collection

Environmental biofilm samples were collected from superficial rocks submersed in surface water at the same three longitudinal points along the Machángara and San Pedro Rivers (see **Figure 1**). Briefly, samples from point 0 were collected from a site with little or no anthropogenic impact, while samples from points 1 and 2 were taken from sites with high anthropogenic impact in both rivers, such as urban, industrial, or agricultural areas. These biofilm samples were collected twice during three different seasons (rainy season 1, rainy season 2, and dry season) between November 2022 and July 2023 (see **Table 1**). Water temperature, pH, and other physicochemical parameters were measured *in situ* using a ProDSS Multiparameter Digital Water Quality Meter (YSI, Xylem INC; United States). Biofilm samples were collected following the protocol outlined by Rimet and colleagues (2020) (Rimet et al., 2020) with slight modifications.

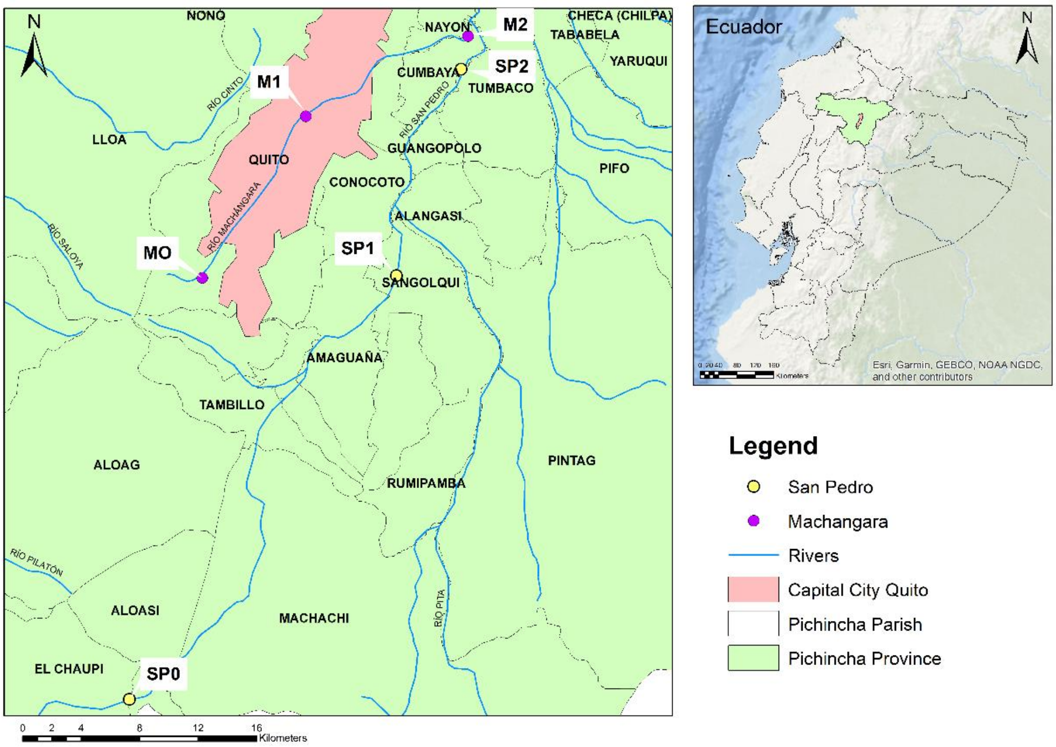

Figure 1. General map of the sample collection points in both Machángara and San Pedro Rivers.

Machángara River (purple dots) includes the following sample collection points: M0-Guamaní (control point), M1-Puengasí, and M2-Nayón. Similarly, the San Pedro River (yellow dots) includes the following sample collection points: SP0-Chaupi (control point), SP1-Sangolquí, and SP2-Cumbayá. The map was created using ArcGIS Desktop software (https://doc.arcgis.com/en/archive/#?q=10.8; version 10.8, accessible online).

Prior to each sampling collection, a plastic tray was cleaned with 75% ethanol and then rinsed with sterile distilled water. A set of three stones was collected at a depth of 20 to 50 cm from the water level and left to drain for a few min. The stones were placed on the tray, and an area of 100 cm^2 was rinsed using 50 mL of sterile water and scraped with the help of a sterile plastic spoon. This mixture was used to fill a 50 mL sterile tube and stored at 4°C in a cooler until arrival at the Microbiology Institute at the Universidad San Francisco de Quito (IM-USFQ) for further processing.

Table 1. Biofilm sample data and corresponding meteorological data by season

Sample code	River	GPS Coordinates	Parish (Province)	Region	Season	Collection date	Average water temperature (ºC)	Monthly Precipitation (mm) [a]	Name of INAMHI Stations [a]	GPS Coordinates of INAMHI Stations [a]	Height of INAMHI Stations (m)[a]
M0	Machángara	0°20'8"S 78°34'58"W	Guamaní (Pichincha)	Andean	Rainy Season 1	12/11/2022 26/11/2022	7.65	111.7	M0024 Iñaquito	0°10'41.9"S 78°29'15.7"W	2789
					Rainy Season 2	12/03/2023 18/03/2023	7.95	145.9			
					Dry Season	17/06/2023 01/07/2023	8.65	38.8			
M1	Machángara	0°13'19"S 78°29'14"W	Puengasí (Pichincha)	Andean	Rainy Season 1	12/11/2022 26/11/2022	16.60	111.7	M0024 Iñaquito	0°10'41.9"S 78°29'15.7"W	2789
					Rainy Season 2	12/03/2023 18/03/2023	14.70	145.9			
					Dry Season	17/06/2023 01/07/2023	16.50	38.8			
M2	Machángara	0°11'07.0"S 78°24'54"W	Nayón (Pichincha)	Andean	Rainy Season 1	14/11/2022 28/11/2022	18.60	103.4	M0002 La Tola	0°13'54.5"S 78°22'13.4"W	2480
					Rainy Season 2	10/03/2023 17/03/2023	15.20	120.4			
					Dry Season	16/06/2023 30/06/2023	16.53	29.1			
SP0	San Pedro	0°35'44"S 78°37'26"W	Chaupi (Pichincha)	Andean	Rainy Season 1	11/11/2022 25/11/2022	9.60	149.5	M0003 Izobamba	0°21'57.0"S 78°33'18"W	3058
					Rainy Season 2	11/03/2023 19/03/2023	10.28	180.9			
					Dry Season	15/06/2023 29/06/2023	10.45	67.9			
SP1	San Pedro	0°19'48"S 78°27'35"W	Sangolquí (Pichincha)	Andean	Rainy Season 1	11/11/2022 25/11/2022	14.70	149.5	M0003 Izobamba	0°21'57.0"S 78°33'18"W	3058
					Rainy Season 2	11/03/2023 19/03/2023	13.65	180.9			
					Dry Season	15/06/2023 29/06/2023	14.30	67.9			
SP2	San Pedro	0°12'29"S 78°25'13"W	Cumbayá (Pichincha)	Andean	Rainy Season 1	14/11/2022 28/11/2022	15.95	103.4	M0002 La Tola	0°13'54.5"S 78°22'13.4"W	2480
					Rainy Season 2	10/03/2023 17/03/2023	15.23	120.4			
					Dry Season	16/06/2023 30/06/2023	15.60	29.1			

[a] Data provided by the National Institute of Meteorology and Hydrology from Ecuador (INHAMI) in October 2023 (https://www.inamhi.gob.ec/).

Furthermore, for the analysis of major and minor elements to assess environmental variables, biofilm samples were obtained from the same pool of rocks previously mentioned by scraping their surface with a sterile plastic spoon. Approximately 0.5 to 2 grams of biofilms were collected and placed into small plastic bags. The samples were stored at 4°C in a cooler until they were transported to the Core Lab for Environmental Sciences at Universidad San Francisco de Quito (USFQ) for further analysis.

Culture of microorganisms from biofilm samples and isolation

For the quantification of *Escherichia coli* and total coliforms, serial dilutions of the samples were cultured in Chromocult®Agar medium (Merck KGaA, Darmstadt, Germany) following the protocol established by previous studies (Borja-Serrano et al., 2020; Vinueza et al., 2021). In addition, 1 mL of the same samples was cultured on Chromocult Agar medium supplemented with 2 µg/mL of ceftriaxone (third-generation cephalosporin) using the streak-plate method to quantify and isolate resistant bacteria (Sanders, 2012). The petri dishes were incubated at 37°C for 24 to 48 hours. All colony-forming unit (CFU)/mL calculations were adjusted using average biofilm density to provide an approximate estimate of the values per gram of biofilm.

To obtain environmental-resistant bacteria, a maximum of 5 colonies of *E. coli* (blue/violet) and 5 colonies of coliforms (red/pink) were randomly selected from the Chromocult agar supplemented with ceftriaxone at each sampling point. At some sampling points (M0 rainy season 1, SP0 rainy season 1, and SP0 rainy season 2), some white colonies grew on the media supplemented with ceftriaxone being also isolated for further analysis and identification. All isolated colonies were subsequently cultured on MacConkey agar and then utilized for DNA extraction.

DNA extraction from colonies

DNA extraction was performed according to previously published protocols with slight modifications (Dashti et al., 2009; Machado et al., 2017; Salinas et al., 2020). Briefly, two to five colonies were placed in a 1.5 mL autoclaved tube containing 500 μL distilled autoclaved water and boiled for 15 min in a water bath. Subsequently, the tubes were stored at -20°C for 24 h to ensure thermal shock. After the 24-hour period, the samples were centrifuged for 5 min at 208×g (RCF) and the supernatant was transferred to new 1.5 mL autoclaved tubes. The DNA concentration and quality were measured using a Nanodrop One Spectrophotometer (ThermoFisher, Madison, USA). Samples with a concentration above 50 ng/μL were diluted to a final concentration of 25 ng/μL for PCR analysis.

Colonies identification

The molecular identification of unknown white colonies and some *E. coli* colonies (as reference) was performed through Sanger sequencing. Isolated DNA was amplified with 16S conserved rRNA genes following the protocol outlined by previous studies (Borja-Serrano et al., 2020) (see **Supplementary Table S1**). Subsequently, PCR products were sequenced at Macrogen (Seul, Korea) using ABI 3730xl Instruments. The sequences obtained from both forward and reverse primers were overlapped using PreGap4 and Gap4 software (Staden Package, Cambridge, England) (Staden et al., 2003), and, finally, primer sequences were removed. The resulting nucleotide sequences underwent comparative analysis against the GenBank database using the Standard Nucleotide Basic Local Alignment Search Tool (BLAST) to determine bacterial identities. Data accuracy was assessed based on sequence identity levels with the bacterial identification defined as >92% of sequence identity homology.

Molecular identification of different β-lactamase genes

Once microbial DNA was extracted from the colonies, the *bla*$_{CTX\text{-}M}$ gene was amplified following the protocol outlined by Hasibuan and colleagues (Hasibuan et al., 2018) with a few modifications. The specific primers used for this amplification are detailed in **Table 2**. The PCR mixture consisted of a final volume of 25 μL and contained 5 μL of 5X GoTaq Flexi Buffer (Promega, Madison, USA), 2 μL of 25 mM $MgCl_2$ (Promega, Madison, USA), 1.25 μL of 10 μM for each PCR primer, 0.5 μL of 10 mM dNTP Mix (Promega, Madison, USA), 0.10 μL of 5U GoTaq Flexi DNA polymerase (Promega, Madison, USA), 2 μL of template DNA, and the remaining volume of DNA-free water. The thermocycling procedure was conducted in a thermocycler (Bio-Rad Laboratories, Inc, California, USA) with an initial denaturation of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, an annealing at 57°C for 30 seconds, an extension of 72°C for 45 seconds, and a final extension of 72°C for 5 min. For the remaining genes (*bla*$_{OXA}$, *bla*$_{TEM}$, and *bla*$_{SHV}$), a multiplex PCR was conducted following the protocol outlined by (Fang et al., 2008) with slight modifications. The PCR mixture was similar to the one used for *bla*$_{CTX\text{-}M}$ gene amplification, except that the volume used for each primer was adjusted to 0.50 μL of 10 μM (see primers in **Table 2**). The thermocycler procedure was carried out using the same equipment as previously mentioned, with an initial denaturation step of 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 90 seconds, extension of 72°C for 60 seconds, and a final extension of 72°C for 10 min. Positive controls were provided by the IM-USFQ, while negative control consisted of DNA-free water, all samples were analyzed in duplicate or triplicate assays. The PCR products were visualized using electrophoresis with a 1.5% agarose gel and SybrSafe staining for 40 to 45 min.

Table 2. Primers and PCR cycling parameters for the detection of beta-lactamase genes (*bla*$_{CTX-M}$, *bla*$_{OXA}$, *bla*$_{TEM}$, and *bla*$_{SHV}$).

Gene	Primer sequence (5′–3′)	PCR cycling parameters	Gene (size [bp])	References
Single PCR assays				
bla$_{CTX-M}$	ATGTGCAGYACCAGTAARGTKATGGC	2 min at 94°C; 30 cycles of 94°C for 1 min, 57°C for 30 s, 72°C for 45 seconds	593	(Hasibuan et al., 2018)
	TGGGTRAARTARGTSACCAGAAYCAGCGG			
Multiplex PCR assays				
bla$_{OXA}$	ACACAATACATATCAACTTCGC	15 min at 95°C; 30 cycles of 94°C for 30 s, 62°C for 1.5 mins, 72°C for 1 min	813	(Fang et al., 2008)
	AGTGTGTTTAGAATGGTGATC			
bla$_{TEM}$	CGCCGCATACACTATTCTCAGAATGA		445	
	ACGCTCACCGGCTCCAGATTTAT			
bla$_{SHV}$	CTTTATCGGCCCTCACTCAA		237	
	AGGTGCTCATCATGGGAAAG			

Total DNA extraction from biofilm samples

Total DNA was extracted from biofilm samples using the Dneasy PowerSoil ProKit (Qiagen, Germany) according to the manufacturer's protocol. DNA concentrations and quality were assessed using a Nanodrop One Spectrophotometer (ThermoFisher, Madison, USA).

Molecular identification of potential pathogens

The molecular identification of potential pathogens was determined using PCR. First, *16S* rRNA genes were amplified to confirm the presence of prokaryotic DNA in the samples. Then, a series of amplifications were carried out to identify the following potential pathogens: *E. coli* pathotypes (more exactly: enteroaggregative *E. coli*, EAEC; enterohemorrhagic *E. coli*, EHEC; enteropathogenic *E. coli*, EPEC; and enteroinvasive *E. coli*, EIEC), *Helicobacter pylori*, *Mycobacterium tuberculosis*, *M. leprae*, *Campylobacter jejuni*, *C. coli*, *C. upsaliensis*, *Giardia* spp., and *Cryptosporidium* spp. All of these amplifications were conducted under the conditions previously described in (Borja-Serrano et al., 2020; Vinueza et al., 2021), and the primers and thermocycler conditions are specified in the Supplementary Material section (see **Supplementary Table 1**).

Amplicon sequencing analysis

Following the successful amplification of PCR products for *Giardia* spp., *Cryptosporidium* spp., *H. pylori*, *M. tuberculosis*, and *M. leprae*, amplicons were sequenced at Macrogen (Seul, Korea) using ABI 3730xl Instruments, following the same methodology described in the previous **section of Colonies identification.**

Results

Total coliforms and E. coli in river biofilms

The quantification of total coliforms and *E. coli* was assessed in all biofilm samples from both Machángara and San Pedro Rivers along three different longitudinal sampling points during three seasons, as shown in **Figure 1** and **Table 1**. The average and standard deviation values of *E. coli* and total coliforms for the Machángara and San Pedro Rivers are shown in **Figure 2.** Each bar represents the bacterial concentration measured in CFU/g of biofilm humid weight, with black bars indicating measurements in Chromocult agar with ceftriaxone and grey bars indicating measurements without ceftriaxone, detailed information can be found in Supplementary Material (see **Supplementary Table S2**).

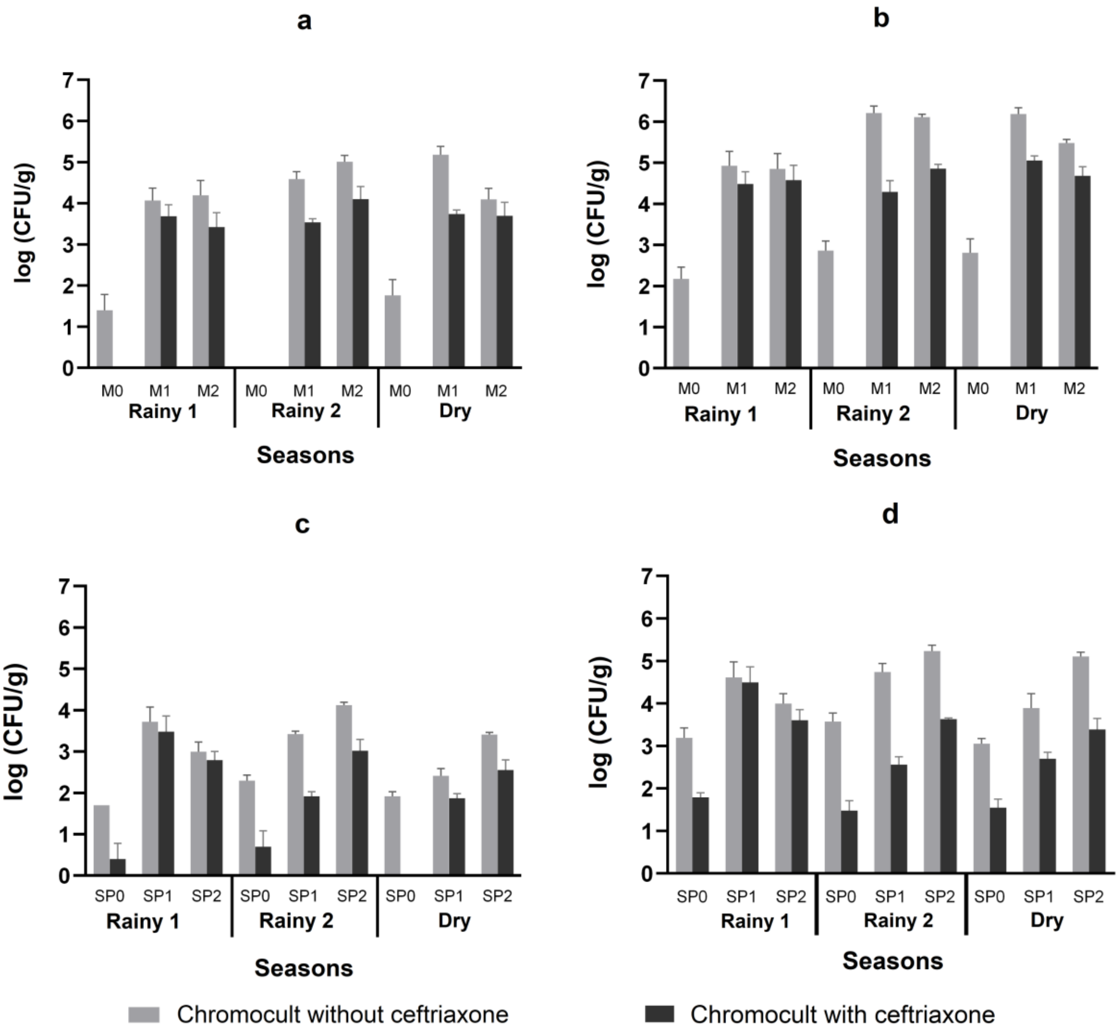

Figure 2. Average and standard deviation values of *Escherichia coli* and total coliforms.

Average and standard deviation values of *Escherichia coli* (a) and total coliforms (b) in the Machángara River, and average values of *E. coli* (c) and total coliforms (d) in the San Pedro River in Chromocult agar with ceftriaxone (black bars) and without (grey bars). All values are presented for the three different longitudinal sampling points during the rainy season 1, the rainy season 2, and the dry season. The sample collection points for the Machángara River were: M0-Guamaní (control point), M1-Puengasí, and M2-Nayón. For the San Pedro River, the sample collection points were: SP0-Chaupi (control point), SP1-Sangolquí, and SP2-Cumbayá. Data represents CFU/g of biofilm humid weight.

In the Machángara River (**Figure 2a and b**), both *E. coli* and total coliform concentrations demonstrated a consistent trend across the three seasons with sampling points M1 and M2 showing higher levels compared to the control point M0. When ceftriaxone was present, during the rainy season 1, point M1 exhibited a higher *E. coli* concentration ($4.8x10^3$ CFU/g), while point M2 had a higher total coliform concentration ($3.8x10^4$ CFU/g). Similarly, during the rainy season 2, point M2 recorded a higher concentration for both *E. coli* and total coliforms ($1.3x10^4$ CFU/g and $7.2x10^4$ CFU/g, respectively). Finally, during the dry season, point M1 had the higher concentrations for both *E. coli* ($5.5x10^3$ CFU/g) and total coliforms ($1.1x10^5$ CFU/g). On the other hand, in the absence of ceftriaxone, the trend slightly differed. In the rainy season 1, point M2 had the highest *E. coli* concentration ($1.6x10^4$ CFU/g), while point M1 had the highest total coliform concentration ($8.4x10^4$ CFU/g), meanwhile on the rainy season 2, points M2 and M1 evidenced the highest *E. coli* concentration ($1.0x10^5$ CFU/g) and total coliforms ($1.6x10^6$ CFU/g), respectively. Lastly, during the dry season, point M1 exhibited the highest concentrations of *E. coli* ($1.5x10^5$ CFU/g) and total coliforms ($1.5x10^6$ CFU/g). To compare the results between quantification with and without ceftriaxone, on average, 24% of *E. coli* and 27% of total coliforms were resistant to the antibiotic. In general, the highest concentrations of *E. coli* and total coliforms were detected during the rainy season 2 and the dry season, whereas the rainy season 1 exhibited lower concentrations. As expected, the control point M0, consistently showed the lowest microbial load across all the seasons, with no growth of *E. coli* observed.

In the San Pedro River (**Figure 2c and d**), a similar trend was observed in which the points located in urban zones (SP1 and SP2) presented the highest concentrations of *E. coli* and total coliforms through the three seasons when compared with the control point SP0. In the presence of ceftriaxone, during the rainy season 1, point SP1 showed the highest concentration of both *E. coli* and total coliforms ($3.0x10^3$ CFU/g and $3.1x10^4$ CFU/g, respectively). Contrary, during the rainy season 2, point SP2 presented the highest concentration for both *E. coli* and total coliforms ($1.0x10^3$ CFU/g and $4.3x10^3$ CFU/g, respectively). Finally, in the dry season, point SP2 presented the highest concentration for both *E. coli* and total coliforms ($3.62x10^2$

CFU/g and $2.4x10^3$ CFU/g, respectively). In the same way, in the absence of antibiotics, the trend remained consistent. During the rainy season 1, point SP1 presented the highest concentration of both *E. coli* and total coliforms ($5.2x10^3$ CFU/g and $4.1x10^4$ CFU/g, respectively). Meanwhile, during the rainy season 2, points SP2 and SP1 demonstrated the highest *E. coli* concentration ($1.3x10^4$ CFU/g) and total coliforms ($5.5x10^4$ CFU/g), respectively. Finally, in the dry season, point SP2 presented the highest concentration for both *E. coli* and total coliforms ($2.6x10^3$ CFU/g and $1.3x10^5$ CFU/g, respectively). To compare the results obtained between quantification with and without ceftriaxone, on average, 19% of *E. coli* and 16% of total coliforms exhibited resistance to the antibiotic. Similarly, to the Machángara River, the San Pedro River also presents higher microbial concentrations during rainy season 2 and the dry season compared to the rainy season 1, highlighting the impact of seasonal variations on microbial dynamics within river biofilms. Notably, the control point SP0 constantly demonstrated the lowest values of total coliforms and *E. coli* across all seasons, indicating relatively lower fecal contamination levels at this site.

Molecular identification of potential pathogens on biofilm samples

The molecular identification of potential pathogens was carried out using PCR on biofilm samples collected from both rivers to confirm the presence or absence of various microorganisms, including *E. coli* pathotypes (EHEC – enteropathogenic *E. coli*; EAEC-enteroaggregative *E. coli*; EIEC – enteroinvasive *E. coli*; EPEC – enteropathogenic *E. coli*), *M. tuberculosis*, *M. leprae*, *C. coli*, *C. upsaliensis*, *C. jejuni*, *Helicobacter pylori* and parasites such as *Cryptosporidium* spp., and *Giardia* spp. The obtained results are presented in **Figure 3** and detailed in the Supplementary Material (see **Supplementary Table S3**).

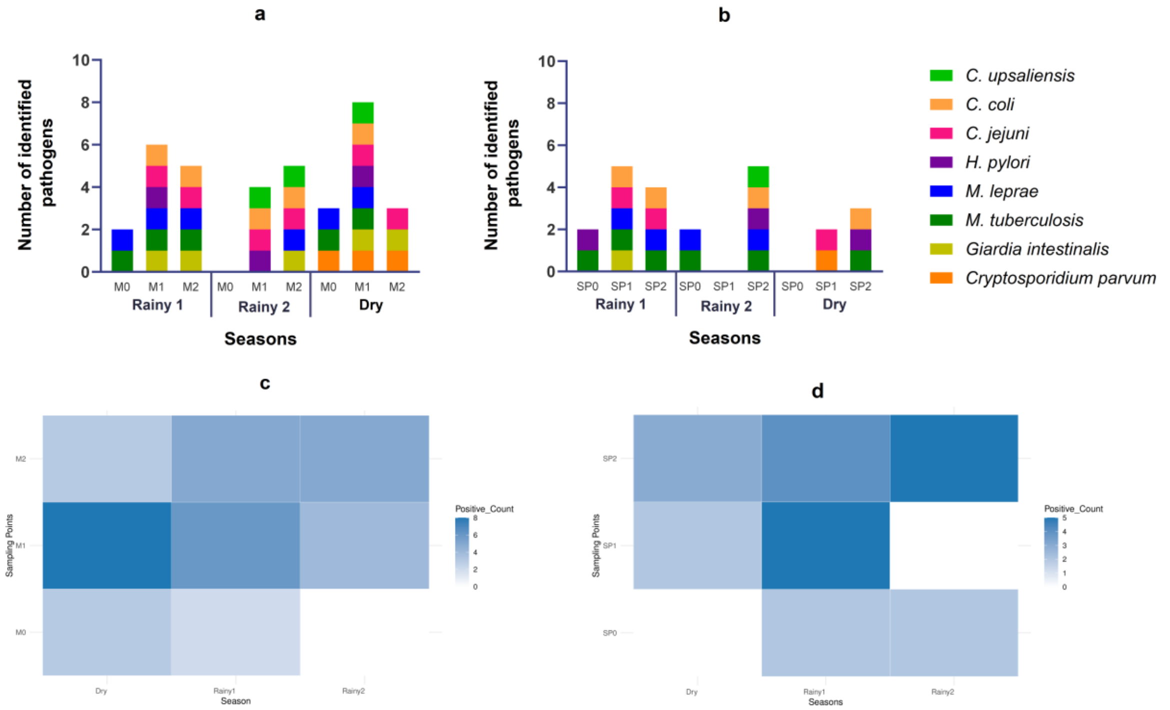

Figure 3. Molecular identification of potential pathogens.

Potential pathogens molecular identified by conventional PCR on biofilm samples in the Machángara River (a) and San Pedro River (b). All pathogens are presented for the three different longitudinal sampling points during the rainy season 1, the rainy season 2, and the dry season; and a heatmap showing the general abundance identified for the Machángara River (b) and San Pedro River (b).

In the Machángara River, as shown in **Figure 3a**, sampling points M1 and M2 showed a higher number of potential pathogens when compared to point M0 across all analyzed seasons. *Campylobacter* species were detected at points M1 and M2, displaying distinct patterns depending on the analyzed season. Notably, *H. pylori* was consistently detected on point M1 throughout all the seasons. Interestingly, *M. leprae* and *M. tuberculosis* were detected at all three points during the rainy season 1, with only *M. leprae* found at M2 during the rainy season 2. Moreover, during the dry season, both *Mycobacterium* species were detected exclusively at M0 and M1. Regarding parasites, *Giardia* spp. was identified at points M1 and M2 during the rainy season 1 and the dry season, but it was only detected at point M2 during the rainy season

2. In contrast, *Cryptosporidium* spp. was exclusively detected during the dry season across all longitudinal points. Overall, as indicated by the heatmap from **Figure 3c**, the dry season presented the highest abundance of identified potential pathogens in the Machángara River.

In the San Pedro River, as depicted in **Figure 3b**, a distinct pattern was observed compared to the Machángara River. Nonetheless, both points located in the urban zone (SP1 and SP2) exhibited the highest diversity of potential pathogens in comparison to the control point SP0, except for SP1 during the rainy season 2, where no pathogens were identified. In the case of *C. coli*, it was detected at points SP1 and SP2 during the rainy season 1 and the dry season, while it was only identified at point SP2 during the rainy season 2. *C. jejuni* was detected at points SP1 and SP2 during the rainy season 1 and only at SP1 during the dry season. In contrast, *C. upsaliensis* was only detected at SP2 during the rainy season 2. *H. pylori* was detected at SP0 during the rainy season 1 and SP2 during the rainy season 2 and the dry season. *M. tuberculosis* was detected at all sampling points during the rainy season 1, points SP0 and SP2 during the rainy season 2, and solely at SP2 during the dry season. *M. leprae* was detected at points SP1 and SP2 during the rainy season 1 and at SP0 and SP2 in the rainy season 2. Finally, *Giardia* spp. was detected only at SP1 during the rainy season 1 and *Cryptosporidium* was only detected at SP1 during the dry season. Overall, the rainy season 1 presented the highest abundance of identified microorganisms in the San Pedro River, as indicated by the heatmap in **Figure 3d**.

Comparing both rivers, it is evident that each presents a unique pattern of abundance across the longitudinal points and seasons analyzed. It is important to mention that none of the four pathotypes of *E. coli* (EHEC, EAEC, EIEC, and EPEC) were detected on these biofilm samples analyzed.

Amplicon sequencing analysis

In order to verify the results obtained from the molecular identification of potential pathogens, positive amplicons were randomly selected for Sanger sequencing analysis at Macrogen (Seoul, Korea). Comparing these sequences to the GenBank nucleotide collection using BLASTN, it was possible to confirm the presence of *M. tuberculosis* in both biofilm samples with a 100% identity. Also, DNA sequencing confirmed the presence of *Giardia intestinalis* with 100% identity, and positive amplicons of *Cryptosporidium* spp. were associated with *C. parvum* with an identity percentage ranging from 98% to 99%.

However, analysis of *H. pylori* and *M. leprae* did not yield significant similarities when compared to the GenBank database. Despite the sequencing analysis failing to validate the similarity of these species in the tested biofilm samples, likely due to compromised DNA quality resulting from environmental factors and concentrations, conventional PCR-based molecular analysis demonstrated the presence of both microorganisms, as shown in **Figure 3.**

Analysis of trace metals and major elements on biofilm samples

The concentration of trace metals and major elements in the biofilm samples from the Machángara and San Pedro Rivers, across the three seasons, are shown in **Figure 4**. The trace elements analyzed in the present study included copper (Cu), chromium (Cr), manganese (Mn), lead (Pb), zinc (Zn), nickel (Ni), arsenic (As), cadmium (Cd), phosphorus (P), barium (Ba), titanium (Ti), cobalt (Co), tin (Sn), antimony (Sb), beryllium (Be), strontium (Sr), vanadium (V), and molybdenum (Mo), while the major elements evaluated were aluminum (Al), iron (Fe), magnesium (Mg), calcium (Ca), sodium (Na), and potassium (K). Further information can be found in Supplementary Material (see **Supplementary Table S4**).

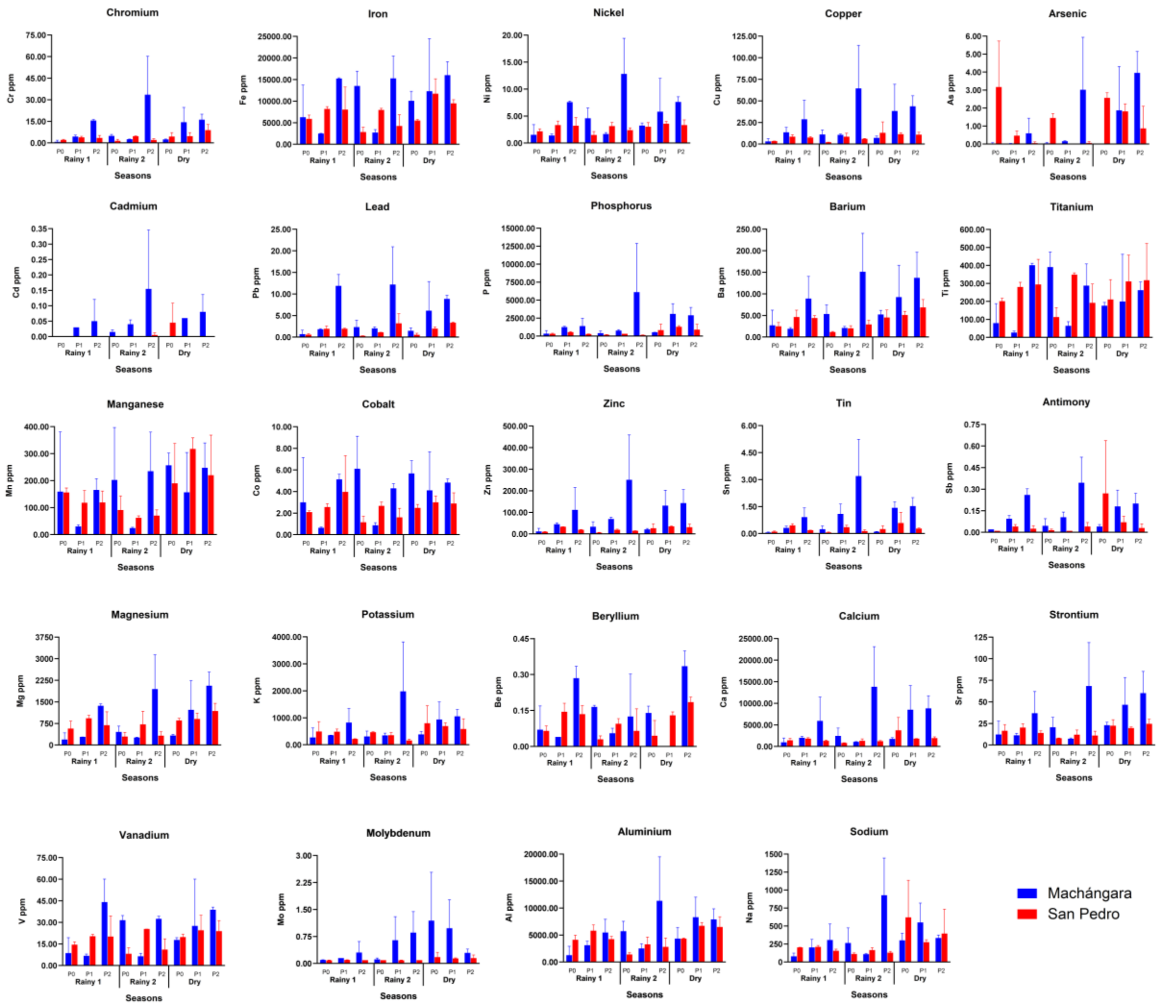

Figure 4. Average and standard deviation values of major and trace elements.

Average and standard deviation values of major and trace elements on biofilm samples from the Machángara River (blue bars) and San Pedro River (red bars). All values are presented for the three different longitudinal sampling points (P0=M0 and SP0, P1=M1 and SP1, and P2=M2 and SP2) during the rainy season 1, the rainy season 2, and the dry season. The values are presented on ppm (mg/kg of humid weight biofilm).

In the Machángara River, during the rainy season 2, point M2 evidenced the highest concentrations of several trace elements, including Cu (64.41 ppm), Cr (33.52 ppm), Pb (12.20 ppm), Zn (250.84 ppm), Ni (12.81 ppm), Cd (0.16 ppm), P (6,107.95 ppm), Ba (151.40 ppm), Sn (3.20 ppm), Sb (0.35 ppm), and Sr (68.38 ppm). The dry season showed the highest concentrations of Mn (256.73 ppm), Co (5.68 ppm), and Mo (1.19 ppm) at point M0, and levels of As (3.96 ppm) and Be (0.34 ppm) at point M2. Finally, during the rainy season 1, point M2 exhibited again the highest concentrations of Ti (401.16 ppm) and V (44.11 ppm). In the case of major elements, a similar trend was observed with point M2 during the rainy season 2 demonstrating highest concentrations of Al (11,352.12 ppm), Ca (13,843.44 ppm), Na (928.02 ppm), and K (1,981.48 ppm); while, during the dry season, point M2 showed the highest concentrations of Mg (2,061.67 ppm) and Fe (16,016.34 ppm).

Concerning the San Pedro River during the dry season, point SP2 showed the highest concentrations of Cr (8.88 ppm), Pb (3.35 ppm), Ba (68.73 ppm), Sr (24.61 ppm), Co (3.97 ppm), and Be (0.19 ppm); while, point SP1 had the highest concentrations of Zn (34.88 ppm), Ni (3.60 ppm), P (1302.15 ppm), Sn (0.59 ppm), and Mn (317.66 ppm); and, finally, point SP0 possessed the highest concentrations of Cu (13.00 ppm), Cd (0.05 ppm), Sb (0.27 ppm), and Mo (0.13 ppm). During the rainy season 2, point SP1 exhibited the highest values for Ti (349.23 ppm) and V (25.36 ppm). Finally, during the rainy season 1, point SP0 had the highest concentration of As (3.17 ppm). Major elements also demonstrated higher concentrations during the dry season, exhibiting the highest concentrations of Ca (3,747.69 ppm), Na (619.92 ppm), and K (796.13 ppm) in point SP0. On the other hand, point SP1 showed the highest values of Al (6,701.25 ppm) and Fe (11,699.24 ppm), and lastly point SP2 evidenced the highest concentration of Mg (1,181.58 ppm).

Molecular identification of different β-lactamase genes on isolates from biofilm samples

From the samples cultivated on Chromocult agar medium supplement with ceftriaxone, a total of 266 isolates were obtained, comprising 199 *E. coli*, 133 coliforms, and 14 unidentified bacteria. These unknown bacteria were later identified through Sanger sequencing as *Ralstonia* sp. in the Machángara River with 90 to 98% identity and *Aeromonas* sp. in the San Pedro River with 94% identity. Overall, the prevalence of *E. coli* isolates from both rivers, carrying β-lactamase was 95% for *bla*$_{\text{CTX-M}}$, 55% for *bla*$_{\text{TEM}}$, 13% for *bla*$_{\text{OXA}}$, and 7% for *bla*$_{\text{SHV}}$. In coliforms, the prevalence was 44% for *bla*$_{\text{TEM}}$, 41% for *bla*$_{\text{CTX-M}}$, 31% for *bla*$_{\text{SHV}}$, and 29% for *bla*$_{\text{OXA}}$. Detailed results are shown in **Figure 5** and Supplementary Material (see **Supplementary Tables S5 and S6**).

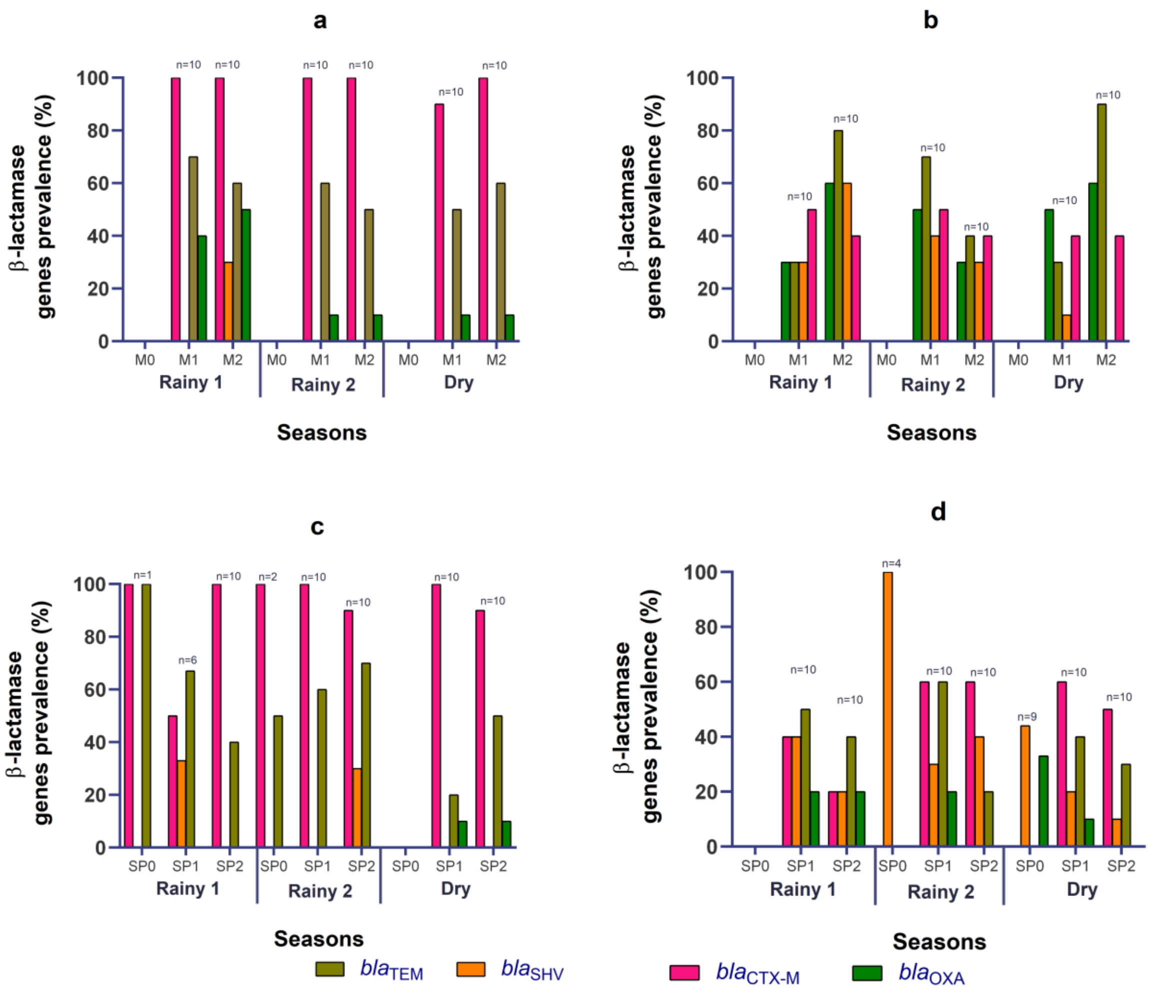

Figure 5. Prevalence of beta-lactamase genes.

Prevalence of four beta-lactamase genes on *E. coli* isolates from biofilm samples collected from the Machángara River (a) and San Pedro River (c), and prevalence in coliforms isolates from biofilm samples from the Machángara River (b) and San Pedro River (d).

Among the analyzed β-lactamase encoding genes, as depicted in **Figures 5a** and **5c**, *bla*$_{\text{CTX-M}}$ was the most prevalent in *E. coli* isolates, being detected in 98 (59/60) and 92% (54/59) of the isolates from the Machángara and San Pedro Rivers, respectively. *bla*$_{\text{TEM}}$ gene was identified in 58% (35/60) and 51% (30/59) of the isolates from the Machángara and San Pedro Rivers, respectively. Furthermore, the Machángara River also exhibited a prevalence of 22% (13/60) for *bla*$_{OXA}$ and 5% (3/60) for *bla*$_{SHV}$, meanwhile the San Pedro River showed a prevalence of 8% (5/59) for *bla*$_{SHV}$ and 4% (2/59) for *bla*$_{OXA}$. Regarding coliform isolates, as shown in **Figures 5b** and **5d** for the Machángara River, *bla*$_{TEM}$ was the most prevalent at 57% (34/60), followed by *bla*$_{OXA}$ at 47% (28/60), *bla*$_{CTX-M}$ at 43% (26/60) and *bla*$_{SHV}$ at 28% (17/60) of the isolates. In the San Pedro River, *bla*$_{CTX-M}$ was the most prevalent at 40% (29/73), followed by *bla*$_{TEM}$ at 33% (24/73), *bla*$_{SHV}$ at 33% (24/73), and *bla*$_{OXA}$ at 14% (10/73).

Discussion

As environmental degradation continues to present significant threats to nature and public health, there is an urgent need to intensify the monitoring of environmental contamination, especially in freshwater resources (UN-Water et al., 2023). Given the escalating concerns surrounding the persistence of antimicrobial resistance in the environment and pollution in water bodies (Matviichuk et al., 2023), it is essential to adopt a holistic approach that transcends disciplinary boundaries. The One Health Approach seeks to understand the interconnection between humans, animals, and environmental health (Prata, 2022). This approach facilitates the understanding and management of contamination spread and antimicrobial resistance on a broader scale. Through ongoing monitoring of environmental contamination, particularly in freshwater ecosystems, we can identify potential sources of pathogens and resistant bacteria, thereby enhancing our ability to mitigate the risk of serious public health diseases.

Furthermore, to contextualize our findings, it is crucial to consider the surrounding activities and population densities near the sampling points of each river. In the Machángara River, the presence of highly dense urban and industrial areas might contribute to elevated levels of pollution and microbial contamination (Borja-Serrano et al., 2020; Ibarra et al., 2024). This is attributed to the interception of 76% of wastewater discharges originating from households, industries, and other anthropogenic activities (Reinoso, 2015). Similarly, the San Pedro River may be influenced by agricultural practices or other anthropogenic activities including industries located nearby that discharge effluents directly into the river, as well as highly urbanized areas (Carrera, 2011; Ramirez-Cando et al., 2019). Understanding these factors helps to elucidate the potential sources of microbial contamination and antibiotic resistance in the studied rivers. It is also important to acknowledge the geographical formations near the area and the location of the rivers near volcanic zones. Several studies have indicated that past volcanic activities may influence the concentrations of metals, increasing their levels (Borja-Serrano et al., 2020; Vargas-Solano et al., 2019; Vinueza et al., 2021). Both rivers originate in the highlands and are situated near areas with previous volcanic activity (Borja-Serrano et al., 2020; Ibarra et al., 2024; Ramirez-Cando et al., 2019).

Escherichia coli and total coliforms counts in biofilm samples

In this study, we found that sampling points located within industrial or urban zones along both the Machángara and San Pedro Rivers, more exactly, M1, M2, SP1, and SP2, consistently showed higher concentrations of *E. coli* and total coliforms compared to control points M0 and SP0. *E. coli* is commonly used as a fecal indicator to assess water quality, as its presence often indicates the presence of potentially harmful bacteria or intestinal pathogens. While total coliforms by themselves are indicative of environmental contamination (Boni et al., 2021). In general, the levels of *E. coli* ranged from magnitudes of 10^5 CFU/g for the Machángara River and 10^4 CFU/g for the San Pedro River, with total coliform concentrations typically an order of magnitude higher in both cases. These findings align with similar studies conducted on biofilm samples from rivers in South Africa (Fosso-Kankeu et al., 2014) and streams in Germany (Balzer et al., 2010). Notably, the comparable magnitudes of total coliforms and *E. coli* suggest that fecal discharges without any prior treatment from both human and animal sources are likely the primary contamination source. In addition, agricultural runoffs can transport microbial contaminants to rivers, which can then adhere to biofilms on rocks (Bastos et al., 2023; UN-Water et al., 2023) Interestingly, bacterial cultures grown on media with and without ceftriaxone did not display significant differences in their loads, suggesting a substantial proportion of antibiotic-resistant *E. coli* and total coliforms within environmental biofilms. This data suggests that anthropogenic activities along both rivers may exert selective pressure on bacteria to exchange genetic material and acquire resistance to antibiotics commonly used in human and veterinary medicine (Matviichuk et al., 2023; Reichert et al., 2021). According to the literature, the microorganisms found within biofilm communities can potentially facilitate the spread of resistance among bacteria in the environment (Mao et al., 2021).

Despite efforts to improve water treatment infrastructure in Quito over the past decade, existing treatment plants currently only process a small fraction (less than 3.5%) of the city´s wastewater (Borja-Serrano et al., 2020; Vinueza et al., 2021). Moving forward, Quito’s municipal government has proposed the construction of three additional treatment plants, aiming to treat at least 55% of wastewater discharges (EPMAPS, 2023).

Molecular identification of potential pathogens on biofilm samples

The results of molecular identification of potential pathogens using PCR on biofilm samples provide valuable insights into the microbial dynamics within the Machángara and San Pedro Rivers. In the Machángara River, sampling points M1 and M2 consistently presented a higher diversity of potential pathogens compared to control point M0 across all seasons, with distinctive seasonal patterns observed for *Campylobacter* species and *H. pylori*. Notably, *M.*

leprae and *M. tuberculosis* were detected during the rainy season 1 at all three points, indicating a potential season influence on their prevalence and a source of contamination outside the densely populated city of Quito. Furthermore, *Giardia* and *Cryptosporidium* spp. showed differential distributions with higher detection rates during the dry season. Overall, the Machángara River biofilm samples presented a higher abundance of potential pathogens during the dry season at most sampling points. Conversely, in the San Pedro River, urban zone points SP1 and SP2 demonstrated a higher diversity of potential pathogens, except during the rainy season 2 for SP1, where no pathogens were identified. Seasonal variations were evident for most microorganisms with certain pathogens exhibiting peak abundances during specific seasons, as in the case of *M. tuberculosis* which was found across all the sampling points during the rainy season 1. Similarly, *Giardia* and *Cryptosporidium* spp. were only detected at point SP1 during the rainy season 1 and the dry season, respectively. Overall, the rainy season 1 displayed the highest abundance of identified microorganisms in the San Pedro River.

Campylobacter species, known as the leading cause of acute bacterial gastroenteritis globally, are commonly found in surface water due to contamination from animal feces, sewage effluents, and agricultural runoffs (Mughini-Gras et al., 2016). Despite its importance, few studies have focused on analyzing their presence in environmental biofilms (Ma et al., 2022; Maal-Bared et al., 2012). *Campylobacter* species thrive in environmental biofilms, suggesting the application of environmental biofilms as a biomarker of different contamination sources. *C. jejuni* was detected on biofilms samples from points M1 and M2 throughout all the seasons, as well as points SP1 and SP2 during the rainy season 1 and also point SP1 during the dry season. The presence of this bacterium can be attributed to discharges from animal or human origins containing feces. Literature suggests that this species, in particular, can survive and proliferate in multispecies biofilms from natural aquatic environments to survive harsh conditions (Bronowski et al., 2014; Maal-Bared et al., 2012). Meanwhile, *C. upsaliensis* was identified at points M1, M2, and SP2 during the rainy season 2, as well as point M1 during the dry season. A study revealed its ability to form biofilms under laboratory conditions and is mainly associated with dogs' feces, as a natural reservoir, possibly explaining its presence in the urban points of the present study (Elmonir et al., 2022; Ma et al., 2022). On the other hand, *C. coli* was detected at points M1 and M2 during both rainy seasons, while it was only detected at M1 during the dry season. In the San Pedro River, it was detected at points SP1 and SP2 during the rainy season 1 and also point SP2 during the rainy season 2 and the dry season. This bacterium is associated with poultry, cattle, and avian industries but has also been found related to agricultural discharges in freshwaters, which may explain its presence in this study (Denis et al., 2011; Mughini-Gras et al., 2016). Notably, point SP2 of the San Pedro River is situated in an area known for small-scale cultivation of crops such as maize, beans, and other vegetables. Additionally, this agricultural production includes fruits like guava, avocado, lemon, lime, and peaches. Moreover, there is still a limited portion of soil around this point dedicated to pasture (Simbaña Pillajo, 2023). Recently, a study assessed its presence on biofilm under laboratory conditions, but they did not find any visible biofilm formation on substrates such as glass, stainless steel, and polystyrene coupons (Ma et al., 2022). Another *in vitro* study demonstrated lower adhesion levels of *C. coli* when compared to *C. jejuni* (Sulaeman et al., 2010). Further studies are needed to understand the presence of *C. coli* and *C. upsaliensis* in environmental biofilms as primary or secondary colonizers within biofilm formation as similarly observed in some clinically relevant biofilms (Machado & Cerca, 2015).

H. pylori was consistently identified at point M1 throughout all the analyzed seasons in the Machángara River biofilm samples and also punctually detected at point SP0 during the rainy season 1 and point SP2 during the rainy season 2 and the dry season in the San Pedro River. *H. pylori* is recognized as the etiological agent of gastritis and peptic ulcer and is associated with gastric cancer in humans. More than half of the world's population is infected with this bacterium (Horiuchi et al., 2021; Mezmale et al., 2020). Despite its significance, the main route of transmission remains incompletely understood. Many studies suggest that person-to-person transmission or ingestion of contaminated water, raw vegetables, or even milk may be the most important routes of transmission (Mezmale et al., 2020). Moreover, *H. pylori* has been detected in various environmental sources, including water sources, soil, and animals such as livestock and pets (cats and dogs) (Horiuchi et al., 2021). Several studies have emphasized the association between contaminated water sources and the occurrence of *H. pylori* infections (Duarte et al., 2021; Xie et al., 2022). Additionally, research has shown that biofilms can serve as reservoirs of these bacteria within water distribution systems (Watson et al., 2004). The detection of *H. pylori* in biofilm samples from both rivers aligns with these findings, suggesting that untreated water may serve as a potential source of *H. pylori* contamination in biofilms collected from urban or industrial zones. Furthermore, the presence of *H. pylori* at control point SP0 may be attributed to the proximity of cattle farms in the area.

Mycobacterial species are also well-known to form biofilms in different environments, which typically confer their advantages and they have been frequently reported in water systems (Esteban & García-Coca, 2018). The presence of *M. leprae* in biofilm samples, especially at control points M0 and SP0, can be attributed to natural reservoirs such as armadillos, which are known to be susceptible to leprosy (Chavarro-Portillo et al., 2019). Other potential reservoirs due to the presence of armadillos include arthropods or free-living amoeba, which have been reported to provide conditions that maintain their cell viability (Chavarro-Portillo et al., 2019). On the other hand, its presence at urban points such as M1, M2, SP1, and SP2, could be attributed to the untreated discharge of contaminated fluids from patients through wastewater discharges (Arraes et al., 2017). According to the latest report of the Ministry of Public Health of Ecuador, no new cases of leprosy have been reported in Quito, indicating that the presence of *M. leprae* on biofilm could represent cases not officially reported or merely the remains of

DNA material from these bacteria within biofilm samples (Ministry of Public Health of Ecuador (MSP), 2024). In the same way, *M. tuberculosis* was identified at control points M0 and SP0, suggesting sources other than humans that could perhaps be attributed to the livestock activity near those areas (Scantlebury et al., 2004). The detection of *M. tuberculosis* in industrial or urban zones such as M1, M2, SP1, and SP2, could be explained by the lack of wastewater treatment plants and a combination of domestic discharges, industrial wastewater, mainly from slaughterhouses, and runoff from agricultural activities, which end up in rivers, having the potential to infect both humans and animals when they come into contact with these waters (Kesarwani et al., 2022; Mtetwa et al., 2022). However, it is important to mention that the detection of mycobacterial species was strictly realized through molecular and sequencing analyses being a limitation of the present study and further evaluation is needed to confirm the spread of these pathogens on environmental biofilms.

Interestingly, none of the four pathotypes of *E. coli* were detected in the analyzed biofilm samples from both rivers. Literature indicates that the survival of EHEC in soil is greatly influenced by microbial diversity, suggesting that this pathotype can survive for longer periods when diversity is low. Similarly, the survival of STEC (Shiga-toxin-producing *E. coli*) is associated with the absence of several species of protozoa that usually prey on these microorganisms (Ravva et al., 2010; van Elsas et al., 2011; Vogeleer et al., 2014). Therefore, the survival of pathotypes is greatly influenced by the environmental microcosm suggesting perhaps that the environmental biofilms in this study were not favorable for the colonization or survival of *E. coli* pathotypes, especially since *E. coli* pathotypes were detected in water samples (Cabrera-Ontaneda et al., 2024).

Regarding parasites, *C. parvum* was detected at points M0, M1, M2, and SP1 during the dry season, while *G. intestinalis* was identified at points M1, M2, and SP1 during the rainy season 1, point M2 during the rainy season 2, and points M1 and M2 during the dry season.

Both parasites have been associated with gastrointestinal disease outbreaks reported worldwide (Fradette et al., 2022; Hamilton et al., 2018), primarily attributed to contaminated water sources (Hamilton et al., 2018; Jellison et al., 2020; Sammarro Silva & Sabogal-Paz, 2021). It is crucial to analyze these parasites in freshwater environments where they are predominantly found in their infective forms, more exactly *C. parvum* as oocyst and *G. intestinalis* as cysts (Sammarro Silva & Sabogal-Paz, 2021). Numerous studies suggested that environmental biofilms serve as conducive habitats for the attachment of protozoan (oo)cysts and provide protection from various environmental stressors such as UV light (Jellison et al., 2020; Lefebvre et al., 2020; Masangkay et al., 2020). The presence of these parasites in biofilm samples could be attributed to anthropogenic activities, including inadequate sanitation practices and improper domestic wastewater treatment. Additionally, the presence of wildlife, domestic animals, and livestock in the vicinity of rivers plays a significant role in the zoonotic transmission of these parasites and their persistence in the environment (Fradette et al., 2022; Hamilton et al., 2018). These results highlight the importance of assessing the presence of waterborne protozoan pathogens in biofilms as a complementary approach to water quality analysis.

Understanding the dynamics of pathogen prevalence in biofilms can inform strategies for water quality management and public health interventions. Further research is needed to evaluate the viability of these microorganisms, elucidate the factors driving these patterns, and assess the potential risks posed by pathogenic microorganisms in river biofilms.

Trace metals and major elements in biofilm samples

Trace metals and major elements were also assessed for all biofilm samples. Overall, when comparing the results obtained from both rivers, notable variations in the concentration of trace elements were observed across different seasons. Specifically, during the rainy season 2, point M2 in the Machángara River exhibited the highest concentrations of several trace elements, more exactly Cu, Cr, Pb, Zn, Ni, Cd, P, Ba, Sn, Sb, and Sr. Meanwhile, during the dry season, the highest concentrations of Co and Mo were detected at point M0, while point M2 showed the highest concentration of As and Be. Furthermore, during the rainy season 1, point M2 demonstrated elevated levels of Ti and V. Additionally, during the dry season, point SP1 in the San Pedro River evidenced the highest concentration of Mn. It is important to mention that throughout the study period, point SP0 consistently presented the highest levels of As in the San Pedro River. Moreover, biofilm samples from the Machángara River consistently showed higher concentrations of major elements when compared to those from the San Pedro River. Notably, during the rainy season 1, point M2 exhibited the highest concentrations of Al, Ca, Na, and K; while, during the dry season, point M2 showed high levels of Mg and Fe.

The variations in trace elements concentration across different seasons and sampling points suggest different interactions with the environment and anthropogenic activities such as land use practices and industrial activities, which may contribute to these fluctuations. Additionally, differences in the geological composition of the riverbed and surrounding areas may also influence the availability and distribution of trace elements in water column and biofilm samples (Guerrieri et al., 2022). On the other hand, higher concentrations of major elements in biofilm samples from the Machángara River indicate a potentially greater influence of anthropogenic activities and land use practices in this river when compared to the San Pedro River, also agreeing with previous physicochemical analyses realized on water samples of these rivers (Borja-Serrano et al., 2020; Vinueza et al., 2021). Environmental biofilms are sensitive to various pollutants, including pesticides, pharmaceuticals, and heavy metals (Carafa et al., 2021). Our findings regarding the concentration of trace elements in river biofilms corroborate with previous studies highlighting the impact of metals on biofilm communities and acting as natural reservoirs (Guerrieri et al., 2022; Serra et al., 2010; Tien & Chen, 2013). Further research is needed to elucidate the specific sources and mechanisms driving trace metal and major metal accumulation in biofilms, in particular at points M2 and SP1, and their potential impact on aquatic ecosystems and human health. In our previous study conducted during 2017-2018 (Borja-Serrano et al., 2020), we observed that the Machángara River exhibited higher concentrations of several trace and major elements compared to the San Pedro River. Specifically, elevated levels of Cu, Pb, Cr, Mn, Cd, Ni, Zn, Al, and Fe were detected in the Machángara River, while the San Pedro River showed higher concentrations of Ba, Ca, V, Na, and Mg. However, upon comparing these findings with the results of the present study, some variations were noted. For instance, at point M2 of the Machángara River, we observed higher concentrations of Ba, Ca, V, Na, and Mg, contrasting with the San Pedro River. Conversely, at point SP1 in the San Pedro River, higher levels of Mn were quantified, differing from the Machángara River. The remaining elements examined in the previous study yielded consistent results, with higher concentrations observed in the Machángara River. It is essential to note that our 2020 study had limitations, such as being conducted at a single river point and without considering seasonality (Borja-Serrano et al., 2020). This highlights the significance of investigating these parameters under varying seasonal influences and longitudinally to gain a comprehensive understating of trace and major element concentrations.

Prevalence of beta-lactamase coding genes on biofilm samples

The isolation of colonies on Chromocult agar medium supplemented with ceftriaxone yielded a total of 266 isolates comprising 199 *E. coli*, 133 coliforms, and 14 other bacteria, which were further identified through DNA sequencing using the Sanger technique as *Ralstonia* sp. for the Machángara River and *Aeromonas* sp. for the San Pedro River. Overall, 95% of the *E. coli* isolates from both rivers harbored the $bla_{\text{CTX-M}}$ gene, followed by bla_{TEM} (55%), bla_{OXA} (13%), and bla_{SHV} (7%). In contrast, the coliforms exhibited a more equitable prevalence of the same four genes, more exactly *bla*$_{TEM}$ (44%), followed by *bla*$_{CTX-M}$ (41%), *bla*$_{SHV}$ (31%), and *bla*$_{OXA}$ (29%).

The prevalence of β-lactamase genes in isolated *E. coli* of biofilm samples agrees with findings from studies conducted in various regions. For instance, a study in the Yamuna River in India reported that 88 and 61% of the enterobacterial isolates carried *bla*$_{CTX-M}$ and *bla*$_{TEM}$ (Siddiqui et al., 2018), respectively. In 2019, another Ecuadorian study conducted on irrigation waters, fruits, and vegetables found several resistant *E. coli* isolates (Montero et al., 2021), more exactly *bla*$_{CTX-M}$ (98%) and *bla*$_{TEM}$ (92%). In Poland, a study on Dunajec, Czarny Dunajec, Bialy Dunajec, and Bialka Rivers reported that 46% of the *E. coli* isolates were positive for the *bla*$_{TEM}$ gene (Lenart-Boroń, 2017). Overall reports emphasize the widespread dissemination of β-lactamase genes, particularly *bla*$_{CTX-M}$ and *bla*$_{TEM}$, among environmental isolates, including those from environmental biofilms in numerous rivers.

The high prevalence of both genes in *E. coli* and coliforms isolates suggests the emergence of extended spectrum β-lactamase (ESBL) producing strains, which pose a significant public health concern due to their resistance to multiple β-lactam antibiotics. It is noteworthy that before the 1990s, the *bla*$_{TEM}$ gene was the most commonly detected β-lactamase in Gram-negative bacteria (Cantón et al., 2012). However, in recent years, the prevalence of *bla*$_{TEM}$ has been declining, with *bla*$_{CTX-M}$ becoming the predominant gene (Seyedjavadi et al., 2016). This shift may be attributed to the dissemination of *bla*$_{CTX-M}$ via mobile genetic elements such as plasmids, transposons, and integrons, making it the most prevalent β-lactamase (Cangui-Panchi et al., 2023; Cantón et al., 2012; Girlich et al., 2020; A. Machado et al., 2023). Furthermore, the equitable distribution of β-lactamase genes among coliform isolates (environment potentially bacteria) suggests a diverse reservoir of antibiotic resistance determinants within environmental biofilms, such as in rivers, with implications for the spread of resistance genes and the persistence of multidrug resistance (MDR) bacteria in the environment. These results highlight the importance of surveying antibiotic resistance genes in environmental reservoirs, as well as the need to develop strategies to mitigate its dissemination in the environment through the One Health approach. Efforts to address this challenge should focus on measures to reduce antibiotic usage, improve water treatment practices, monitor resistance genes in human, animal, plant, and ecosystem health, and so preserve the efficacy of antibiotics.

Conclusions and limitations

In conclusion, this study unveils a substantial presence of *E. coli* and total coliforms within biofilms samples collected from the Machángara and San Pedro Rivers, particularly concentrated in urban/industrial areas. The identification of pathogenic microorganisms evidenced a diverse array of infectious agents inhabiting freshwater resources of Quito and exhibiting variable season patterns unique to each river. Furthermore, the analysis of major and trace elements revealed a notable presence of contaminants, suggesting anthropogenic activities as the primary source of impact affecting the quality of the rivers. Regarding antibiotic resistance, a higher prevalence of the $bla_{\text{CTX-M}}$ gene was observed in *E. coli* isolates, whereas the bla_{TEM} gene predominated in coliforms. These findings strongly suggest that environmental biofilms can serve as effective biomarkers, reflecting the complex interaction between microbial communities, pollution by major and trace elements, and environmental factors in freshwater ecosystems. Furthermore, it highlights the necessity for ongoing surveillance of water quality by monitoring antibiotic resistance, pathogens, and hazardous chemicals in aquatic ecosystems to establish effective strategies for contamination reduction, safeguarding public health, and preserving natural environments.

A limitation of our study is that the selection of seasons for the experimental design was based on historical data, with the rainy season in Quito typically starting in September, with another peak of rain in March, and the driest months mainly occurring in June (Cazorla & Juncosa, 2015). Additionally, we considered the decrease in rainfall from December onwards, influenced by the El Niño phenomenon in this region (Portilla Farfán, 2018; Zambrano-Barragán et al., 2011) when choosing the sampling months for the study. However, it is important to recognize that the study was conducted during an unusual year that deviated from historical patterns. This discrepancy is evident when comparing our findings to the report provided by the National Institute of Meteorology and Hydrology from Ecuador (INHAMI; Table 1), which indicates higher precipitation values during the sampling month for the rainy season 2 compared to the rainy season 1. Furthermore, defining seasons in the highlands presents a challenge due to continuous and less pronounced season variations attributable to the presence of the Andean Mountain range and valleys (Portilla Farfán, 2018; Zambrano-Barragán et al., 2011). Further studies should also be performed on the emergent contaminants in both rivers, such as pharmaceuticals and personal care products, hormones, microplastics, and flame retardants that are not often controlled or monitored in the environment.

PART 3: SHORT COMMUNICATION

Exploring Antibiotic Resistance Genes (ARGs) and their dynamics in environmental biofilms: A Sequencing Approach

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Main text

After the discovery of penicillin in 1928, antibiotics emerged as a novel treatment for certain infections (Jiang et al., 2021). Nowadays, antibiotics are widely used in human and veterinary medicine to treat infectious diseases, in livestock industries as growth promoters, and in agriculture for crop improvement programs (Jiang et al., 2021; Velazquez-Meza et al., 2022). According to a report by the World Health Organization, the annual consumption of antibiotics ranges from 1 to 2,225 tons in 65 countries (World Health Organization (WHO), 2019). Consequently, the indiscriminate use of antibiotics has increased rapidly in recent years, leading to a rise in antibiotic resistance genes (ARGs) in bacteria (Haenelt et al., 2023; Machado & Cerca, 2015).

Studies have documented the widespread dissemination of ARGs across various ecosystems, particularly in aquatic environments. Moreover, the presence of ARGs in bacteria is associated with various mechanisms that aid in their spread, primarily in bacterial communities found within biofilms (Balcázar et al., 2015; Matviichuk et al., 2023). Several horizontal gene transfer (HGT) mechanisms, such as conjugation, transduction, and transformation, have been reported to facilitate the dissemination of ARGs (Machado et al., 2023; Matviichuk et al., 2023). According to the literature, conjugation is the predominant HGT mechanism due to the proximity of bacterial cells within biofilms (Machado et al., 2023; Michaelis & Grohmann, 2023).

One of the most significant resistance genes encodes for extended-spectrum β-lactamases (ESBLs), which produce enzymes capable of hydrolyzing third-generation cephalosporins (Salinas et al., 2021; Siddiqui et al., 2018). These genes are often carried on mobile genetic elements (MGE) such as plasmids, which can facilitate their spread between bacterial species. One of the ESBLs that has rapidly disseminated through the environment is the *bla*$_{CTX-M}$ gene (Cantón et al., 2012; Girlich et al., 2020). Literature indicates that this gene has proliferated mainly in *Escherichia coli,* since the 2000s, and aided by its association with plasmids (Cantón et al., 2012; Girlich et al., 2020; Salinas et al., 2024). Although the transmission of this gene has been studied on *E. coli* strains, there is a need to understand if this transmission can occur between different related species, like the coliform group in natural environments such as rivers (Montero et al., 2021; Salinas et al., 2021, 2024; Siddiqui et al., 2018). Therefore, the main goal of this study was to characterize *bla*$_{CTX-M}$ gene diversity in *E. coli* and coliform isolates from environmental biofilms and to assess the possible occurrence of HGT events between resistant *E. coli* and environmental bacteria.

Methods

Sample site and collection

Environmental biofilm samples were collected from superficial rocks submerged in surface water at the three previously mentioned longitudinal points along the Machángara and San Pedro Rivers (see **Figure 1**). However, the collection dates were limited to the rainy season (referred to as rainy season 2 in **Part 2**) and the dry season. Biofilm samples were obtained by scraping a 100 cm^2 surface area of a single rock using a sterilized swab. Subsequently, the swab was placed in a tube containing sterile distilled water and stored at 4°C in a cooler until arrival at the Microbiology Institute at the Universidad San Francisco de Quito (IM-USFQ) for further processing.

Cultivation of microorganisms from biofilm samples and isolation

The quantification of *E. coli* and total coliforms was performed as previously described in **Part 2.** Briefly, serial dilutions of the samples were cultured in Chromocult Agar medium with and without a 2 µg/mL supplement of ceftriaxone (third-generation cephalosporin), following the established protocol of previous studies (Borja-Serrano et al., 2020; Sanders, 2012; Vinueza et al., 2021). The petri dishes were then incubated at 37°C for 24 to 48 h. All colony-forming unit (CFU/mL) calculations were adjusted using average biofilm density to provide an approximate estimate of the values per gram of biofilm (CFU/g).

To obtain environmental-resistant bacteria, a maximum of 3 colonies of *E. coli* (blue/violet) and 3 colonies of coliforms (red/pink) were randomly selected from the Chromocult Agar supplemented with ceftriaxone at each sampling point. Subsequently, all isolated colonies were cultured on MacConkey agar for DNA extraction.

DNA extraction from colonies

DNA extraction was carried out as previously described in **Part 2.** Two to five isolated colonies were placed in a 1.5 mL autoclaved tube containing 500 μL distilled autoclaved water and boiled for 15 min in a water bath. The tubes were then stored at -20°C for 24 h to ensure thermal shock. After the 24 h period, the samples were centrifuged for five min at 208×g (RCF) and the supernatant was transferred to new 1.5 mL autoclaved tubes. The DNA concentration and quality were measured using a Nanodrop One Spectrophotometer (ThermoFisher, Madison, USA). Samples with a concentration above 50 ng/μL were diluted to a final concentration of 25 ng/μL for PCR analysis.

Molecular identification of bla*$_{\mathbf{CTX\text{-}M}}$ *gene

After obtaining microbial DNA, the *bla*$_{\mathrm{CTX\text{-}M}}$ gene was amplified following the protocol outlined by Hasibuan and colleagues with slight modifications (Hasibuan et al., 2018). The specific primers and conditions are detailed in **Table 2.** The PCR mixture consisted of a final volume of 25 μL and contained 5 μL of 5X GoTaq Flexi Buffer (Promega, Madison, USA), 2 μL of 25 mM $MgCl_2$ (Promega, Madison, USA), 1.25 μL of 10 μM for each PCR primer, 0.5

μL of 10 mM dNTP Mix (Promega, Madison, USA), 0.10 μL of 5U GoTaq Flexi DNA polymerase (Promega, Madison, USA), 2 μL of template DNA, and the remaining volume of DNA-free water. The thermocycling procedure was conducted in a thermocycler (Bio-Rad Laboratories, Inc, California, USA) with an initial denaturation of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, an annealing at 57°C for 30 seconds, an extension of 72°C for 45 seconds, and a final extension of 72°C for 5 min. Positive controls were provided by the IM-USFQ, while negative control consisted of DNA-free water, all samples were analyzed in duplicate or triplicate assays. The PCR products were visualized using electrophoresis with a 1.5% agarose gel and SybrSafe staining for 40 to 45 min.

Allelic variant analysis

Following the successful amplification of PCR products for *bla*$_{CTX-M}$, amplicons were sequenced at Macrogen (Seul, Korea) using ABI 3730xl Instruments. The sequences obtained from both forward and reverse primers were overlapped using PreGap4 and Gap4 software (Staden Package, Cambridge, England) (Staden et al., 2003), and, finally, primer sequences were removed from the consensus sequences. The resulting nucleotide sequences were then analyzed against the ResFinder database (Center for Genomic Epidemiology) with a threshold of >80% of identity and a minimum length of 60% for Acquired Antimicrobial Resistance to identify the allelic variants of the *bla*$_{CTX-M}$ gene.

Results and Discussion

Total coliforms and E. coli in environmental biofilms

The quantification of *E. coli* and total coliforms was assessed from the biofilm samples collected from both Machángara and San Pedro Rivers along three different longitudinal sampling points during two seasons. The average and standard deviation values of *E. coli* and total coliforms are shown in **Figure 6.** Each bar represents the bacterial concentration measured in CFU/g of biofilm humid weight with black bars indicating measurements in Chromocult agar with ceftriaxone and grey bars indicating measurements without ceftriaxone.

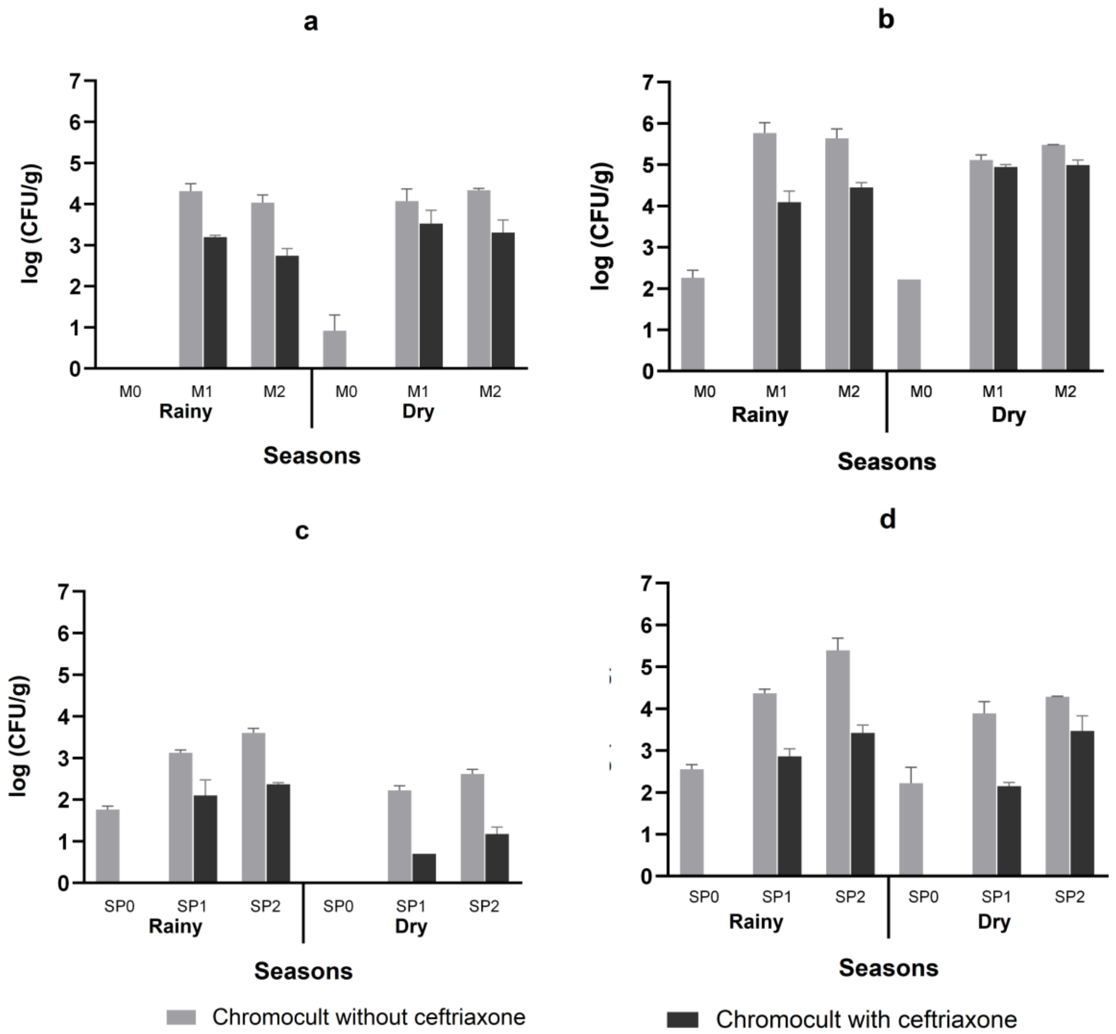

Figure 6. Average and standard deviation values of *Escherichia coli* and total coliforms.

Average and standard deviation values of *Escherichia coli* (a) and total coliforms (b) in the Machángara River, and average values of *E. coli* (c) and total coliforms (d) in the San Pedro River in Chromocult agar with ceftriaxone (black bars) and without (grey bars). All values are presented for the three different longitudinal sampling points during the rainy and dry seasons. The sample collection points for the Machángara River were: M0-Guamaní (control point), M1-Puengasí, and M2-Nayón. For the San Pedro River, the sample collection points were: SP0-Chaupi (control point), SP1-Sangolquí, and SP2-Cumbayá. Data represents CFU/g of biofilm humid weight.

The results obtained from the collection of biofilm samples using a swab show a similar trend to those where biofilm was collected from a pool of rocks. In both Machángara and San Pedro Rivers, *E. coli* and total coliform were higher at sampling points located in urban or industrial zones (M1, M2, SP1, and SP2) when compared to control points (M0 and SP0). In general, *E. coli* concentrations ranged from 10^3 CFU/g in the San Pedro River to 10^4 CFU/g in the Machángara River also evidencing a superior order of magnitude for total coliform loads in both rivers. These results are consistent with our previous study (**Part 2**) and suggest that a significant amount of contamination originates from untreated discharges containing fecal matter (Balzer et al., 2010; Bastos et al., 2023). Furthermore, these findings suggested that collecting biofilm samples with a swab can yield results consistent with those obtained from washed rocks, highlighting the dependence of the biofilm sampling method on the specific objectives of the aimed research. Future research could focus on further elucidating the sources and pathways of microbial contamination in rivers and evaluating the efficacy of different biofilm sampling techniques in various environmental contexts.

Molecular identification of $bla_{CTX\text{-}M}$ gene in isolates

From the samples cultured on Chromocult agar medium supplemented with ceftriaxone, a total of 91 isolates were obtained, comprising 41 *E. coli*, 47 coliforms, and 3 unidentified bacteria. Overall, 55% of the isolated colonies tested positive for the $bla_{CTX\text{-}M}$ gene. Specifically, 85 and 32% of *E. coli* and coliform isolates carried this gene, respectively. Interestingly, none of the unidentified bacteria tested positive for the $bla_{CTX\text{-}M}$ gene when conventional PCR analysis was performed. Detailed results are illustrated in **Figure 7,** where black bars represent the percentage of *E. coli* and grey bars indicate the percentage of coliforms for the Machángara River (**Figure 7a)** and the San Pedro River (**Figure 7b).**

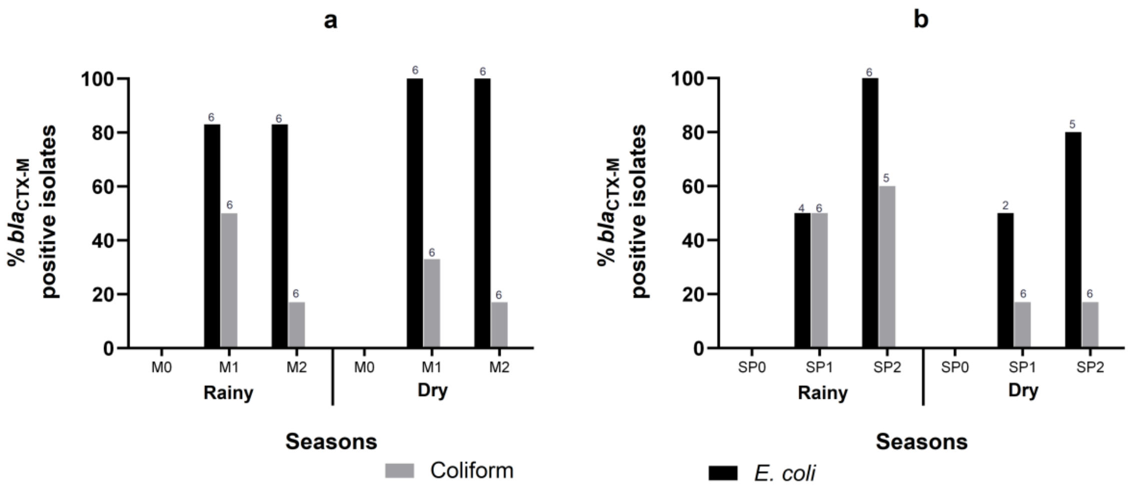

Figure 7. Prevalence of *bla*$_{CTX-M}$ gene.

Prevalence of *blaCTX-M* gene on *E. coli* (black bars) and coliform (grey bars) isolates from biofilm samples collected from the Machángara River (a) and San Pedro River (b).

These findings shed light on the prevalence of the *bla*$_{CTX-M}$ gene in *E. coli* and coliforms isolated from environmental biofilms, highlighting differences in gene distribution between them. The high percentage of *bla*$_{CTX-M}$ positive *E. coli* isolates suggests a potential reservoir for antibiotic resistance genes in these bacteria. However, the lower prevalence of the same gene in coliforms indicates variations in antibiotic resistance profiles among different bacterial species within the same environmental niche. Comparing these results with our previous study (**Part 2**), where most of the *E. coli* isolates evidenced this gene but only less than half of the coliforms did, the present work suggests that the dissemination of this gene has already begun among environmental bacteria. These findings also align with another study conducted on irrigation waters from Ecuador, where 98% of the *E. coli* isolates were positive for the *bla*$_{CTX-M}$ gene (Montero et al., 2021).

Allelic variants of bla$_{\text{CTX-M}}$ *gene in isolates*

The consensus sequences obtained from the *E. coli* and coliform isolates are shown in **Table 3**. Moreover, samples labeled with the code EC belong to *E. coli* isolates, while those labeled with the code CO belong to coliform isolates. Upon observation, it is evident that isolates from sampling points M1 and M2 in the Machángara River exhibited different allelic variants for the *bla*$_{\text{CTX-M}}$ gene in most cases. Overall, the most predominant allelic variants for the *bla*$_{\text{CTX-M}}$ gene in the Machángara River were found to be *bla*$_{\text{CTX-M 27}}$ and *bla*$_{\text{CTX-M 55}}$, with 24% (5/21) and 19% (4/21), respectively. Although it was expected to identify pairs of isolates of *E. coli* and environmental coliforms originating from the same rock with the same allelic variant, the results demonstrated that only one pair of bacterial species presented the same allelic variant. This particular pair was from point M2 during the dry season, where both BM2.2 EC1 and BM2.2 CO1 isolates contained the same allelic variant *bla*$_{\text{CTX-M 55}}$.

On the other hand, the results from the San Pedro River evidenced analogous trends with isolates from *E. coli* and coliforms showing different allelic variants. In this river, the dominant allelic variants were *bla*$_{\text{CTX-M 8}}$ and *bla*$_{\text{CTX-M 55}}$, with 33% (6/18) and 22% (4/18), respectively. Once again, only one pair of *E. coli* and coliform isolates was identified with an allelic variant match in the San Pedro River at point SP2 during the dry season. More exactly, BSP2.2 EC2 and BSP2.2 CO1 isolates shared the same *bla*$_{\text{CTX-M 55}}$ allelic variant.

Regarding these dominant allelic variants, the results obtained in this study align with those reported in a previous study conducted by Salinas and colleagues (Salinas et al., 2024). In their study, the authors analyzed *E. coli* isolates obtained from humans and animals in a rural community near the capital city of Ecuador, Quito, where the more prevalent allelic variants for the *bla*$_{\text{CTX-M}}$ gene were *bla*$_{\text{CTX-M 55}}$, *bla*$_{\text{CTX-M 65}}$, *bla*$_{\text{CTX-M 27}}$, and *bla*$_{\text{CTX-M 8}}$ (Salinas et al., 2024).

Table 3. Allelic variants of *bla*$_{CTX-M}$ gene in *E. coli* and coliform isolates.

Rivers	Seasons	Sampling points	Sample codes	Allelic variants
Machángara	Rainy	M1	BM1.1 EC1	*bla*$_{CTX-M\ 27\ (or\ 196\ or\ 174)}$
			BM1.1 EC2	*bla*$_{CTX-M\ 15\ (or\ 216\ or\ 210\ or\ 202)}$
			BM1.1 CO3	N/A
			BM1.2 EC1	*bla*$_{CTX-M\ 136}$
			BM1.2 EC2	*bla*$_{CTX-M\ 65\ (or\ 90)}$
			BM1.2 EC3	*bla*$_{CTX-M\ 27\ (or\ 196\ or\ 174)}$
			BM1.2 CO1	*bla*$_{CTX-M\ 213}$
			BM1.2 CO3	*bla*$_{OXY\ 1-5\ (or\ 1-4,\ or\ 1-3)}$
		M2	BM2.1 EC1	*bla*$_{CTX-M\ 55\ (or\ 79\ or\ 179)}$
			BM2.1 EC2	*bla*$_{CTX-M\ 55\ (or\ 79\ or\ 179)}$
			BM2.1 EC3	*bla*$_{CTX-M\ 12}$
			BM2.1 CO1	*bla*$_{OXY\ 1-3\ (or\ 1-4\ or\ 1-5)}$
	Dry	M1	BM1.1 EC1	*bla*$_{CTX-M\ 65\ (or\ 90)}$
			BM1.1 EC2	*bla*$_{CTX-M\ 27\ (or\ 219)}$
			BM1.1 EC3	*bla*$_{CTX-M\ 65\ (or\ 90)}$
			BM1.1 CO1	*bla*$_{CTX-M\ 15\ (or\ 219\ or\ 210\ or\ 202)}$
			BM1.1 CO2	*bla*$_{CTX-M\ 15\ (or\ 219\ or\ 210\ or\ 202)}$
		M2	BM2.2 EC1	*bla*$_{CTX-M\ 55\ (or\ 79\ or\ 179)}$
			BM2.2 EC2	*bla*$_{CTX-M\ 27\ (or\ 196\ or\ 175)}$
			BM2.2 EC3	*bla*$_{CTX-M\ 27\ (or\ 196\ or\ 174)}$
			BM2.2 CO1	*bla*$_{CTX-M\ 55\ (or\ 79\ or\ 179)}$
San Pedro	Rainy	SP1	BSP1.1 EC1	*bla*$_{CTX-M\ 55\ (or\ 79\ or\ 179)}$
			BSP1.1 CO2	N/A
			BSP1.2 EC1	*bla*$_{CTX-M\ 55\ (or\ 202)}$
			BSP1.2 CO1	*bla*$_{CTX-M\ 8}$
			BSP1.2 CO2	N/A
		SP2	BSP2.1 EC1	*bla*$_{CTX-M\ 8}$
			BSP2.1 EC2	*bla*$_{CTX-M\ 8}$
			BSP2.1 EC3	*bla*$_{CTX-M\ 15\ (or\ 216\ or\ 210\ or\ 202)}$
			BSP2.1 CO1	No identification
			BSP2.2 EC1	*bla*$_{CTX-M\ 8}$
			BSP2.2 EC2	*bla*$_{CTX-M\ 8}$
			BSP2.2 EC3	*bla*$_{CTX-M\ 8}$
			BSP2.2 CO1	*bla*$_{CTX-M\ 22\ (or\ 3\ or\ 211\ or\ 162)}$
			BSP2.2 CO3	N/A
	Dry	SP1	BSP1.2 EC1	*bla*$_{CTX-M\ 15\ (or\ 216\ or\ 210\ or\ 202)}$
			BSP1.2 CO2	N/A
		SP2	BSP2.2 EC2	*bla*$_{CTX-M\ 55\ (or\ 79\ or\ 179)}$
			BSP2.2 CO1	*bla*$_{CTX-M\ 55\ (or\ 79\ or\ 179)}$

N/A: data not available due to unspecific amplification during conventional PCR.

Future Perspectives

Further analysis will be conducted on the four candidate isolates, two from the Machángara River and two from the San Pedro River. The next steps to assess the genetic environment of the bla_{CTX-M} gene will involve following the outlined protocol by Salinas and colleagues (2024) with slight modifications (Salinas et al., 2024). Briefly, plasmid DNA extraction will be performed from the four candidate isolates using the Pure Yield Plasmid Miniprep System Kit (Promega, Madison, USA). The concentration of the extracted DNA will be measured using the Qubit 1x dsDNA High Sensitivity assay kit and Qubit 4.0 fluorometer (Thermo Fisher Scientific, Madison, USA). Nanopore sequencing will be conducted with the extracted plasmid DNA, following the instructions for library preparation using the Rapid Barcoding Sequencing Kit (Oxford Nanopore Technologies, Oxford, UK). Finally, further bioinformatics analysis will be performed on the obtained reads to assemble the plasmids and compare the genetic structures flanking the bla_{CTX-M} gene to identify possible evidence of horizontal gene transfer events between resistant *E. coli* and environmental coliform bacteria.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Primers and PCR cycling parameters for the detection of various potential pathogens (Cabrera-Ontaneda et al., 2024).

Organisms	Primers	Primer sequence (5′–3′)	PCR cycling parameters	Gene (size [bp])	References
Single PCR assays					
Universal	Forward: fDD2	CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG	3 min at 94°C; 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1.5 min	*16S* rRNA (1,600)	(Dobrowsky et al., 2014)
	Reverse: rPP2	CCAAGCTTCTAGACGGITACCTTGTTACGACTT			
Helicobacter pylori	Forward: FHpyl	GCGGGATAGTCAGTCAGGTG	2 min at 94°C; 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min	*16S* rRNA (706)	(Valenzuela & Machado, 2016)
	Reverse: RHpyl	AAGATTGGCTCCACTTCGCA			
Campylobacter coli	Forward: IpxA*C*	AGACAAATAAGAGAGAATCAG	2 min at 94°C; 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 45 s	*lpxA* gene (391)	(Klena et al., 2004)
	Reverse: lpxARKK2m	CAATCATGDGCDATATGASAATAHGCCAT			
Campylobacter jejuni	Forward: IpxAC	ACAACTTGGTGACGATGTTGTA		*lpxA* gene (331)	
	Reverse: lpxARKK2m	CAATCATGDGCDATATGASAATAHGCCAT			
Campylobacter upsaliensis	Forward: pxAC	AAGTCGTATATTTTCYTACGCTTGTGTG		*lpxA* gene (206)	
	Reverse: lpxARKK2m	CAATCATGDGCDATATGASAATAHGCCAT			
EAEC	Forward: AggRKs1	GTATACACAAAAGAAGGAAGC	2 min at 95°C; 35 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min	*aggR* (254)	(Ramírez Castillo et al., 2013)
	Reverse: AggRkas2	ACAGAATCGTCAGCATCAGC			
EHEC	Forward: VTcomU	GAGCGAAATAATTTATATGTG		*stx* (518)	
	Reverse: Vtcomd	TGATGATGGCAATTCAGTAT			
EPEC	Forward: SK1	CCCGAATTCGGCACAAGCATAAGC		*eae* (881)	

	Reverse: SK2	CCCGGATCCGTCTCGCCAGTATTCG			
EIEC	Forward: IpaIII	GTTCCTTGACCGCCTTTCCGATACCGTC		*ipaH* (619)	
	Reverse: IpaIV	GCCGGTCAGCCACCCTCTGAGAGTAC			
Mycobacterium leprae	Forward: S13	CTCCACCTGGACCGGCGAT	5 min at 95°C; 30 cycles of 94°C for 2 min, 58°C for 1 min, 72°C for 2min	*pra* (531)	(Arunagiri et al., 2017)
	Reverse: S62	GACTAGCCTGCCAAGTCG			
Nested PCR assays					
Mycobacterium tuberculosis	Forward: Mpb1	TCCGCTGCCAGTCGTCTTCC	5 min at 95°C; 30 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 30 s	*MPB64* (240)	(Madhavan et al., 2000)
	Reverse: Mpb2	GTCCTCGCGAGTCTAGGCCA			
	Forward: Mpb3	ATTGTGCAAGGTGAACTGAG	5 min at 95°C; 35cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s	*MPB64* (200)	
	Reverse: Mpb4	AGCATCGAGTCGATCGCGGA			
Cryptosporidium spp.	Forward: Cry 15	GTAGATAATGGAAGAGATTGTG	10 min at 95°C; 45 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 50 seconds	*COWP* (550)	(Salza, 2014; Yu et al., 2009)
	Reverse: Cry 9	GGACTGAAATACAGGCATTATCTT			
	Forward: Cowpnest F	TGTGTTCAATCAGACACAGC	10 min at 95°C; 32 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 50 s.	*COWP* (311)	
	Reverse: Cowpnest R	TCTGTATATCCTGGTGGG			
Giardia spp.	Forward: AL3543	AAATTATGCCTGCTCGTCG	5 min at 94°C; 35 cycles of 94°C for 45s, 50°C for 45 s, 72°C for 1 min	*TPI* (605)	(Salza, 2014)
	Reverse: AL3546	CAAACCTTTTCCGCAAACC			
	Forward: AL3544	CCCTTCATCGGTGGTAACTT	5 min at 94°C; 35 cycles of 94°C for 45s, 53°C for 30 s, 72°C for 1 min	*TPI* (530)	
	Reverse: AL3545	GTGGCCACCACTCCCGTGCC			

Supplementary Table S2. *E. coli* and total coliforms quantification on media with and without ceftriaxone (antibiotic) in both Machángara and San Pedro Rivers biofilm samples.

		Medium Chromocult agar with Ceftriaxone						Medium Chromocult agar without Ceftriaxone					
		E. coli **(CFU/g ± SD)**			**Coliforms (CFU/g ± SD)**			***E. coli*** **(CFU/g ± SD)**			**Coliforms (CFU/g ± SD)**		
Rivers	**Sampling points/Seasons**	**Rainy Season 1**	**Rainy Season 2**	**Dry Season**	**Rainy Season 1**	**Rainy Season 2**	**Dry Season**	**Rainy Season 1**	**Rainy Season 2**	**Dry Season**	**Rainy Season 1**	**Rainy Season 2**	**Dry Season**
Machángara	**M0**	0	0	0	0	0	0	$2.5x10^{1}$ ($3.5x10^{1}$)	0	$5.8x10^{1}$ ($8.2x10^{1}$)	$1.5x10^{2}$ ($1.4x10^{2}$)	$7.3x10^{2}$ ($5.1x10^{2}$)	$6.5x10^{2}$ ($7.5x10^{2}$)
	M1	$4.8x10^{3}$ ($4.4 x10^{3}$)	$3.5x10^{3}$ ($7.4x10^{2}$)	$5.5x10^{3}$ ($1.4x10^{3}$)	$3.0x10^{4}$ ($3.0x10^{4}$)	$1.9x10^{4}$ ($1.7x10^{4}$)	$1.1x10^{5}$ ($3.3x10^{4}$)	$1.2x10^{4}$ ($1.2x10^{4}$)	$3.9x10^{4}$ ($1.9x10^{4}$)	$1.5x10^{5}$ ($9.0x10^{4}$)	$8.4x10^{4}$ ($1.1x10^{5}$)	$1.6x10^{6}$ ($7.6x10^{5}$)	$1.5x10^{6}$ ($6.3x10^{5}$)
	M2	$2.6x10^{3}$ ($3.3x10^{3}$)	$1.3x10^{4}$ ($1.3x10^{4}$)	$4.9x10^{3}$ ($5.6x10^{3}$)	$3.8x10^{4}$ ($4.9x10^{4}$)	$7.2x10^{4}$ ($1.9x10^{4}$)	$4.8x10^{4}$ ($3.2x10^{4}$)	$1.6x10^{4}$ ($2.0x10^{4}$)	$1.0x10^{5}$ ($4.2x10^{4}$)	$1.2x10^{4}$ ($1.0x10^{4}$)	$7.1x10^{4}$ ($9.6x10^{4}$)	$1.3x10^{6}$ ($2.3x10^{5}$)	$3.0x10^{5}$ ($6.6x10^{4}$)
San Pedro	**SP0**	$2.5x10^{0}$ ($3.5x10^{0}$)	$5.0x10^{0}$ ($7.1x10^{0}$)	0	$6.2x10^{1}$ ($1.7x10^{1}$)	$3.0x10^{1}$ ($2.1x10^{1}$)	$3.5x10^{1}$ ($2.1x10^{1}$)	$5.0x10^{1}$ ($0.0x10^{0}$)	$2.0x10^{2}$ ($7.0x10^{1}$)	$8.3x10^{1}$ ($2.3x10^{1}$)	$1.5x10^{3}$ ($1.1x10^{3}$)	$3.7x10^{3}$ ($2.2x10^{3}$)	$1.1x10^{3}$ ($3.7x10^{2}$)
	SP1	$3.0x10^{3}$ ($4.2x10^{3}$)	$8.2x10^{1}$ ($2.4x10^{1}$)	$7.5x10^{1}$ ($2.1x10^{1}$)	$3.1x10^{4}$ ($4.2x10^{4}$)	$3.6x10^{2}$ ($1.9x10^{2}$)	$5.0x10^{2}$ ($2.1x10^{2}$)	$5.2x10^{3}$ ($6.7x10^{3}$)	$2.6x10^{3}$ ($4.7x10^{2}$)	$2.6x10^{2}$ ($1.3x10^{2}$)	$4.1x10^{4}$ ($5.4x10^{4}$)	$5.5x10^{4}$ ($3.2x10^{4}$)	$7.7x10^{3}$ ($9.2x10^{3}$)
	SP2	$6.2x10^{2}$ ($3.9x10^{2}$)	$1.0x10^{3}$ ($9.1x10^{2}$)	$3.6x10^{2}$ ($2.7x10^{2}$)	$4.0x10^{3}$ ($3.1x10^{3}$)	$4.3x10^{3}$ ($2.8x10^{2}$)	$2.4x10^{3}$ ($2.0x10^{3}$)	$9.9x10^{2}$ ($7.0x10^{2}$)	$1.3x10^{4}$ ($2.3x10^{3}$)	$2.6x10^{3}$ ($3.5x10^{2}$)	$9.9x10^{3}$ ($7.0x10^{3}$)	$9.9x10^{3}$ ($7.0x10^{3}$)	$1.3x10^{5}$ ($3.4x10^{4}$)

Supplementary Table S3. Detection of potential pathogens by PCR in biofilm samples of Machángara and San Pedro Rivers.

Microorganisms	**Gene**	**Machángara River**									**San Pedro River**								
		Seasons									**Seasons**								
		Rainy Season 1			**Rainy Season 2**			**Dry Season**			**Rainy Season 1**			**Rainy Season 2**			**Dry Season**		
		M0	**M1**	**M2**	**M0**	**M1**	**M2**	**M0**	**M1**	**M2**	**SP0**	**SP1**	**SP2**	**SP0**	**SP1**	**SP2**	**SP0**	**SP1**	**SP2**
Universal	***16srRNA***	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
***Cryptosporidium* spp.**	***COWP***	-	-	-	-	-	-	X	X	X	-	-	-	-	-	-	-	X	-
***Giardia* spp.**	***TPI***	-	X	X	-	-	X	-	X	X	-	X	-	-	-	-	-	-	-
EAEC	***aggR***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EHEC	***stx***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPEC	***ege***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EIEC	***ipaH***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Helicobacter pylori	***16srRNA***	-	X	-	-	X	-	-	X	-	X	-	-	-	-	X	-	-	X
Campylobacter jejuni	***IpxAC***	-	X	X	-	X	X	-	X	X	-	X	X	-	-	-	-	X	-
C. coli	***IpxAC***	-	X	X	-	X	X	-	X	-	-	X	X	-	-	X	-	-	X
C. upsaliensis	***IpxAC***	-	-	-	-	X	X	-	X	-	-	-	-	-	-	X	-	-	-
Mycobacterium tuberculosis	***Mpb64***	X	X	X	-	-	-	X	X	-	X	X	X	X	-	X	-	-	X
M. leprae	***pra***	X	X	X	-	-	X	X	X	-	-	X	X	X	-	X	-	-	-

Supplementary Table S4. Average and standard deviation values of major and trace elements in biofilm samples of Machángara and San Pedro Rivers.

Rivers	Seasons	Sampling points	Al	Fe	Mg	Ca	Na	K	Cu	Cr	Mn	Pb	Zn	Ni	As	Cd	P	Ba	Ti	Co	Sn	Sb	Be	Sr	V	Mo
			(ppm ± SD)																							
MCL (mL/L)			0.10[a]	0.3[a]	N/A	N/A	N/A	N/A	0.005[a]	0.032[a]	0.10[a]	0.001[a]	0.03[a]	0.025[a]	0.05[a]	0.001[a]	N/A	1.0[a]	N/A	0.20[a]	N/A	N/A	0.10[a]	-	0.10[b]	0.01[b]
LOD (mg/Kg)			0.05	0.10	0.02	0.00	0.05	0.01	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.09
Machángara	Rainy Season 1	M0	1254.30 ±1667.78	6261.87 ±7476.01	193.36 ±230.26	963.96 ±1004.19	79.64 ±44.49	272.06 ±352.53	3.11 ±3.08	0.82 ±1.03	159.45 ±221.25	0.70 ±0.91	11.47 ±14.07	1.53 ±1.87	0.03 ±0.04	<LOD	332.63 ±437.21	27.15 ±35.14	79.49 ±108.12	2.99 ±4.15	0.06 ±0.03	0.02 ±0.00	0.07 ±0.10	12.31 ±15.39	8.59 ±10.76	0.10 ±0.01
		M1	3110.67 ±742.24	2465.43 ±76.30	281.86 ±0.38	2001.67 ±336.70	202.87 ±111.80	353.81 ±5.47	13.60 ±5.76	4.42 ±1.00	30.35 ±5.80	1.81 ±0.16	44.73 ±6.54	1.40 ±0.27	0.02 ±0.02	0.03 ±0.00	1262.63 ±101.89	19.07 ±3.02	26.43 ±8.56	0.64 ±0.07	0.31 ±0.10	0.10 ±0.02	0.04 ±0.00	11.29 ±2.21	6.81 ±1.17	0.15 ±0.00
		M2	5461.86 ±2467.88	15238.78 ±177.90	1362.92 ±66.23	5924.62 ±5544.40	306.12 ±221.32	825.41 ±517.51	28.70 ±22.10	15.51 ±0.77	165.82 ±4.54	11.88 ±2.64	111.13 ±104.29	7.58 ±0.23	0.60 ±0.84	0.05 ±0.07	1400.01 ±1081.50	89.09 ±51.19	401.16 ±10.71	5.12 ±0.48	0.92 ±0.52	0.26 ±0.04	0.29 ±0.05	36.90 ±25.10	44.11 ±16.00	0.31 ±0.30
	Rainy Season 2	M0	5712.24 ±1839.02	13502.76 ±3427.93	456.48 ±198.03	2455.04 ±1798.36	264.45 ±209.13	307.68 ±201.72	11.14 ±5.11	4.84 ±1.08	202.55 ±194.57	2.32 ±1.60	33.34 ±23.31	4.58 ±1.93	0.04 ±0.06	0.02 ±0.01	411.04 ± 299.13	53.25 ±21.51	390.99 ±84.24	6.11 ±3.01	0.24 ±0.18	0.05 ±0.05	0.17 ±0.01	20.61 ±11.54	31.52 ±3.22	0.12 ±0.04
		M1	2514.41 ±8711.14	2723.26 ±697.24	258.75 ±13.64	1062.84 ±87.09	108.37 ±9.58	348.77 ±85.14	10.59 ±0.98	2.52 ±0.16	23.93 ±3.15	2.03 ±0.34	69.29 ±8.50	1.67 ±0.28	0.16 ±0.04	0.04 ±0.01	774.00 ±123.79	21.06 ±3.61	64.24 ±22.80	0.88 ±0.23	1.10 ±0.54	0.11 ±0.04	0.06 ±0.02	7.39 ±0.90	6.45 ±2.05	0.65 ±0.66
		M2	11352.12 ±8144.87	15280.56 ±5175.42	1945.25 ±1194.30	13843.44 ±9292.39	928.02 ±518.34	1981.48 ±1833.49	64.41 ±49.84	33.52 ±26.70	235.28 ±144.87	12.20 ±8.75	250.84 ±207.70	12.81 ±6.60	3.02 ±2.92	0.16 ±0.19	6107.95 ±6772.02	151.40 ±88.93	288.17 ±119.85	4.30 ±0.43	3.20 ±2.04	0.35 ±0.18	0.13 ±0.18	68.38 ±50.36	32.43 ±1.81	0.86 ±0.59
	Dry Season	M0	4324.92 ±2054.13	10090.32 ±2184.71	332.78 ±34.26	1743.97 ±299.39	301.33 ±96.69	383.22 ±111.28	7.12 ±2.08	2.39 ±0.28	256.73 ±45.76	1.48 ±0.66	20.88 ±4.51	3.23 ±0.49	<LOD	<LOD	533.16 ±6.80	52.32 ±9.56	175.68 ±18.72	5.68 ±1.17	0.10 ±0.01	0.04 ±0.01	0.14 ±0.03	23.02 ±3.39	17.81 ±1.55	1.19 ±1.34
		M1	8301.48 ±3715.15	12308.13 ±12125.83	1222.85 ±1018.76	8515.20 ±5568.47	549.78 ±270.27	937.77 ±654.37	38.25 ±30.97	14.42 ±10.28	157.05 ±147.26	6.14 ±6.75	131.44 ±71.02	5.82 ±6.19	1.88 ±2.44	0.06 ±0.00	3094.70 ±1405.78	92.67 ±73.80	199.62 ±262.04	4.11 ±3.59	1.43 ±0.34	0.18 ±0.11	<LOD	46.62 ±31.71	27.57 ±32.51	0.98 ±0.79
		M2	7901.88 ±1958.08	16016.34 ±3111.39	2061.67 ±476.31	8829.40 ±2911.29	333.92 ±41.32	1056.92 ±249.96	43.76 ±12.16	16.08 ±3.92	247.86 ±91.75	8.88 ±0.81	142.63 ±63.26	7.62 ±0.95	3.96 ±1.19	0.08 ±0.06	2887.51 ±1091.65	137.15 ±59.32	262.55 ±45.16	4.83 ±0.35	1.53 ±0.47	0.20 ±0.07	0.34 ±0.06	60.14 ±25.2	38.79 ±1.77	0.30 ±0.11
San Pedro	Rainy Season 1	SP0	4121.46 ±844.36	5875.70 ±874.22	574.45 ±260.90	1442.82 ±439.77	202.96 ±1.75	493.61 ±364.05	3.45 ±0.04	2.06 ±0.45	156.21 ±16.61	0.57 ±0.23	9.78 ±1.08	2.16 ±0.47	3.17 ±2.56	<LOD	319.86 ±84.17	24.93 ±8.71	201.74 ±16.29	2.09 ±0.12	0.11 ±0.04	0.01 ±0.00	0.07 ±0.02	16.53 ±7.12	14.49 ±1.96	0.09 ±0.01
		SP1	5810.18 ±1061.26	8203.86 ±521.92	927.66 ±467.12	1818.53 ±204.93	211.07 ±19.52	482.10 ±102.61	8.51 ±2.18	3.84 ±0.71	118.30 ±45.00	1.96 ±0.63	33.04 ±0.25	3.32 ±0.74	0.47 ±0.26	<LOD	562.73 ±68.67	46.48 ±15.84	280.42 ±25.96	2.56 ±0.30	0.46 ±0.09	0.04 ±0.01	0.15 ±0.04	20.22 ±4.37	20.33 ±1.27	0.10 ±0.01
		SP2	4237.93 ±1061.26	8055.72 ±5224.42	687.84 ±467.12	1308.24 ±167.37	157.23 ±22.13	217.93 ±4.41	7.45 ±1.15	3.46 ±1.74	118.95 ±42.36	1.95 ±0.12	19.11 ±1.04	3.20 ±1.54	0.05 ±0.07	<LOD	287.46 ±12.64	44.16 ±5.71	294.15 ±139.70	3.97 ±3.35	0.19 ±0.01	0.03 ±0.02	0.14 ±0.04	13.92 ±2.50	20.19 ±14.29	0.09 ±0.00
	Rainy Season 2	SP0	1396.32 ±325.67	2846.45 ±1122.40	295.91 ±143.25	806.01 ±70.20	110.49 ±16.98	463.45 ±19.18	2.02 ±0.14	1.43 ±1.05	90.89 ±52.52	0.28 ±0.05	5.98 ±1.25	1.50 ±0.64	1.45 ±0.24	<LOD	183.26 ±29.92	11.53 ±1.24	112.83 ±51.99	1.15 ±0.57	0.07 ±0.02	0.02 ±0.01	0.03 ±0.01	7.83 ±0.48	8.21 ±4.07	0.09 ±0.00
		SP1	3283.16 ±1348.18	7947.41 ±403.23	716.25 ±457.52	1292.91 ±452.29	166.33 ±34.12	356.36 ±97.42	8.46 ±4.09	4.60 ±0.33	62.05 ±7.44	1.12 ±0.01	18.96 ±3.02	3.14 ±0.66	<LOD	<LOD	326.48 ±1.17	20.05 ±6.29	349.23 ±9.30	2.67 ±0.36	0.36 ±0.11	0.01 ±0.00	0.10 ±0.02	11.99 ±5.41	25.36 ±0.04	0.09 ±0.001

		SP2	2806.26 ±1653.19	4229.43 ±2607.85	326.63 ±136.90	1229.71 ±145.80	130.03 ±18.66	161.64 ±42.62	6.05 ±0.45	2.02 ±0.88	70.18 ±21.22	3.21 ±2.21	14.27 ±0.53	2.39 ±0.43	0.06 ±0.08	0.01 ±0.01	143.28 ±26.78	29.17 ±9.92	192.08 ±105.10	1.61 ±0.84	0.14 ±0.07	0.04 ±0.03	0.07 ±0.09	10.98	11.05 ±7.54	0.09 ±0.00
	Dry Season	SP0	4328.94 ±111.40	5498.98 ±233.56	854.56 ±76.30	3747.69 ±2962.06	619.92 ±515.16	796.13 ±651.51	13.00 ±12.61	4.42 ±2.68	189.92 ±148.38	0.53 ±0.37	26.41 ±20.34	3.02 ±0.77	2.57 ±0.28	0.05 ±0.06	834.20 ±839.28	45.38 ±17.32	210.60 ±110.58	2.48 ±0.33	0.25 ±0.19	0.27 ±0.37	0.05 ±0.06	22.39 ±6.89	19.79 ±2.09	0.18 ±0.13
		SP1	6701.25 ±621.36	11699.24 ±3497.96	906.25 ±203.15	1816.06 ±20.88	275.59 ±34.32	693.93 ±117.51	11.40 ±1.68	4.67 ±2.48	317.66 ±41.20	1.96 ±0.40	34.88 ±2.08	3.60 ±0.40	1.82 ±0.42	<LOD	1302.15 ±149.56	51.20 ±8.17	311.37 ±145.66	3.00 ±0.58	0.59 ±0.60	0.07 ±0.04	0.13 ±0.01	19.66 ±1.20	24.50 ±10.51	0.14 ±0.01
		SP2	6481.43 ±1903.16	9475.31 ±847.11	1181.58 ±269.70	1943.68 ±274.04	394.65 ±339.45	583.84 ±379.95	10.88 ±2.72	8.88 ±3.88	220.06 ±148.84	3.35 ±0.13	31.04 ±14.50	3.33 ±0.95	0.87 ±1.23	<LOD	896.25 ±754.73	68.73 ±18.29	317.59 ±204.17	2.89 ±0.99	0.28 ±0.04	0.03 ±0.03	0.19 ±0.02	24.61 ±5.34	23.94 ±7.26	0.15 ±0.08

[a] Table 2. Quality criteria acceptable for the preservation of aquatic and wildlife in fresh waters, cold or warm, and marine waters and estuaries. Texto Unificado Legislación Secundaria del Medio Ambiente (TULSMA), Book VI, Annex I (Ministry of Environment of Ecuador (MAE), 2015).
[b] Table 3. Quality criteria for water for agricultural irrigation. TULSMA, Book VI, Annex I (Ministry of Environment of Ecuador (MAE), 2015).
MCL: Maximum Contaminant Level; * Values that exceed the quality criteria; <LOD: below the limit of detection; N/A: not available. The reported values were obtained by triplicate measurements of each analyzed river sample.

Supplementary Table S5. Prevalence of antibiotic resistance genes in *E. coli* isolates obtained from biofilm samples of Machángara and San Pedro Rivers.

		***Escherichia coli* isolates**														
Rivers	**Seasons**	**Rainy Season 1**					**Rainy Season 2**					**Dry Season**				
	Sampling points	**Genes prevalence**				**Total isolates (N)**	**Genes prevalence (%)**				**Total isolates (N)**	**Genes prevalence (%)**				**Total isolates (N)**
		$\mathit{bla}_{\text{CTX-M}}$	$\mathit{bla}_{\text{SHV}}$	$\mathit{bla}_{\text{TEM}}$	$\mathit{bla}_{\text{OXA}}$		$\mathit{bla}_{\text{CTX-M}}$	$\mathit{bla}_{\text{SHV}}$	$\mathit{bla}_{\text{TEM}}$	$\mathit{bla}_{\text{OXA}}$		$\mathit{bla}_{\text{CTX-M}}$	$\mathit{bla}_{\text{SHV}}$	$\mathit{bla}_{\text{TEM}}$	$\mathit{bla}_{\text{OXA}}$	
Machángara	**M0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	M1	10/10 (100%)	0/10 (0%)	7/10 (70%)	4/10 (40%)	**10**	10/10 (100%)	0/10 (0%)	6/10 (60%)	1/10 (10%)	**10**	9/10 (90%)	0/10 (0%)	5/10 (50%)	1/10 (10%)	**10**
	M2	10/10 (100%)	3/10 (30%)	6/10 (60%)	5/10 (50%)	**10**	10/10 (100%)	0/10 (0%)	5/10 (50%)	1/10 (10%)	**10**	10/10 (100%)	0/10 (0%)	6/10 (60%)	1/10 (10%)	**10**
San Pedro	**SP0**	1/1 (100%)	0	1/1 (100%)	0	**1**	2/2 (100%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	**2**	0	0	0	0	**0**
	SP1	3/6 (50%)	2/6 (33%)	4/6 (67%)	0/6 (0%)	**6**	10/10 (100%)	0/10 (0%)	6/10 (60%)	0/10 (0%)	**10**	10/10 (100%)	0/10 (0%)	2/10 (20%)	1/10 (10%)	**10**
	SP2	10/10 (100%)	0/10 (0%)	4/10 (40%)	0/10 (0%)	**10**	9/10 (90%)	3/10 (30%)	7/10 (70%)	0/10 (0%)	**10**	9/10 (90%)	0/10 (0%)	5/10 (50%)	1/10 (10%)	**10**
TOTAL		34/37 (92%)	5/37 (14%)	22/37 (59%)	9/37 (24%)	**37**	41/42 (98%)	3/42 (7%)	25/42 (60%)	2/42 (5%)	**42**	38/40 (95%)	0/40 (0%)	18/40 (45%)	4/40 (10%)	**40**

Supplementary Table S6. Prevalence of antibiotic resistance genes in coliform isolates obtained from biofilm samples of Machángara and San Pedro Rivers.

		Coliforms isolates														
Rivers	**Seasons**	**Rainy Season 1**					**Rainy Season 2**					**Dry Season**				
	Sampling points	**Genes prevalence**				**Total isolates (N)**	**Genes prevalence (%)**				**Total isolates (N)**	**Genes prevalence (%)**				**Total isolates (N)**
		bla$_{\text{CTX-M}}$	***bla***$_{\text{SHV}}$	***bla***$_{\text{TEM}}$	***bla***$_{\text{OXA}}$		***bla***$_{\text{CTX-M}}$	***bla***$_{\text{SHV}}$	***bla***$_{\text{TEM}}$	***bla***$_{\text{OXA}}$		***bla***$_{\text{CTX-M}}$	***bla***$_{\text{SHV}}$	***bla***$_{\text{TEM}}$	***bla***$_{\text{OXA}}$	
Machángara	**M0**	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
	M1	5/10 (50%)	3/10 (30%)	3/10 (30%)	3/10 (30%)	**10**	5/10 (50%)	4/10 (40%)	7/10 (70%)	5/10 (50%)	**10**	4/10 (40%)	1/10 (10%)	3/10 (30%)	5/10 (50%)	**10**
	M2	4/10 (40%)	6/10 (60%)	8/10 (80%)	6/10 (60%)	**10**	4/10 (40%)	3/10 (30%)	4/10 (40%)	3/10 (30%)	**10**	4/10 (40%)	0/10 (0%)	9/10 (90%)	6/10 (60%)	**10**
San Pedro	**SP0**	0	0	0	0	**0**	0/4 (0%)	4/4 (100%)	0/4 (0%)	0/4 (0%)	**4**	0/9 (0%)	4/9 (44%)	0/9 (0%)	3/9 (33%)	**9**
	SP1	4/10 (40%)	4/10 (40%)	5/10 (50%)	2/10 (20%)	**10**	6/10 (60%)	3/10 (30%)	6/10 (60%)	2/10 (20%)	**10**	6/10 (60%)	2/10 (20%)	4/10 (40%)	1/10 (10%)	**10**
	SP2	2/10 (20%)	2/10 (20%)	4/10 (40%)	2/10 (20%)	**10**	6/10 (60%)	4/10 (40%)	2/10 (20%)	0/10 (0%)	**10**	5/10 (50%)	1/10 (10%)	3/10 (30%)	0/10 (0%)	**10**
TOTAL		15/40 (38%)	15/40 (38%)	20/40 (50%)	13/40 (33%)	**40**	21/44 (48%)	18/42 (43%)	19/42 (45%)	10/42 (24%)	**44**	19/49 (39%)	8/49 (16%)	19/49 (39%)	15/49 (31%)	**49**