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First detection of diarrheagenic *Escherichia coli* pathotypes in the Mearim River Watershed, Maranhão State, Brazil

Primeira detecção de patótipos diarreiogênicos de *Escherichia coli* na Bacia Hidrográfica do Rio Mearim, Estado do Maranhão, Brasil

Vitórya Mendes da Silva Monteiro[1]; Edianez dos Santos Mendes[2]; Kelyane da Costa Silva Menezes[3]; Carlos Alberto Guimarães[4]; Hanna Gabriely Pinto Gonçalves[5]; Nancyleni Pinto Chaves Bezerra[6]*; Danilo Cutrim Bezerra[7]; Larissa Sarmento dos Santos Ribeiro[6]; Alcina Vieira de Carvalho Neta[8]; Viviane Correa Silva Coimbra[6]

Highlights

ETEC, typical EPEC and atypical EPEC were isolated from the analyzed samples.
Consumption water from the evaluated river stretch may pose to unique risk health.
Water quality surveillance is important for the management of water resources.

Abstract

The Mearim River Watershed has multiple uses e.g. leisure, navigation, fishing and subsistence agriculture and constitutes the main source of supply for the populations of municipalities situated along its course. In addition to being a water supply source, the existence of the 'pororoca' (tidal bore) effect in a stretch of the lower course of the Mearim River attracts people from several Brazilian states and different countries, as it

[1] Graduate Student of the Course in Biological Sciences Degree, Universidade Estadual do Maranhão, UEMA, São Luís, MA, Brazil. E-mail: vymendes224@gmail.com
[2] Graduate Student of the Course in Fisheries Engineering, UEMA, São Luís, MA, Brazil. E-mail: edianezmendes@hotmail.com
[3] Graduate Student of the Course in Animal Science, UEMA, São Luís, MA, Brazil. E-mail: kelyanemenezes@hotmail.com
[4] Student of the Master's Course of the Professional Postgraduate Program in Animal Health Defense, UEMA, São Luís, MA, Brazil. E-mail: guimaraesmedvet@hotmail.com
[5] Student of the Master's Course of the Postgraduate Program in Animal Science, UEMA, São Luís, MA, Brazil. E-mail: hanna_gabriely@hotmail.com
[6] Profs. Drs. of the Masters Course of the Professional Postgraduate Program in Animal Health Defense, UEMA, São Luís, MA, Brazil. E-mail: nancylenichaves@hotmail.com; larissa.sarmento@uema.br; vivianecorrea@yahoo.com
[7] Prof. Dr. of the Graduate Course in Animal Science, UEMA, São Luís, MA, Brazil. E-mail: danilocbezerra15@gmail.com
[8] Prof. Dr. of the Master and Doctoral Course of the Postgraduate Program in Animal Science, UEMA, São Luís, MA, Brazil. E-mail: alcinavcn@yahoo.com
* Author for correspondence

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offers excellent conditions for surfing in fresh water. In this respect, given the importance of the watershed, this study was developed to report the detection of diarrheagenic *Escherichia coli* pathotypes in a stretch of the lower course of the Mearim River, located in the state of Maranhão, Brazil. Thirty water samples were collected from 10 sampling points. To quantify *E. coli,* the chromogenic enzymatic system was used and positive samples were isolated and biochemically identified. Pure cultures underwent DNA extraction by heating followed by polymerase chain reaction (PCR) characterization. At the time of the collections, an observation schedule was used to record information on the existence of rearing of livestock and domestic animals; businesses; residences; and fruit and vegetable farming on the riverbanks. The samples were analyzed for the mean populations of *E. coli,* which ranged from 444 to 2,585 MPN mL^{-1}. Twenty bacterial isolates were identified and the diarrheal pathotypes ETEC, typical EPEC and atypical EPEC were detected. The detection of these pathotypes can represent an epidemiological risk and compromise several uses of this water resource, such as irrigation of fruits and vegetables consumed raw, fishing, animal watering and recreation. Structural investments in basic sanitation are essential to minimize environmental degradation resulting from anthropic activities and to act preventively in public health. In addition, the recovery of riparian forests along the watershed and the maintenance of vegetation in these areas are measures to reduce the transport of particles from the soil to the watercourses, improving the qualitative and quantitative characteristics of this water resource.
Key words: Animal health. Human health. Enteropathogens. Environmental microbiology.

Resumo

A Bacia Hidrográfica do Rio Mearim apresenta múltiplos usos - lazer, navegação, pesca e agricultura de subsistência - e constitui a principal fonte de abastecimento para as populações dos municípios inseridos em seu curso. Além de fonte hídrica de abastecimento, a existência do efeito pororoca em um trecho do baixo curso do Rio Mearim atrai pessoas de vários estados brasileiros e diferentes países, pois apresenta excelentes condições para a prática de surfe em água doce. Nesse sentido, considerando a importância da Bacia Hidrográfica, objetivou-se relatar a detecção de patótipos diarreiogênicos de *Escherichia coli* em um trecho do baixo curso do rio Mearim, localizado no estado do Maranhão. Para isso, foram realizadas coletas de 30 amostras de água em 10 pontos amostrais. Para a quantificação de *E. coli* utilizou-se o sistema cromogênico enzimático e das amostras positivas procedeu-se ao isolamento e identificação bioquímica dos isolados. A extração do DNA das culturas puras foi realizada por aquecimento seguido da caracterização por reação em cadeia da polimerase (PCR). No momento das coletas utilizou-se uma pauta de observação para anotação de informações sobre a existência de criação de animais de interesse pecuário e doméstico, empreendimentos comerciais, residências e cultivo de frutas e hortaliças nas margens do rio. Nas amostras analisadas, foram quantificadas populações médias de *E. coli* que variaram de 444 a 2.585 $NMP.mL^{-1}$, identificados 20 isolados bacterianos e detectados os patótipos diarreiogênicos ETEC, EPEC-típica e EPEC-atípica. A detecção destes patótipos pode representar risco epidemiológico e compromete diversos usos desse recurso hídrico, como a irrigação de frutas e hortaliças ingeridas cruas, pesca, dessedentação animal e recreação. Investimentos estruturais em saneamento básico são fundamentais para minimizar a degradação ambiental resultante das atividades antrópicas e para atuar preventivamente na saúde pública. Adicionalmente, a recuperação das matas ciliares ao longo da Bacia Hidrográfica e manutenção da vegetação nestas áreas são medidas para a redução do transporte de

partículas do solo para os cursos d'água, e em consequência, acarretará na melhoria das características quali-quantitativas desse recurso hídrico.

Palavras-chave: Enteropatógenos. Microbiologia ambiental. Sanidade animal. Saúde humana.

Introduction

The importance of water is related not only to its functions in nature, but also the role it plays in human health, economy and quality of life (F. S. de Andrade, Silva, Aride, & Oliveira, 2016). Climate change, the population explosion and the increased pollution of water bodies are the main factors causing water scarcity and the consolidation of a water crisis (Al-Batsh et al., 2019; Clark, Jamal, & Weidhaas, 2019).

The state of Maranhão, Brazil, is located in a strategic geographic position for the flow of grain production, with great biodiversity and water potential (ten basins and two hydrographic systems). This last characteristic is the basis for the development of several activities, such as agriculture, recreation, tourism and transport. However, the large water network of the state is not sufficient to ensure the supply of quality water for the diverse needs of its population (Universidade Estadual do Maranhão [UEMA], 2016).

With a total area of 98,289.05 km^2, the Mearim Watershed is the largest in Maranhão, occupying 29.6% of the total area of the state (UEMA, 2016). It comprises 84 municipalities, of which 50 are fully inserted in the valley and the others are partially located in the watershed (Companhia de Desenvolvimento dos Vales do São Francisco e do Paraíba [CODEVASF], 2016). A stretch of the lower course of the Mearim River is known for the 'pororoca' (tidal bore) phenomenon, which attracts surfers from various parts of the world and is considered the tourism hub of the region (Associação Brasileira de Surf na Pororoca [ABRASPO], 2021).

Nevertheless, the human development index in the region is low as a consequence the precarious sanitary and infrastructure conditions of most municipalities along the Mearim River Watershed. With respect to water supply to the municipalities, a large part of the population suffers from lack of water, which forces them to collect it directly from rivers in this watershed for multiple purposes.

Studies show that there is a strong correlation between human action and impacts on water bodies (Chanapathi, Thatikonda, Keesara, & Ponguru, 2020; Xie, Jiang, Zhang, & Huang, 2020). In the investigation of water quality, *Escherichia coli* has microbiological relevance, as it is a microorganism that indicates fecal contamination and an etiological agent of waterborne diseases. This bacterium is indicative of the presence of other enteric pathogens, besides its diarrheagenic types (Shiga toxin-producing *E. coli* - STEC, enteropathogenic *E. coli* - EPEC, enterotoxigenic *E. coli* - ETEC, enteroaggregative E. coli - EAEC, enteroinvasive *E. coli* - EIEC and diffuse adherent *E. coli* - DAEC). The last ones, despite not having the same epidemiological profile, have an enormous potential to cause diseases (Croxen et al., 2013) and, therefore, pose challenges to public health. Water diagnosed with these pathogenic microorganisms is unsuitable for multiple uses, such as direct consumption, recreation and irrigation of fruits and vegetables consumed raw.

The severity of diseases caused by pathogenic *E. coli* warrants international and national surveillance programs that monitor and track outbreaks caused by this microorganism. Studies on this topic are important for establishing parameters that can be used to support through microbiological and epidemiological data the environmental conditions of a given area, providing criteria for the control and monitoring of environmental degradation that affects the fauna, flora and the quality of life of animals (domestic and livestock) and humans. In view of this, the present study was developed to report the detection of diarrheagenic *Escherichia coli* pathotypes in a stretch of the lower course of Mearim River, part of the Mearim River Watershed, located in the state of Maranhão, Brazil.

Material and Methods

Study site

The study area comprised a stretch of the lower course of Mearim River, state of Maranhão, Brazil. This area belongs to the Legal Amazon and is located in the sedimentary plain of the Mearim River Watershed, covering an area of 1,816,994 km^2 (Instituto Brasileiro de Geografia e Estatística [IBGE], 2017).

The dominant climate in the region is humid tropical with a dry winter season. The study area typically has a dry period of six to seven months, of which three to four months are considered very dry, with less than 8% of the annual precipitation. In the rainy period, from five to six months, at least two months can be considered very rainy, with over 30% of the total precipitation (Cunha & Silva, 2002).

Sampling

For the collection of water samples, the following factors were considered: ease of access; type of riparian vegetation found on the banks of the river; and sinuosity of the river course. Thus, the collection of environmental samples consisted of 10 points (P1 to P10) chosen strategically along the river course. The geographic location of the collection points was determined using electronic equipment for global positioning that uses a satellite signal called Global Position System (GPS).

Three water samples were collected at each sampled point (P1 to P10), totaling 30 samples. Collections were carried out during the period of lower rainfall, at monthly intervals (August, September and October 2020). The samples were collected in sterile borosilicate glass bottles with a capacity of 500 mL, following the procedures for water sample collection described in the Water Sample Collection and Preservation Guide of the Environmental Company of the State of São Paulo (Companhia de Tecnologia de Saneamento Ambiental [CETESB], 2011). All samples were stored under protection from sunlight in isothermal boxes with recyclable ice, and the respective collection containers were identified with the sample data (date and time of collection, point evaluated and municipality sampled). At the time of collections, an observation schedule was used to record information on the existence of livestock or domestic animals, businesses, residences and fruit and vegetable growing in the stretch of the sampled river.

Laboratory analyses

For the quantification of *E. coli,* the chromogenic enzymatic system (Colilert, Idexx, USA) was used, following the manufacturer's instructions. From each collected sample, 10 mL of water was diluted in 90 mL of sterilized distilled water and poured into sterilized flasks containing the substrate. Then, the solution was incubated in an oven at 35±0.5 °C for 24 h. The presence of *E. coli* was confirmed by the emission of blue fluorescence from the sample when exposed to ultraviolet light at a wavelength of 365 nm (IDEXX Laboratories Inc.).

For the isolation and confirmation of *E. coli,* the conventional methodology was adopted, which was divided into two steps:

• *First step: sample enrichment*

A 10-µL aliquot of the water samples cultivated in the Colilert enzyme substrate that was positive for *E. coli* was inoculated in MacConkey Agar (MCK). The culture was incubated at 37 ºC for 24 h. From the plates representing each point, all colonies with different morphotypes and, at most, five colonies with the same morphotype were transferred. Each of them was inoculated in tryptic soy agar (TSA) and in brain-heart infusion broth (BHI) and incubated at 37 ºC for 24 h.

• *Second step: identification of isolated colonies*

The identification of the *E. coli* species in each pure culture was confirmed by biochemical-physiological tests, using Rugai medium with lysine: (i) gas production from glucose; (ii) lactose fermentation; (iii) lysine decarboxylation; (iv) indole production from tryptophan; (v) non-degradation of urea; and, (vi) absence of hydrogen sulfide production from sulfur amino acids.

Pure cultures of *E. coli* underwent DNA extraction by heating according to the protocol proposed by Rowlands, Papasidero, Paula, Cano and Gelli (2006). Initially, *E. coli* suspensions grown in BHI broth (1.5 mL) were precipitated by centrifugation (10,000 rotations per minute – rpm, for 10 min). Subsequently, the cells were washed with ultrapure water (Milli-Q) and resuspended again in 100 µL of ultrapure water; at each resuspension, the samples were vortexed. Then, these were placed in a boiling bath (95 °C) for 5 min and taken to an ice bath for the same time. The last two procedures were repeated once more and then the samples were centrifuged at 10,000 rpm for 10 min. At the end of this step, 100 µL of the supernatant were removed and the precipitate was discarded.

The extracted DNA was quantified in a spectrophotometer by reading the absorbance at 260 nm and the 260/280 nm ratio was used to determine the purity of the samples. Then, the samples were stored at -20 °C until molecular analysis.

The DNA of the *E. coli* isolates was characterized by PCR using the GoTaq® Colorless Master Mix Kit (Promega®) according to the manufacturer's recommendations, generating a final volume of 25 µL per reaction. Table 1 summarizes the target primers and amplification conditions. Positive controls for *eae, Stx1* and *Stx2* (*E. coli* CDC EDL-933, INCQS 00171), bfpA (*E. coli* CDC O126, INCQS 000184), elt (*E. coli* 0761-2) and est (*E. coli* 0122-4), as well as the internal negative control (ultrapure water), were included in each reaction batch.

Table 1
Sequence of oligonucleotides used as primers in polymerase chain reaction (PCR) and amplification conditions

Primer designation	Primers (5'-3')	Program	Amplicon size (bp)	References
eae1	CTGAACGGCGATTACGCGAA CCAGACGATACGATCCAG	94 ºC/5 min 94 ºC/1 min 53 ºC/2 min 72 ºC/3min 30x 72 ºC/7 min	917	Reid, Betting and Whittam (1999)
bfpA	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	94 ºC/5 min 94 ºC/30 s 56 ºC/1 min 72 ºC/2 min 29x 72 ºC/7 min	326	Gunzburg, Tornieport and Riley (1995)
Stx1	CAGTTAATGTGGTGGGGAAGG CACCAGACAATGTAACCGCTG	95 ºC/5 min 95 ºC/20 s 61 ºC/40 s 72 ºC/90 s 30x 72 ºC/7 min	348	Vidal, Vidal, Lagos, Levine and Prado (2004)
Stx2	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	95 ºC/5 min 95 ºC/20 s 61 ºC/40 s 72 ºC/90 s 30x 72 ºC/7 min	584	Vidal et al. (2004)
elt	GGCGACAGATTATACCGTGC CGG TCT CTA TAT TCC CCT GTT	95 ºC/5 min 95 ºC/45 s 50 ºC/1 min 72 ºC/1 min 40x 72 ºC/7 min	450	Aranda, Fagundes-Neto and Scaletsky (2004)
est	ATTTTTMTTTCTGTATTRTCTT CACCCGGTACARGCAGGATT	95 ºC/5 min 95 ºC/45 s 50 ºC/1 min 72 ºC/1 min 40x 72 ºC/7 min	190	Aranda et al. (2004)

bp = base pair; s = seconds; min = minutes.

The PCR products were visualized by applying 5 µL of the amplified product on 2% agarose gel, staining with SYBR Safe® and horizontal electrophoresis for 30 min at 90V in 1X TBE buffer. The bands were visualized under ultraviolet light and digital images were recorded with the L-PIX Image EX image capturing system (Locus Biotechnology, Brazil).

Results and Discussion

At the 10 collection points, the average populations of *E. coli* ranged from 444 to 2,585 MPN.mL^{-1}. Other studies have already been carried out in Brazil on the microbiological quality of surface water and indicated high levels of contamination by microorganisms of the coliform group, resulting in inadequate sanitary conditions (Costa et al., 2020; Gonçalves et al., 2020; Marques, Nunes-Gutjahr, & Braga, 2020) and risk for multiple uses. Lack of adequate monitoring and control systems is one of the main factors that compromise water quality. Thus, stricter quality standards must be adopted to ensure that some water sources in use do not become unsuitable for consumption and other major uses, which would result in risks to public health.

The evaluated stretch showed fluctuations in the bacterial population counts, which constitutes an epidemiological risk for the multiple purposes used. Because the evaluated waters from the entire stretch of the Mearim River are used for fishing and livestock farming, especially cattle, microbiological contamination results are of great importance for the local population and other users of this water resource.

In the 30 samples, two different bacterial morphotypes characterized by (i) pink colonies, and (ii) colorless/transparent colonies were isolated on MacConkey agar (differential and selective medium). Of these, 20 different bacterial isolates with biochemical characteristics compatible with *E. coli* were isolated and, for the first time in the state of Maranhão, virulence genes characteristic of enterotoxigenic *E. coli* (ETEC) and typical and atypical enteropathogenic E. coli (EPEC) (Table 2) were detected.

Table 2
Diarrheagenic *Escherichia coli* pathotypes detected in water samples from a stretch of the lower course of Mearim River, Maranhão state

Collection point	Gene	Diarrheagenic *E. coli* pathotype
01	*bfpA, est*	EPEC-t, ETEC
02	*Est*	ETEC
03	*est, elt*	ETEC
04	*bfpA, est, elt*	EPEC-t, ETEC
05	*bfpA, eae1, elt*	EPEC-t, EPEC-a, ETEC
06	*bfpA*	EPEC-t
07	*eae1, est*	EPEC-a, ETEC
08	*bfpA, eae1, est, elt*	EPEC-t, EPEC-a, ETEC
09	*bfpA*	EPEC-t
10	*bfpA, est*	EPEC-t, ETEC

EPEC-t = typical enteropathogenic E. coli; ETEC = enterotoxigenic *E. coli;* EPEC-a = atypical enteropathogenic *E. coli.*

Akter et al. (2013) analyzed water samples from 46 rivers in the People's Republic of Bangladesh and identified the EPEC, STEC and ETEC pathotypes in the country's waterways. Titilawo, Obi and Okoh (2015) developed a study on surface waters in southwestern Nigeria at 10 sampling points and observed that, of the 300 isolated colonies, 91% had virulence genes related to the EPEC, EHEC and ETEC pathotypes. In a study carried out in the Xopotó Watershed - Minas Gerais, Brazil, Drumond, Santiago, Moreira, Lanna and Roeser (2018) detected the STEC and EPEC pathotypes in three of the 13 evaluated points and the ETEC strain in one sampling point. The results of the aforementioned studies show that the EPEC, STEC and ETEC pathotypes are the most recurrent in microbiological contamination of surface waters in different countries, including Brazil, which the present study corroborates regarding the detection of the EPEC and ETEC pathotypes.

In the 10 sampled points, virulence genes characteristic of ETEC (*elt* and *est* genes) were detected, alone or combined. Due to the characteristics of the anthropic activities developed in the vicinity of the sampled area, it is possible that contamination by this pathotype originates in cattle, dog and human feces. Mainil (2013) mentioned that the main hosts of ETEC are humans, cattle, swine and dogs. In the state of Minas Gerais, G. I. Andrade et al. (2012) detected virulence genes characteristic of this pathotype in both healthy calves and animals with diarrhea. In a study with calves in the Kashmir Valley, India, Manzoor et al. (2015) diagnosed ETEC in 15% of the 200 evaluated animals.

Virulence genes characteristic of EPEC (*eae1* and/or *bfpA*) were detected in eight sampled points. At the points termed P5, P7 and P8, the virulent gene *eae1* was identified, which shows atypical characteristics. According to Nguyen, Taylor, Tauschek and Robins-Browne (2006), this pathotype has high pathogenicity for humans, with a prolongation of the diarrheal state in affected patients and the possibility of evolution to death. It is worth mentioning that P5 is near a public hospital.

Sidhu, Ahmed, Hodgers and Toze (2013) analyzed the microbiological quality of rivers in Australia and found that 3% of diarrheagenic strains were atypical EPEC. Schuroff, Lima, Burgos, Lopes and Pelayo (2014) diagnosed 2% of the *eae* gene in lakes in Paraná, Brazil. Therefore, compared with molecular detection studies of diarrheagenic EPEC in surface waters already developed in Brazil and in other countries, the results obtained in the present study revealed similarity in the diagnosis of atypical EPEC.

Detection of the bacterium EPEC, a recurrent cause of hospitalizations due to gastroenteritis, in its atypical form, is a reason for caution in the use of local water resources. Moreover, according to Sidhu et al. (2013), there is an increasing trend for the occurrence of the *eae* gene in surface water. Detection of ETEC in places where livestock activities are carried out, such as the studied site, highlights the need to implement hygienic-sanitary measures for workers in handling animals and in the use of cattle manure for fertilizing food crops, especially fruits and vegetables consumed raw.

The *Stx1* and *Stx2* genes were not detected during the study period. This is not indicative of absence of STEC throughout the year in the same evaluated stretch, since seasonality is considered the main influencing variable in the degradation of the

microbiological quality of surface waters, due to the intensification of surface runoff, which accentuates the transport of particles contaminated by fecal material to water bodies. Brennan et al. (2013) inferred that the ability of *E. coli* to survive due to its simple nutritional requirements and adaptability within the soil transforms the environment into a spreader of microbiological contamination for water bodies, increasing the possibility of carrying of diarrheagenic pathotypes.

In a study conducted in the Xopotó River Watershed in the Alto Rio Doce region, Minas Gerais, Brazil, for molecular identification of diarrheagenic *E. coli* at two times of the year, Drumond, Santiago, Moreira, Lanna and Roeser (2018) detected the *Stx1* virulence gene in two of the 13 sampling points, only at the time of higher rainfall.

Based on the *in locu* observations of the sampled points, separately or combined, we can infer that the main polluting sources in the evaluated stretch are the rearing of livestock (cattle, horses and swine) and domestic animals (dogs), population centers, businesses and the presence of a hospital, which occur near the banks of the river. Additionally, the observations revealed that the population of the sampled area uses the local water resources for various purposes, which shows that the economy of the municipalities located in the Mearim River Watershed is closely related to the activities developed along it, including small subsistence crops such as rice, maize and watermelon, in addition to artisanal fishing.

Although the development and quality of life of civilizations are closely linked to the availability of water, as highlighted by Londe, Coutinho, Di Gregório, Santos and Soriano (2014), research shows that pollution and degradation of water resources are recurrent realities associated with accelerated population growth. For L. C. de Andrade, Rodrigues, Andreazza and Camargo (2019), the lack of planning in urban development, coupled with the population density, can cause the contamination and pollution of water resources, which are often the only source of supply for the population.

According to Pereira et al. (2016), for a sustainable process to occur that allows the development of agricultural production and the growth of urbanization, along with the preservation of the environment, public policy actions that enable economic development and the maintenance of natural ecosystems are necessary. For the National Water Agency (Agência Nacional das Águas [ANA], 2017), the surveillance and analysis of the quality of surface water are essential for the sustainable management of water resources, as this will indicate the situation of water bodies and the main changes over time. Furthermore, these measures provide the possibility of identifying trends and supporting the formulation of diagnoses that can support inspection, environmental licensing and the formulation of public policies aimed at public health.

Conclusions

The microbiological quality of water in the lower stretch of Mearim River, belonging to the Mearim Watershed in the state of Maranhão, Brazil, suffers deterioration characterized by high average populations of *E. coli* and diversified diarrheagenic pathotypes (ETEC, typical EPEC and atypical EPEC). The confirmation of this group of pathogenic

microorganisms along the evaluated stretch represents a microbiological risk to public health, compromising various uses such as irrigation of vegetables and other crops consumed raw, fishing, animal watering, recreation and tourism.

Structural investments in basic sanitation are essential to minimize environmental degradation stemming from human activities and to act preventively on human health, animal health and biodiversity. Additionally, the recovery of riparian forests along the watershed and the maintenance of vegetation in these areas are effective measures to reduce the transport of particles from the soil to the watercourses. As a result, they can improve the qualitative and quantitative characteristics of the water in the evaluated stretch.

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