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Determination of mercury concentration by a new spectrophotometric method and evaluation of bacterial diversity in river water samples from Brazil

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ABSTRACT

One of the main micropollutants reported in river water is mercury (Hg), a heavy metal toxic to human and animal organisms that can promote bacterial resistance to antimicrobials used in the clinical practice. Having done this in consideration, monitoring the concentration of Hg in the river is an important indicator of physical-chemical and microbiological quality of water. Thus, in this study, the Hg concentration was determined using a new spectrophotometric method in river water samples recovered from Minas Gerais, Brazil. Furthermore, the diversity and antimicrobial resistance of Gram-positive and Gram-negative bacteria isolated from these samples were also reported. A new ultraviolet-visible spectrophotometric method was validated and applied to quantify Hg in water and revealed high concentrations in the samples (0.13–0.35 $\mu g{\cdot}mL^{-1}$), above the limits established by Brazilian standards (0.002 $\mu g{\cdot}mL^{-1}$). Gram-negative bacteria (mainly *Escherichia coli*) were recovered in greater proportion (88.4%) from water samples with high mercury concentration and showed resistance to tetracycline and ampicillin. Our results highlighted that *E. coli* resistant to carbapenems, which are latest generation beta-lactams, were detected. In conclusion, the Hg levels are considerably high in river waters in Brazil, and these mercury-rich water sources are important reservoirs of multi-drug resistant bacteria.

Key words: AmpC betalactamase, extended-spectrum betalactamase (ESBL), *Klebsiella Pneumoniae* carbapenemase (KPC), mercury, river, susceptibility profile

HIGHLIGHTS

- New spectrophotometry method was validated and applied to determine the mercury concentration in river water samples.
- Mercury concentrations above the limits established by Brazilian standards was reported in river from Minas Gerais.
- Resistance to antimicrobials was detected in bacteria isolates from mercury-rich river water sample.
- Carbapenem-resistant *Escherichia coli* were reported in mercury-rich river water sample.

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GRAPHICAL ABSTRACT

1. INTRODUCTION

Water is an indispensable natural resource for life on earth, and rivers play an important socio-economic role. However, rivers undergo the impacts of anthropic activities and constant changes in concentrations of heavy metals, organic matter and microbial diversity are observed (Naidoo & Olaniran 2014). Among the impacts of the presence of components such as heavy metals in river waters, it can be highlighted the alteration of the aquatic bacterial population which, due to its great genomic plasticity, adapts to the environment and becomes tolerant or resistant to them (Pal *et al.* 2015).

Mercury (Hg) is one of the metals most toxic to humans and animals, and it reaches the environment due to its use in industrial and domestic activities and rainwater, being considered a chemical micropollutant (Hölzel *et al.* 2012; Devarajan *et al.* 2015). Depending on its chemical form, Hg can be transported and deposited away from its source, and be rapidly inserted into the food chain through methylation, which can be bioaccumulated and biomagnified along trophic chains (Kehrig *et al.* 2011).

Considering the impacts of the presence of this heavy metal in the aquatic environment, its concentration should be monitored and kept within the limits defined by each country's regulatory agencies. Several analytical techniques for determining and separating mercury have been used such as cold-vapor integrated quartz crystal microbalance (CV-QCM), gas chromatography-triple quadrupole mass spectrometry (GC-MS/MS), electrochemical sensors, mercury analyzers, fluorescence, atomic absorption spectroscopy and atomic fluorescence spectroscopy. However, most of these methods come up against the high cost and the need for a highly qualified technical team for their execution, which reduces the possibility of applying them in the routine analysis of small and medium water quality assessment centers (Saleh *et al.* 2020). An alternative in this case would be the use of spectrophotometric methods such as ultraviolet-visible (UV-VIS) spectrophotometry, which is a well-established technique with a low cost of acquisition and maintenance and it is accessible to routine laboratories (Vieira *et al.* 2014).

The aquatic environment is one of the largest natural reservoirs of microrganisms, with a great diversity of bacterial species from the soil and plants. In this environment, the genetic exchange is favored, with intense transference of several genes, including resistance genes (Proia *et al.* 2016). Bacterial resistance may be related to the accumulation of antimicrobial residues or even some heavy metals or other chemical components. These chemical agents exert a selective pressure on the environment by remodeling the microflora and selecting more and more resistant bacteria (Hirsch *et al.* 1999; Kemper

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2008). For instance, heavy-metal ion resistance genes (for example, against mercury, cadmium, and silver) have been found together with antimicrobial resistance determinants (Nakahara *et al.* 1977; Wireman *et al.* 1997; Skurnik *et al.* 2010). Thus, it is suggested that the environmental load of mercury may promote and maintain antimicrobial resistance together with mercury resistance in several environments. However, most studies have established this relationship using clinical isolates (Nakahara *et al.* 1977; Skurnik *et al.* 2010), which are generally associated with exposure of mercury to personal care products (i.e., dental amalgam fillings). Thus, little is known about the impact of mercury on the selection of bacterial resistance in other environments, especially in river waters (Rahman & Singh 2018).

Thus, considering the presence of mercury in river waters and the challenges related to the methods used in its determination, this study had the objectives of developing and validating a cheaper and accessibly UV-VIS method to measure this micropollutant in river water. In addition, we aim to correlate the mercury concentrations reported in water river with the bacterial diversity and susceptibility to clinically relevant antimicrobials in these environments.

2. MATERIALS AND METHODS

2.1. Sample collection

Three samples of water (1.5 L each) from the Pará River (Minas Gerais, Brazil) were collected. One near to source of the river in Resende Costa city (Rp_1) (Latitude: 20° 55 ‘20 ‘S Longitude: 44° 14′ 15″ W) and others at 90 km and 180 km away from the source, respectively, in the cities of Passa Tempo (Rp_2) (Latitude: 20° 39 ‘02 ‘S, Longitude: 44° 29′ 44″ W) and Divinópolis (Rp_3) (Latitude: 20° 08 ‘20 ‘S, Longitude : 44.53. 02 W). All samples were placed into sterilized polypropylene bottles and taken to the laboratory by refrigerated transport. For determination of mercury concentration, an aliquot of 500 mL of each sample was acidified with HNO_3 at pH 2.0 and stored in an amber flask at 2 to 8 °C. Samples were processed at the Laboratório de Diagnóstico Laboratorial e Microbiologia Clínica at the Universidade Federal de São João del-Rei (Divinópolis-MG/Brazil).

2.2. Determination of mercury

2.2.1. Spectrophotometric method

The method developed for mercury determination by UV-VIS spectrophotometry consisted of the addition of 3.0 mL of buffer pH 9.0 (H_3BO_3/KCl), deionized water to 9.9 mL and 100 μL of the complexant 2-(5-Bromo-2-pyridylazo)-5-(diethylamino) phenol (Br-PADAP) 4.98×10^{-4} mol L^{-1}.

In order to optimize the experimental conditions of the method, a plan was carried out to evaluate the following variables: complexing agent (Methylene blue, Calcon, Thymol blue, Methyl orange, Erlym black, and Br-PADAP, all being prepared at a concentration of 2.49×10–5 mol L^{-1} and diluted in water), medium pH (2.0–12.0) and Hg^{2+}/Br-PADAP (1: 1–4: 1) stoichiometry.

The absorbance at 566 nm, corresponding to maximum of absorption, was measured through a UV-VIS spectrophotometer (Thermo Scientific, Genesys 10S). This methodology was validated according to the Eurachem Guide (Magnusson & Örnemark 2014) and the *Instituto Nacional de Normalização da Metrologia e Qualidade da Indústria* (INMETRO) Guide of 2011.

2.2.2. Method validation

To evaluate the linearity, analytical curves were prepared with mercury concentrations in the range from 0.1 to 1.0 μg·mL^{-1}, using a mercury standard solution. Each level was prepared in triplicate, independently and the absorbance were determined randomly. The Ordinary Least Squares Regression Method (OLSM) was applied in order to estimate the linear regression equation.

The limit of detection (LOD) and the limit of quantification (LOQ) were estimated according to the following equations (Oliveira e Silva *et al.* 2018):

$$LOD = X + t \times s \quad (1)$$

$$LOQ = X + 10 \times s \quad (2)$$

were X=average of 10 analytical blank concentrations, s=averaged standard deviation of the concentrations, and *t*=*t* student

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($t_{(0,\ 05;\ n\text{-}1)}$). The LOQ was confirmed experimentally. The results were evaluated considering the recovery and coefficient of variation (% CV).

To evaluate the precision and accuracy, analytical blank solutions were prepared and fortified with standard solution of Hg^{2+} at the concentration levels of 0.30, 0.50 and 0.70 μg mL^{-1}. Each concentration was prepared in seven replicates. The intermediate precision was assessed according to the % CV and accuracy was evaluated by addition and recovery methods.

2.3. Microbiological study

2.3.1. Cultures and bacterial isolation

For bacterial isolation, 100 μL of each raw water sample and after serial dilution were inoculated into chromogenic agar (Renylab, Brazil), in duplicate, and incubated at 37 °C for 24 to 48 h. The colonies were identified in the chromogenic medium according to the manufacturer's instructions and additionally they were submitted to Gram staining and classic biochemical-physiological tests. In order to confirm the genus and/or species of the Gram negative bacteria, the modified Rugai test was performed, which evaluates biochemical parameters such as glucose fermentation, sucrose fermentation, motility, citrate utilization tests, lysine decarboxylation, hydrogen sulphide (H_2S), indol production, phenylalaninadesaminase and urease production. For the identification of Gram positive bacteria, catalase production, growth in BHI medium with 6.5% NaCl and coagulase production were investigated.

Subsequently, the isolates were repeatedly streaked onto the nutrient agar (Isofar, Brazil) to check their purity and to confirm the production of specific pigments. Furthermore, production of the enzyme cytochrome oxidase and growth capacity at 42 °C were verified to confirm the Gram-negative species identification. The isolates were stored in nutrient broth (Isofar, Brazil) with 25% glycerol at –80 °C until further use.

2.3.2. Antibiotic susceptibility test and multidrug resistance (MDR) classification

The susceptibility profile to ciprofloxacin, tetracycline, kanamycin and ampicillin (Sigma–Aldrich, USA) of the bacterial isolates was determined by the agar dilution technique according to the Clinical Laboratory Standards Institute (CLSI 2018).

In addition, all *Escherichia coli* isolates were tested for beta-lactam antimicrobial susceptibility (amoxicillin/clavulanic acid (AMC), aztreonan (ATM), ceftazidime (CAZ), cefotaxime (CTX), ceftriaxone (CRO), cefoxitin (CFO), imipenem (IPM) and meropenem (MEM) (CECON®) using the standard disc diffusion method according to the same guideline.

The isolates were classified as MDR when they were resistant to at least one antimicrobial in each of three different classes (Magiorakos *et al.* 2012). The strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 27853 were used as controls of the experiments.

2.3.3. Determination of ESBL, KPC and AmpC production by *E. coli* isolates

E. coli isolates were submitted to a phenotypic test to determine extended spectrum beta-lactamase enzyme (ESBL) production, using the antimicrobial substrates ceftazidime, aztreonan, ceftriaxone and cefotaxime (CECON®). In addition, isolates resistant or with reduced susceptibility to imipenem or meropenem and/or ESBL-positive were subjected to the Hodge test for investigation of *Klebsiella pneumoniae* carbapenemase (KPC) in according to the CLSI instructions (2018).

The production of the enzyme AmpC was performed according to Elsayed *et al.* (2015), using the disc test approach with imipenem, cefoxitin and amoxicillin/clavulanic acid (CECON®) as inducers and ceftazidime (CECON®) as substrate.

3. RESULTS AND DISCUSSION

Mercury (Hg), in high concentrations in aquatic environments such as rivers, poses a risk to public health (Hölzel *et al.* 2012; Devarajan *et al.* 2015). As previously reported, this event also impacts the composition of the aquatic microbiota, favoring the development and maintenance of antimicrobial resistance in potentially pathogenic bacteria for humans (Nakahara *et al.* 1977; Wireman *et al.* 1997; Skurnik *et al.* 2010). The riverside population counts on water from the rivers for their routine activities, both financially and in terms of food. Despite this, the monitoring of Hg concentrations in water is hampered by the unavailability of cost-effective dosage methods accessible to municipalities. Furthermore, in smaller municipalities, knowledge of the risk posed by aquatic environments impacted by Hg regarding the presence of resistant bacteria is scarce and, therefore, protection strategies are non-existent (Rahman & Singh 2018). Thus, the results of this study may fill part of this gap, highlighting the need to implement public health policies.

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3.1. Standardization and validation of method for mercury quantification using UV-VIS

Determination of mercury by spectrophotometry requires the formation of an absorbing complex in the ultraviolet/visible region. For this purpose, different complexants were evaluated at different pH values. Figure 1 shows the spectra of the complexants evaluated at the pH that provided the best absorbance.

It can be seen in Figure 1(a) and 1(b) that the complex formed between the species Hg(II) and the complexant Br-PADAP at pH 9.0 showed the highest absorbance and, consequently, greater analytical sensitivity. When checking the stoichiometry between the Hg(II) ions and the complexant at pH 9.0, it was observed that the 1:2 stoichiometry (i.e., which leads to the formation of the Hg^{2+}-(5-Br-PADAP)$_2$ complex) presents the best analytical responses (Figura 1(c)). In this sense, it is concluded that the best condition for the spectrophotometric determination of Hg(II) consists of the formation of a complex with 1:2 stoichiometry, using 5-Br-PADAP at pH 9.0. Under these conditions, the complex formed has a maximum absorption at 566 nm and a molar absorptivity of 8.8755×10^2 L mol^{-1} cm^{-1}.

Subsequently, the analytical conditions defined earlier were validated according to the Eurachem (Magnusson & Örnemark 2014) and INMETRO (2011) guides. The first parameter evaluated was linearity as showed by the graphs of residues (regression residues versus Hg^{2+} concentration levels) (Figure 2(a)). Analytical curves were prepared, independently, with an Hg^{2+} standard solution in concentration levels of 0.1; 0.2; 0.4; 0.6; 0.8 and 1.0 μg mL^{-1}. Each level was prepared in triplicate and the readings of solutions were analyzed randomly. After that, the Ordinary Least Squares Regression Method (OLSM) was applied in order to estimate the linear regression equation. Dotted lines on graphs of residues correspond to $\pm t_{(1-\alpha/2;n-2)}S_{res}$, which is the acceptable variation range for regression residues (Oliveira e Silva *et al.* 2018). After applying the Jacknife test and examining the residual plot, we observed the presence of two values outside the range $\pm t_{(1-\alpha/2;n-2)}S_{res}$: one in 0.1 μg mL^{-1} and the other in 0.4 μg mL^{-1}. These values are called outliers and were removed from the data set, respecting a limit of 22.4% (Souza & Junqueira 2005), and did not influence the regression, making the equation representative (Figure 2(d)).

Then, the following assumptions required by OLSM were evaluated: normality, independence and homoscedasticity of the variances of the residues. The QQ plots and the respective Ryan–Joiner correlation coefficients are illustrated in Figure 2. According to the Ryan-Joiner test, there is a significant correlation between the two components, one $R_{eq}=0.9584>R_{crit}=0.9411$, which indicates that there is no normality deviation for $\alpha=0.10$ (Figure 2(b)). According to Durbin-Watson statistics, regression residues presented autocorrelation ($D=1.61>dU=1.37$), which indicates residues' dependence. This characteristic is demonstrated through the random distribution of residues on the residuals autocorrelation graph (Figure 2(c)). Homoscedasticity, in turn, was evaluated by the modification proposed by Brown and Forsythe for the Levene test. In this test $t_L=22{,}67>T_{crit}=1.96\times10^{-12}$, to a 95% confidence level. This homoscedastic behavior is also present on residues graphs (Figure 2(a)), where it can be observed a random distribution of the residues. After verifying the premises of OLSM, the following regression equation was retrieved: Abs=0.3961 [Hg^{2+}]–0.0056 ($R^2=0.9994$). The regression was substantial for $p<0.05$ ($F=2.46\times10^4>F_{crit}=4.6$). In this sense, the method linearity was from 0.1 to 1.0 μg mL^{-1}.

Detection limits (LOD) and quantification limits (LOQ) were estimated according to the Equations (1) and (2), respectively (see in Material and Methods). In these equations, X was considered equal to 0, avoiding the impossibility of using a water sample without Hg^{2+} as blank. The theoretical values of detection and quantification limits were, respectively, 0.02 μg mL^{-1} and 0.1 μg mL^{-1}. The quantification limits were experimentally checked from seven replicates of the concentration level

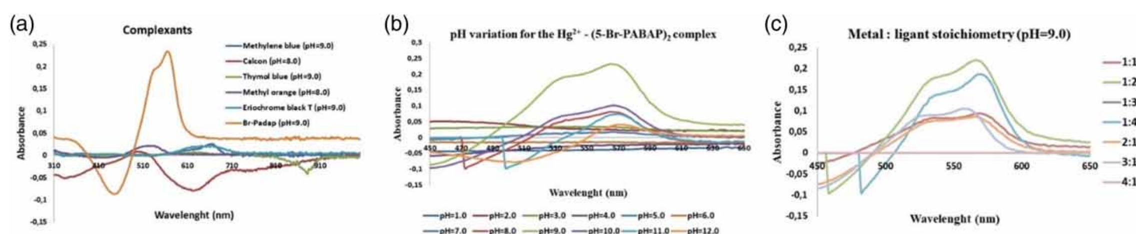

Figure 1 | UV-VIS spectra of the Hg^{2+} complex (0.5 μg mL^{-1}) formed from different complexants (2.49×10^{-5} mol L^{-1}) (a) Effect of pH on the absorptivity of the Hg^{2+}-5-Br-PADAP complex (2.49×10^{-5} mol L^{-1}) in UV-VIS (b) Influence of stoichiometry on the absorptivity of the Hg^{2+}-5-Br-PADAP complex in UV-VIS (2.49×10^{-5} mol L^{-1}).

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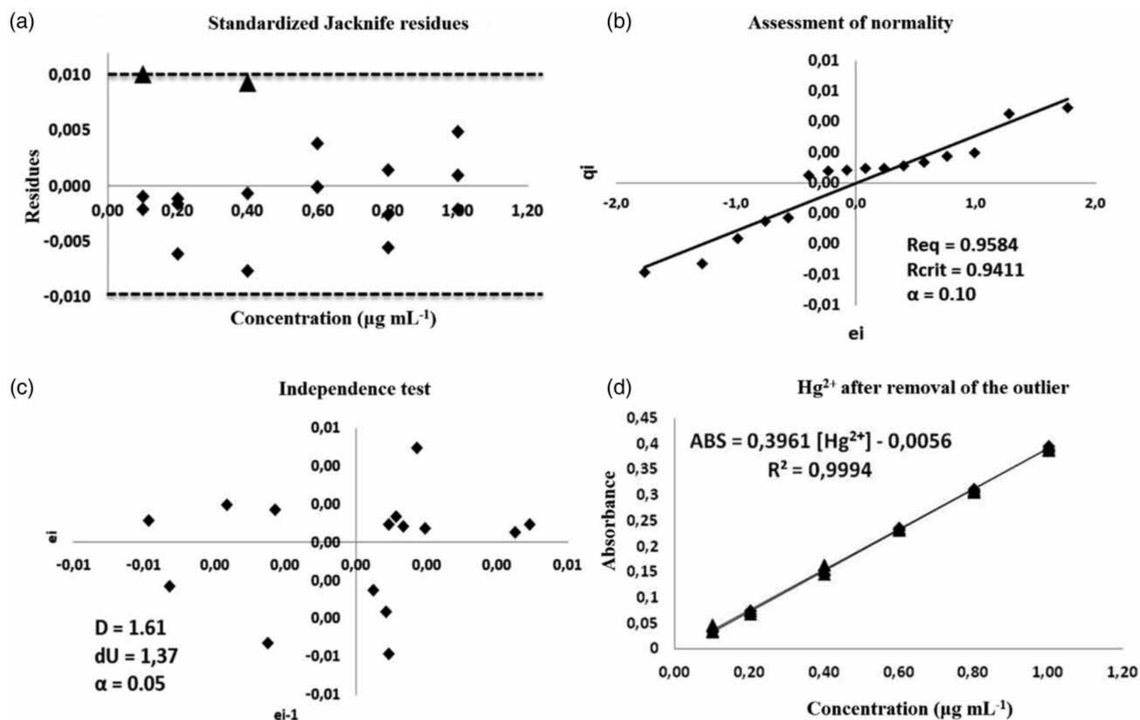

Figure 2 | Parameters used to measure the concentration of mercury in water using UV-VIS spectrophotometry. Residual plots for outlier diagnose by Jacknife standardised residuals test (a). Normal QQ plots of residuals (b). Plots of residuals autocorrelation (c). Analytical curve to mercury determination after outlier removal (d). Dashed lines are $\pm t_{(0.975;n-2)}S_{res}$. ei=residual, R=correlation coefficient of Ryan–Joiner test, d=Durbin–Watson statistic.

0.1 µg mL^{-1}. The limit of quantification was verified experimentally from seven repetitions of the concentration level 0.1. So, the LOQ obtained from this experiment was 0.09. ±0.01 µg mL^{-1} (n=7).

Finally, the precision was evaluated at three concentration levels and it was expressed in terms of repeatability and intermediate precision (Table 1). The variation coefficients were lower than 15%, indicating a good precision of the developed method. In turn, the recoveries varied from 95% to 118%, in the evaluated levels, except at level 0.5 µg mL^{-1} assessed on the third day. Even so, inter-day accuracy showed a recovery of indicating that the method has good accuracy.

According to conditions obtained in this work, it can be said that the analytical sensitivity of the method developed was comparable to the method developed by Al-Bagawi *et al.* (2017), who used the complexing agent 4-(2-thiazolylazo) resorcinol (TAR) and corrected the absorbance complexing using β-correction technique. The detection and quantification limits obtained by these authors were, respectively, 0.024 µg mL^{-1} and 0.081 µg mL^{-1}. It should also be considered that the LOD and LOQ obtained by the authors without the correction for absorbance were 0.12 µg mL^{-1} and 0.42 µg mL^{-1}, respectively.

Table 1 | Repeatability and intermediate precision for mercury determination (n=7)

Levels	0.3 µg mL^{-1}	0.5 µg mL^{-1}	0.7 µg mL^{-1}
Day 1	0.30 ± 0.04 µg mL^{-1} (R=101.4% ; %CV=11.9)	0.51 ± 0.07 µg mL^{-1} (R=102.6% ; %CV=14.2)	0.67 ± 0.06 µg mL^{-1} (R=95.4% ; %CV=9.7)
Day 2	0.32 ± 0.02 µg mL^{-1} (R=105.6% ; %CV=5.0)	0.57 ± 0.08 µg mL^{-1} (R=113.1% ; %CV=13.5)	0.68 ± 0.03 µg mL^{-1} (R=97.1% ; %CV=4.2)
Day 3	0.35 ± 0.01 µg mL^{-1} (R=117.7% ; %CV=3.8)	0.62 ± 0.07 µg mL^{-1} (R=124.3% ; %CV=10.5)	0.72 ± 0.02 µg mL^{-1} (R=103.1% ; %CV=3.1)
Inter-day	0.32 ± 0.03 µg mL^{-1} (R=108.1% ; %CV=9.5)	0.57 ± 0.08 µg mL^{-1} (R=113.3% ; %CV=14.4)	0.69 ± 0.05 µg mL^{-1} (R=98.5% ; %CV=6.8)

R=recovery ; % CV=coefficient of variation.

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3.2. Mercury concentration in river water samples by UV-VIS

Using the method described above, developed and validated through the UV-VIS spectrophotometry technique, mercury concentrations were determined in three river water samples. Considering that the concentration of mercury in river water should be limited up to 0.002 μg mL^{-1} according to Brazilian legislation (CONAMA 2005), at the three sampled points of the Pará River, this limit was exceeded (Table 2).

It is important to note that high concentrations of mercury in waters, sediments and soils, have been frequently related (Alexandre 2006; Tinôco *et al.* 2010). Similar to our findings, a high concentration of mercury (0.021 μg·mL^{-1}) also detected in water from the Ribeirão do Grama-MG basin (Tinôco *et al.* 2010). Possibly anthropic impacts such as mining and agricultural activities with devastation of the riparian forest may be related to the high concentrations of these metals in this environment (Tinôco *et al.* 2010).

The finding of high mercury concentration in the water sample near the source (Rp_1) is stand out. Lin *et al.* (2014) reported that this metal can be transported through the atmosphere, considering its low boiling point, and can be transported and deposited away from its source of origin, and even incorporated into the food chain via methylation. Also, the highest concentration of mercury in Rp_2 compared to Rp_3 should be mentioned, since the latter seems to be more impacted by anthropic activities. However, in Rp_3 it was observed that in most of the surface of the river there were water hyacinths (*Eichhornia crassipes* Mart. (Solms)). Several studies point out that this plant species is tolerant to inhospitable pollution conditions (Caldelas *et al.* 2009) and are able to accumulate nutrients and heavy metals, including mercury (Cordes *et al.* 2000; Jayaweera & Kasturiarachchi 2004; Gardea-Torresdey *et al.* 2005). Thus, this could justify our finding besides corroborating the one suggested by Jayaweera & Kasturiarachchi (2004), that this plant is promising in the bioremediation of mercury present in eutrophic waters.

3.3. Microbial diversity of samples from Rio Pará waters

A total of 69 bacterial colonies were isolated, from which 88.4% (61/69) were Gram-negative and 11.6% (8/69) were Gram-positive (Table 3). In fact, as reviewed by Araujo & Nascimento (2014), in aquatic environments of Brazil the greatest occurrence is of Gram-negative bacteria, possibly related to human interference at these sites.

Of the recovered isolates, most were identified at point 2 (90 km from the source – 38/69; 55.1%), followed by point 1 (near to the source – 17/69; 24.6%) and point 3 (180 km from the source – 14/69; 20.3%). The Gram-positive bacteria (7/8; 87.5%) were mainly recovered from samples from point 1 (Rp_1), near the source and considered the least impacted by anthropic activities. According to Oliveira *et al.* (2012), Gram-positive bacteria are best adapted to environments with low levels of organic carbon dissolved in water, which is considered a marker of anthropic pollution.

Among the Gram-positive bacteria, one isolate of the genus *Streptococcus* (1.5%) and seven of the *Staphylococcus* (10.1%) were recovered, which were not identified at the species level due to methodological limitation (Table 3). These genera are commonly found in the aquatic environment, especially of higher temperature, besides being part of the microbiota of some fish (Salvador *et al.* 2003). Hewson & Fuhrman (2006) reported the finding of *Staphylococcus* spp. in beach water and associated its presence with human microbiota and osmotic pressure due to the presence of an average of 3% NaCl, considering that Gram-positive bacteria are more adapted in hypertonic environments. On the other hand, *Streptococcus* spp. can be found in several environments as it is present in the animal's microbiota (Niewolak 1999; Kabelitz *et al.* 2021). However, the absence of these bacteria in point Rp_3 may be related to the higher level of pollution of the river, and possibly with the greater dissolved carbon concentration, in addition to the greater volume of water at this point at the river, compromising the bacterial isolation.

Table 2 | Concentration of mercury in water samples (μg mL^{-1}) in the water samples of the Pará River

Collection points	Concentration of mercury (μg mL^{-1})
Rp_1	0,13 ± 0,01
Rp_2	0,35 ± 0,01
Rp_3	0,22 ± 0,01

Rp_1: near the source of the river Para (Resende Costa city); Rp_2: 90 km distance from the source (Passa Tempo city); Rp_3: 180 km distance from the source (Divinópolis city).

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Gram-negative bacteria, notably Enterobacteriales such as *Escherichia coli* (33/69; 47.8%), *Enterobacter* sp. (21/69; 30.4%) and *Klebsiella pneumoniae* (4/69; 5.8%) were the most recovered (Table 3). Several studies have related the amount of dissolved organic carbon in water with a greater adaptation of Enterobacteriales in the aquatic environment (Judd *et al.* 2006; Lemke *et al.* 2009; Oliveira *et al.* 2012). Here, *Pseudomonas aeruginosa* (2/69; 2.9%) and *Chromobacterium violaceum* (1/69; 1.5%) were isolated at two collection points. Corroborating with our found, Pontes *et al.* (2009) reported that non-fermenting glucose bacteria of the genera *Pseudomonas*, *Acinetobacter*, and *Stenotrophomonas* were also recovered from Rio Doce – Minas Gerais river basin. *C. violaceum*, in turn, a Gram-negative cocobacillus bacterium that can be found in aquatic environments not impacted in tropical regions, but which may be associated with opportunistic infections in humans such as septicemia, skin lesions and abscesses (Araujo & Nascimento 2013), was isolated only in spring water.

Table 3 | Microbial diversity of water samples recovered from the Pará River and minimum inhibitory concentration (MIC) to some antimicrobials for these isolates

			Minimum Inhibitory Concentration (μg mL^{-1})			
ID	**Identification**	**Collect point**	**TET**	**AMP**	**CIP**	**KAN**
P1_1	*Staphylococcus* sp	RP_1	8 (I)	< 4 (R)	1 (S)	< 8 (S)
P1_2	*Staphylococcus* sp	RP_1	8 (I)	> 64 (R)	< 0,5 (S)	< 8 (S)
P1_3	*Staphylococcus* sp	RP_1	> 32 (R)	16 (R)	< 0,5 (S)	< 8 (S)
P1_4	*Staphylococcus* sp	RP_1	> 32 (R)	> 64 (R)	0,5 (S)	< 8 (S)
P1_5	*Streptococcus* sp	RP_1	> 32 (R)	4 (I)	1 (ND)	< 8 (ND)
P1_6	*Streptococcus* sp	RP_1	> 32 (R)	8 (R)	< 0,5 (ND)	< 8 (ND)
P1_7	*Streptococcus* sp	RP_1	> 32 (R)	16 (R)	1 (ND)	< 8 (ND)
P1_8	*Enterobacter sp*	RP_1	8 (I)	> 64 (R)	1 (S)	< 8 (S)
P1_9	*Klebsiella pneumoniae*	RP_1	> 32 (R)	IR	< 0,5 (S)	< 8 (S)
P1_10	*Klebsiella pneumoniae*	RP_1	> 32 (R)	IR	0,5 (S)	< 8 (S)
P1_11	*Enterobacter sp*	RP_1	> 32 (R)	16 (I)	0,5 (S)	< 8 (S)
P1_12	*Enterobacter sp*	RP_1	8 (I)	> 64 (R)	< 0,5 (S)	< 8 (S)
P1_13	*Enterobacter sp*	RP_1	> 32 (R)	8 (S)	0,5 (S)	8 (S)
P1_14	*Enterobacter sp*	RP_1	> 32 (R)	16 (I)	< 0,5 (S)	< 8 (S)
P1_15	*Chromobacterium violaceum*	RP_1	> 32 (ND)	> 64 (ND)	1 (ND)	8 (ND)
P1_16	*Escherichia coli*	RP_1	>32 (R)	> 64 (R)	< 0,5 (S)	< 8 (S)
P1_17	*Escherichia coli*	RP_1	16 (R)	64 (R)	< 0,5 (S)	< 8 (S)
P2_1	*Streptococcus* sp	RP_2	16 (R)	4 (I)	< 0,5 (ND)	< 8 (ND)
P2_2	*Enterobacter sp*	RP_2	> 32 (R)	> 64 (R)	1 (S)	< 8 (S)
P2_3	*Enterobacter sp*	RP_2	> 32 (R)	> 64 (R)	1 (S)	< 8 (S)
P2_4	*Enterobacter sp*	RP_2	> 32 (R)	> 64 (R)	0,5 (S)	8 (S)
P2_5	*Enterobacter sp*	RP_2	8 (I)	> 64 (R)	< 0,5 (S)	< 8 (S)
P2_6	*Enterobacter sp*	RP_2	> 32 (R)	> 64 (R)	1 (S)	< 8 (S)
P2_7	*Enterobacter sp*	RP_2	> 32 (R)	> 64 (R)	0,5 (S)	< 8 (S)
P2_8	*Enterobacter sp*	RP_2	8 (I)	> 64 (R)	0,5 (S)	< 8 (S)
P2_9	*Enterobacter sp*	RP_2	8 (I)	> 64 (R)	1 (S)	< 8 (S)
P2_10	*Enterobacter sp*	RP_2	> 32 (R)	> 64 (R)	1 (S)	< 8 (S)
P2_11	*Enterobacter sp*	RP_2	> 32 (R)	> 64 (R)	< 0,5 (S)	8 (S)
P2_12	*Enterobacter sp*	RP_2	> 32 (R)	> 64 (R)	1 (S)	< 8 (S)
P2_13	*Enterobacter sp*	RP_2	8 (I)	> 64 (R)	< 0,5 (S)	8 (S)
P2_14	*Enterobacter sp*	RP_2	> 32 (R)	> 64 (R)	< 0,5 (S)	8 (S)

(*Continued.*)

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Table 3 | Continued

			Minimum Inhibitory Concentration (μg mL^{-1})			
ID	**Identification**	**Collect point**	**TET**	**AMP**	**CIP**	**KAN**
P2_15	*Klebsiella pneumoniae*	RP_2	4 (S)	IR	< 0,5 (S)	< 8 (S)
P2_16	*Klebsiella pneumoniae*	RP_2	> 32 (R)	IR	< 0,5 (S)	< 8 (S)
P2_17	*Pseudomonas aeruginosa*	RP_2	> 32 (ND)	>64 (ND)	0,5 (S)	8 (ND)
P2_18	*Pseudomonas aeruginosa*	RP_2	> 32 (ND)	>64 (ND)	< 0,5 (S)	8 (ND)
P2_19	*Escherichia coli*	RP_2	32 (R)	8 (S)	< 0,5 (S)	< 8 (S)
P2_20	*Escherichia coli*	RP_2	< 2 (S)	> 64 (R)	< 0,5 (S)	16 (S)
P2_21	*Escherichia coli*	RP_2	< 2 (S)	8 (S)	< 0,5 (S)	< 8 (S)
P2_22	*Escherichia coli*	RP_2	< 2 (S)	64 (R)	< 0,5 (S)	< 8 (S)
P2_23	*Escherichia coli*	RP_2	16 (R)	> 64 (R)	< 0,5 (S)	< 8 (S)
P2_24	*Escherichia coli*	RP_2	>32 (R)	> 64 (R)	1 (S)	< 8 (S)
P2_25	*Escherichia coli*	RP_2	>32 (R)	32 (R)	1 (S)	< 8 (S)
P2_26	*Escherichia coli*	RP_2	16 (R)	> 64 (R)	< 0,5 (S)	16 (S)
P2_27	*Escherichia coli*	RP_2	16 (R)	64 (R)	< 0,5 (S)	< 8 (S)
P2_28	*Escherichia coli*	RP_2	16 (R)	> 64 (R)	< 0,5 (S)	16 (S)
P2_29	*Escherichia coli*	RP_2	>32 (R)	< 4 (S)	< 0,5 (S)	16 (S)
P2_30	*Escherichia coli*	RP_2	>32 (R)	> 64 (R)	< 0,5 (S)	16 (S)
P2_31	*Escherichia coli*	RP_2	>32 (R)	> 64 (R)	< 0,5 (S)	16 (S)
P2_32	*Escherichia coli*	RP_2	>32 (R)	32 (R)	1 (S)	32 (I)
P2_33	*Escherichia coli*	RP_2	>32 (R)	> 64 (R)	8 (R)	32 (I)
P2_34	*Escherichia coli*	RP_2	>32 (R)	> 64 (R)	< 0,5 (S)	16 (S)
P2_35	*Escherichia coli*	RP_2	16 (R)	16 (I)	< 0,5 (S)	16 (S)
P2_36	*Escherichia coli*	RP_2	< 2 (S)	> 64 (R)	< 0,5 (S)	< 8 (S)
P2_37	*Escherichia coli*	RP_2	< 2 (S)	64 (R)	< 0,5 (S)	16 (S)
P2_38	*Escherichia coli*	RP_2	>32 (R)	>32 (R)	2 (I)	< 8 (S)
P3_1	*Enterobacter sp*	RP_3	> 32 (R)	32 (R)	< 0,5 (S)	< 8 (S)
P3_2	*Enterobacter sp*	RP_3	> 32 (R)	> 64 (R)	0,5 (S)	< 8 (S)
P3_3	*Enterobacter sp*	RP_3	> 32 (R)	> 64 (R)	0,5 (S)	8 (S)
P3_4	*Escherichia coli*	RP_3	4 (S)	< 4 (S)	< 0,5 (S)	< 8 (S)
P3_5	*Escherichia coli*	RP_3	16 (R)	64 (R)	< 0,5 (S)	< 8 (S)
P3_6	*Escherichia coli*	RP_3	>32 (R)	> 64 (R)	< 0,5 (S)	< 8 (S)
P3_7	*Escherichia coli*	RP_3	16 (R)	> 64 (R)	1 (S)	64 (R)
P3_8	*Escherichia coli*	RP_3	16 (R)	> 64 (R)	1 (S)	16 (S)
P3_9	*Escherichia coli*	RP_3	32 (R)	> 64 (R)	2 (I)	16 (S)
P3_10	*Escherichia coli*	RP_3	32 (R)	> 64 (R)	1 (S)	16 (S)
P3_11	*Escherichia coli*	RP_3	32 (R)	> 64 (R)	1 (S)	16 (S)
P3_12	*Escherichia coli*	RP_3	32 (R)	> 64 (R)	< 0,5 (S)	16 (S)
P3_13	*Escherichia coli*	RP_3	16 (R)	> 64 (R)	< 0,5 (S)	16 (S)
P3_14	*Escherichia coli*	RP_3	>32 (R)	32 (R)	2 (I)	< 8 (S)

Breakpoints (μg/mL) according to Clinical Laboratory Standards Institute (CLSI 2018): Enterobacteriales : TET-Tetracycline: S (≤4), I(8) R (≥16); AMP-Ampicillin: S(≤8) I(16) R(≥32); CIP-Ciprofloxacin S (≤1), I(2) (R)(≥4); KAN- Kanamicin: S (≤16), I(32) R (≥64). *Pseudomonas aeruginosa*: CIP-Ciprofloxacin S (≤1), I(2) (R)(≥4). *Staphylococcus* spp.: TET-Tetracycline: S (≤4), I(8) R (≥16), CIP-Ciprofloxacin S (≤1), I(2) (R)(≥4). *Streptococcus* spp.: TET-Tetracycline: S (≤2), I(4) R (≥8), AMP-Ampicillin: S (≤0, 25). IR-Intrinsic resistance to ampicillin, S- susceptible, R-resistant, I-intermediate, ND- not determined.

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3.4. Resistance profile

The antimicrobial susceptibility profile of *Staphylococcus* sp. and *Streptococcus* sp. is shown in Table 3 and the interpretation of the results was performed according to the cut-off points established by CLSI (2018). All *Staphylococcus* spp. were sensitive to ciprofloxacin, while low MICs were observed for *Streptococcus* isolates. Akanbi *et al.* (2017) reported resistance to ciprofloxacin in *S. aureus*, but when recovered from seawater. However, tetracycline resistance was observed in 75% (6/8) of all the recovered Gram-positive bacteria, with MICs up to $>32\ \mu g\ mL^{-1}$. Tetracycline resistance in *Staphylococcus* sp. of natural waters has also been described. It should be emphasized that studies indicate that most of the aquatic bacteria may be related to the microbiota of fish and that, like soil bacteria, harbor tetracycline resistance genes (Lima *et al.* 2006; Akinbowale *et al.* 2007).

In general, high rates of resistance were observed for tetracycline (71.4%) and ampicillin (76.2%) between *E. coli* and *Enterobacter* sp., with MIC up to $>32\ \mu g\ mL^{-1}$ and $>64\ \mu g\ mL^{-1}$, respectively. Furthermore, differences among the antimicrobial susceptibility in Enterobacteriales species recovered from the three collection points of the river were not observed, suggesting that, at least for the antimicrobials tested, the anthropic activities appear to have no impact on bacterial resistance (Table 3). Lower rates of resistance to these antimicrobials (55% to ampicillin and 58% to tetracycline) were reported by Tao *et al.* (2010), who studied Enterobacteriales recovered from China's rivers. In fact, greater resistance to tetracycline has been found in aquatic bacteria (Akinbowale *et al.* 2007; Araujo & Nascimento 2014) since they can harbor the *tet* genes, which encode an efflux pump and can be transferred interspecies via mobile genetic elements (Tao *et al.* 2010). Finally, the high rates of resistance to tetracycline observed at all points of collection may be associated with the selective pressure exerted by this antimicrobial, which is widely used in veterinary medicine for therapy of infections caused by Gram-negative bacteria (Webster *et al.* 2004).

Regarding to ampicillin, Araujo & Nascimento (2014) reported high resistance of Enterobacteriales isolated from aquatic environments in Brazil, and Lima-Bittencourt *et al.* (2007) highlighted the resistance of *Enterobacter* isolates (100%) in a study in Serra do Cipó, Minas Gerais, Brazil. In contrast, Parveen *et al.* (2005) and Schneider *et al.* (2009) reported lower rates of resistance to ampicillin (about 37%) in groundwater and surface waters, respectively, in USA and Brazil. The greatest impact of our findings is that it points to the presence of ampicillin resistance mechanisms circulating in bacterial isolates, including the production of beta-lactamase enzymes, which have potential for dissemination and the possibility of conferring cross-resistance to other beta-lactams (Chen *et al.* 2013). It should be noted that *Klebsiella* spp. present intrinsic resistance to ampicillin (Holt *et al.* 2015) and thus *K. pneumoniae* isolates were not tested for MIC for this antimicrobial.

Considering the high resistance rate to ampicillin (84.8%) and the predominance of *E. coli* among the recovered isolates (47.8%), the susceptibility profile to others beta-lactams, as well beta-lactamases production, were investigated between this Enterobacteriales and are shown in Table 4. The susceptibility rate for all beta-lactams tested other than ampicillin among *E. coli* isolates was 45.5% (15/33), and among them, four showed susceptibility also to ampicillin (P2_19, 21, 29 and P3_4). Among cephalosporins, 24.2% (8/33) of *E. coli* isolates were cefoxitin resistant, markedly at the collection point 3, but most isolates were susceptible to ceftriaxone, ceftazidime and cefotaxime. Resistance (12.1%, 4/33) to aztreonam and amoxacillin-clavulanic acid was also observed among the *E. coli* isolates. Importantly, a higher profile of decreased/intermediate sensitivity (24.2%, 8/33) was observed for amoxicillin/clavulanic acid. None of the isolates was positive for the production of ESBL, AmpC, and KPC according to CLSI phenotypic tests.

On the other hand, susceptibility to ciprofloxacin and kanamycin was markedly high in these Enterobacteriales species, at all points of collection, in agreement with the findings reported by Lima-Bittencourt *et al.* (2007) and Tao *et al.* (2010). Similar results for ciprofloxacin were found in *E. coli* isolates from Rio Athi-Kenya (Wambugu *et al.* 2015), and from Lajeado-Suruví-SC waters (Schneider *et al.* 2009), with rates of resistance of 6.9% and 1.2% respectively. Interestingly, in this study the overall rate of resistance to ciprofloxacin was very low, although active residues of this compound remain in aquatic environments and exert selective pressure on the bacterial community (Devarajan *et al.* 2015). In addition, the kanamycin resistant *E. coli* isolate from the Rp_3 site exhibited a MIC of $64\ \mu g\ mL^{-1}$. Studies in the United States and Australia (Boon & Cattanach 1999; Webster *et al.* 2004) showed low levels of aminoglycoside resistance (<10%), including kanamycin, in bacteria isolated from rural and urban surface water. Possibly, these data reflect the low frequency of veterinary and human use of this antimicrobial (Tzoc *et al.* 2004).

Among other Gram-negative bacteria species, *P. aeruginosa* isolates, recovered only in Rp_2, were susceptible to ciprofloxacin, as observed by Oliveira *et al.* (2017) in species of a sewage treatment plant of the same region studied. This fact, in

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Table 4 | Resistance profile of *Escherichia coli* isolated from water samples from the Pará river to beta-lactam antibiotics using the disk-diffusion method

Collect point	*E. coli* ID	Susceptibility profile							
		CFO	CTX	CAZ	CRO	AMC	ATM	IMP	MEM
Rp_1	P1_16	S	R	S	S	S	R	S	S
	P1_17	S	S	S	S	R	S	R	I
Rp_2	P2_19; P2_20 P2_21; P2_24 P2_25; P2_26 P2_27; P2_28 P2_29; P2_31 P2_33; P2_35	S	S	S	S	S	S	S	S
	P2_22	S	S	S	S	S	S	R	R
	P2_23	R	S	S	S	S	S	S	S
	P2_30	S	S	S	S	I	S	S	S
	P2_32	S	S	S	S	I	S	S	S
	P2_34	S	S	S	S	I	S	S	S
	P2_36	S	S	I	S	R	S	S	I
	P2_37	S	S	S	S	I	S	S	S
	P2_38	R	R	I	R	I	R	S	S
Rp_3	P3_4; P3_6; P3_7	S	S	S	S	S	S	S	S
	P3_5	R	S	S	S	R	S	S	S
	P3_8	R	R	R	R	I	R	S	S
	P3_9	R	S	S	S	I	S	S	S
	P3_10	R	S	S	S	S	S	S	S
	P3_11	R	S	S	S	R	S	S	S
	P3_12	S	S	S	S	S	S	R	I
	P3_13	R	R	R	R	S	R	S	S
	P3_14	R	R	I	R	I	R	S	S

S- Susceptible, R- Resistant, I- Intermediate.

addition to the majority susceptibility of ciprofloxacin in all isolates of this study, suggests that residues of quinolone compounds were not present in these environments, not requiring bacterial adaptive events. The standard MIC cut-off point has not been established for all Gram-negative bacteria or antimicrobials. Nevertheless, *C. violaceum* and *P. aeruginosa* were submitted to the test (Table 3) and exhibited high MICs for tetracycline and ampicillin, suggesting the development of tolerance and/or resistance to these compounds.

Antibiotic resistance determinants are often associated with metal resistance in bacteria. Regarding resistance to Hg, it can be determined by the operon *mer* that is located in plasmid, transposons, integrons and genomic DNA (Nakahara *et al.* 1977; Wireman *et al.* 1997; Skurnik *et al.* 2010; Boyd & Barkay 2012). Consequently, antibiotic resistance genes also disseminate together with mercury resistant genes even in the absence of frequent antibiotics used due to co-selection of the linked markers (Skurnik *et al.* 2010). However, in this study, we did not see a direct relationship between the presence of mercury and resistance to beta-lactams in *E. coli* isolates (Tables 2 and 4). It should be noted that, here, we used phenotypic determination but a molecular approach could reveal the presence of antibiotic resistance genes associated with mercury in these isolates. Thus, this should alert and encourage further studies aiming to contain the spread of bacteria resistance to both heavy metals and clinically relevant antimicrobials.

4. CONCLUSION

High concentrations of mercury are found in Pará River (Minas Gerais, Brazil), which compromises its use and puts the health of the population at risk. Considering its toxicity, mercury must be monitored in aquatic environments and the validation and availability of a quantification method such as UV-VIS, relatively easy to access and low cost, is very important.

Furthermore, the presence of mercury may have an impact on the microbial community, which should be further studied to promote a better understanding of its diversity and susceptibility to antimicrobials, perhaps with potential for mercury bioremediation in these environments.

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CONFLICT INTEREST

The authors report that they do not have any conflicts of interest.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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