

Original Article

Isolation of Mercury-Resistant Bacteria in the Brantas River, Malang, Indonesia

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ABSTRACT

Background: Mercury (Hg) is a toxic heavy metal that can originate from various sources, such as gold mining, the industrial sector, atmospheric deposition, and natural activities. Mercury contamination can cause liver, kidney, and enzyme system damage, as well as DNA damage. Because microorganisms are more sensitive to heavy metal stress than are animals and plants, the characteristics of microorganisms can be potential indicators of environmental health. **Objective:** This study aimed to isolate and identify mercury-resistant bacteria in the Brantas River, Malang city, as potential indicators of Hg pollution. **Methods:** Samples were isolated on nutrient agar (NA) media supplemented with 0.01 ppm HgCl₂ as a selective medium. The growing bacterial colonies were purified, and further resistance tests were carried out by adding HgCl₂ at concentrations of 0.03 ppm, 0.05 ppm, and 0.1 ppm. Bacterial isolates that survived at a concentration of 0.1 ppm HgCl₂ were then tested for sensitivity to concentrations of 0.15 ppm and 0.2 ppm HgCl₂. **Results:** Bacterial isolation via HgCl₂ 0.01 ppm selective media produced 28 bacterial isolates. Further testing revealed that 19 isolates were resistant to 0.03 ppm HgCl₂, 13 isolates (0.05 ppm), and five isolates (0.1 ppm). Sensitivity tests on five bacterial isolates revealed that at a HgCl₂ concentration of 0.15 ppm, isolates RS118, RS1221, and RS1323 showed no growth inhibition, whereas at a concentration of 0.2 ppm, an inhibition zone formed for all five isolates. **Conclusion:** Although mercury-resistant bacteria were detected, this finding does not directly indicate the presence of mercury pollution in the river because bacterial resistance can be caused by various factors, including horizontal gene transfer between bacterial communities. Comprehensive testing of Hg concentrations in rivers is required to accurately determine the status of Hg pollution. This study highlights the importance of considering multiple biomarkers and environmental parameters alongside bacterial resistance patterns when assessing Hg contamination in riverine ecosystems.

KEYWORDS

Bacterial, isolation, mercury-resistant bacteria, pollution, environmental toxicology

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INTRODUCTION

Pollution in the aquatic environment can potentially reduce and damage the existence of aquatic biota, such as aquatic plants, fish, crustaceans, gastropods, benthos, plankton, and periphyton, which is a problem for most water areas in Indonesia [1]. Pollutants in the form of hazardous substances such as hydrocarbons, mercury, ammonium, nitrogen, and plastics can cause poisoning [2–4]. Heavy metals, including Hg, are toxic to living things, including mercury (Hg). Environmental mercury emissions can come from gold mining, industrial sectors (cement production and nonferrous metal production), atmospheric deposition, and natural activities such as forest fires, volcanic eruptions, and erosion [5].

Mercury exposure affects health [6–8]. It can affect nerve development in children and cardiovascular diseases in adults [8]. The effects of mercury exposure include skin irritation, eye irritation, mucous membrane damage, sensory nerve disorders (paresthesia, numbness, difficulty moving fingers and toes, narrowed vision, decreased hearing), motor nerve disorders (muscle coordination, difficulty standing, ease of falling, ataxia, tremors, slow movements, and difficulty speaking), mental disorders, headaches, and hypersalivation [9,10]. In addition, several effects of mercury can interfere with the function of the liver, kidneys, enzyme systems, and synthetic mechanisms, damaging the fetus in pregnant women and causing congenital disabilities, DNA and chromosome damage, disruption of blood vessels to the brain, and brain damage [11]. Microorganisms are more sensitive to heavy metal stress than are animals and plants. The characteristics of microorganisms can be used to evaluate their environmental health [12–14]. Bacteria can decompose hazardous compounds into simpler compounds and can be utilized in bioremediation processes by reducing the pollutants contained in waste [15,16].

The Brantas River is one of the longest rivers in East Java. This river plays an important role in supporting the population and is used to support agricultural, fishery, and industrial activities [17,18]. On the basis of the plankton diversity index as a bioindicator of pollution, the Brantas River is included in the ranks of polluted rivers, indicating that the level of water pollution in the Brantas River is in the moderately polluted category [19]. The Brantas River has experienced water quality degradation with an alert status due to changes in land use from protected to agricultural and household activities that produce domestic and industrial waste [11]. The factors that most influence the decline in river water quality along the Brantas Malang watershed are the large number of industries located close to the river and the use of the river as a waste storage area. The concentrations of pollutants widely received by the Brantas River in Malang city are BOD, nitrate, ammonia and phosphate [20]. Research related to heavy metals (Pb) in water and fish from the Brantas River is known to exceed the maximum threshold value, exceeding the quality standards set by food safety standards [21–23].

The complex interaction between anthropogenic activities and natural processes in river systems necessitates a multifaceted approach for pollution assessment. Although bacterial resistance can serve as an indicator of environmental stress, it must be considered alongside other parameters, such as sediment composition, water chemistry, and the presence of other heavy metals, that may influence bacterial adaptation mechanisms. Understanding these interactions is crucial for developing effective monitoring and remediation strategies for Hg contamination in riverine ecosystems. This is because the condition of a polluted river can affect health and the continuity of life. Specifically, no research has examined the possibility of Hg pollution in the Brantas River. However, considering the activity and conditions of pollution in the Brantas River, a microbiological study was conducted to determine whether bacteria in the Brantas River, Malang, are resistant to Hg and to assess the level of resistance to Hg exposure.

MATERIALS AND METHODS

Study design and setting

This study was conducted in the microbiology laboratory of the Faculty of Mathematics and Natural Sciences, Brawijaya University, through observations based on a descriptive qualitative approach to describe the resistance of bacteria to mercury from the samples to be studied [24,25]. Samples were obtained from the Brantas River, Malang city at coordinate points

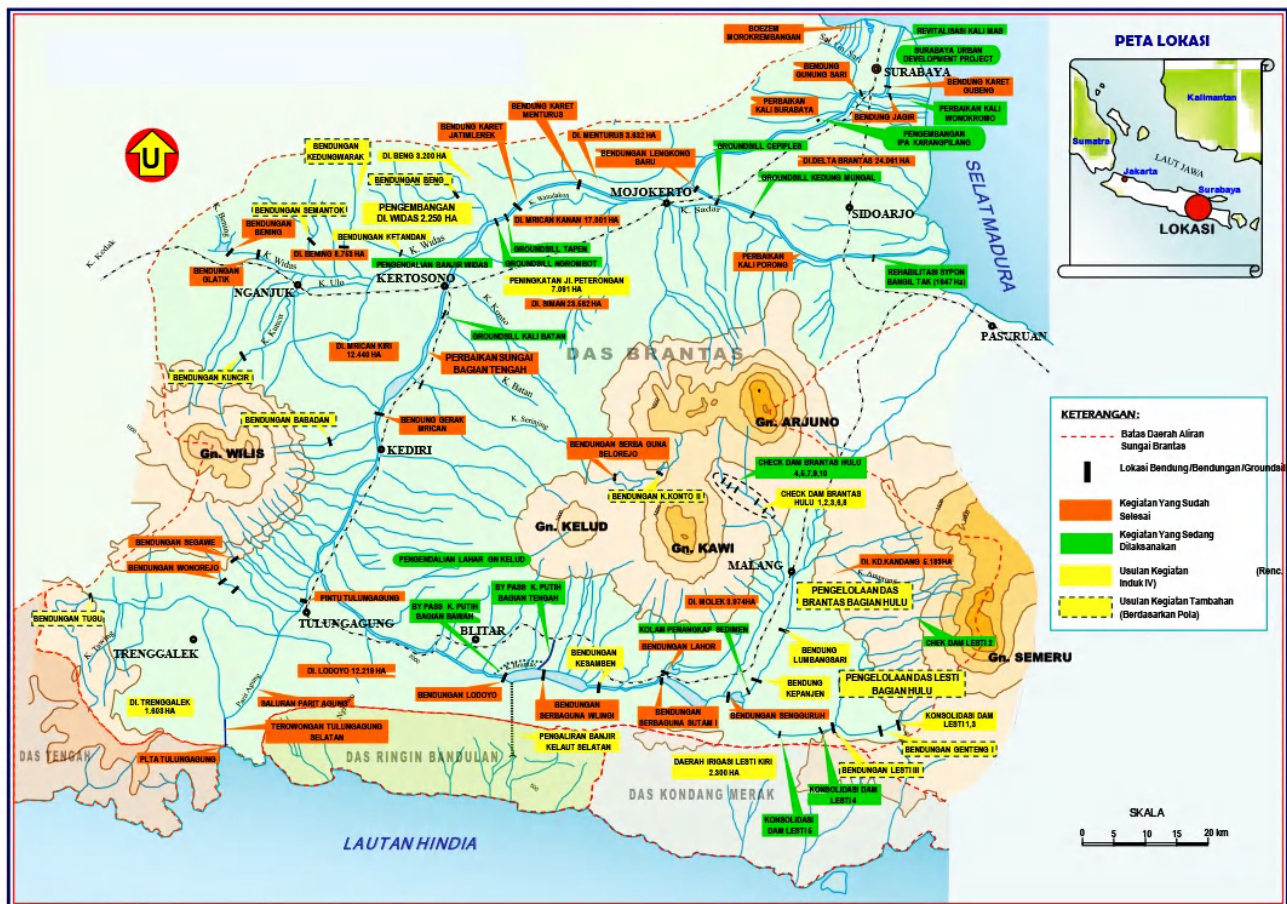


Figure 1. Research sampling location at Brantas River, Malang city, Indonesia. **Remarks:** Maps courtesy of Perusahaan Umum Jasa Tirta I®.

Bacterial isolation stage

The isolation stage was carried out by preparing materials, tools, and samples originating from Brantas River water under aseptic conditions; then, the samples were diluted to 10^{-1} to 10^{-6} . Dilution was carried out via serial dilution by preparing five-tube reactions, with each tube containing 9 mL of sterile 0.85% NaCl solution; then, 1 mL of sample was put into the first tube, homogenized, and 100 μ L was taken to be put into nutrient agar enriched with 2 g/L yeast extract, 5 g/L Bacto peptone, and 5 g/L NaCl, and 0.01 ppm HgCl_2 was added via the spread plate method. The preparation was then incubated for 2×24 h at 37°C [26]. The bacterial colonies that grew were then purified via the streak plate method until single cells were obtained, and the isolates were stored in agar slants for further testing. The number of colonies ranged from 30-300; morphologically, different colonies were collected and purified by repeated subculture on the same medium [5]. If colonies of bacteria live on NA containing additional mercury concentrations, the bacteria can be considered mercury resistant. This is because only certain bacteria can live in media containing mercury.

Resistance testing

NA media with HgCl₂ concentrations of 0.03 ppm, 0.05 ppm, and 0.1 ppm were used. Next, bacterial isolates that were rejuvenated for 2 × 24 h were inoculated into the media. The mixture

was incubated at 37°C for 2 × 24 h. Observations were made every 24 h to facilitate the growth of bacteria with more than 24 h of growth. Observations of the growing bacterial colonies were made. If bacterial colonies grow, the bacterial isolate can survive at a specified HgCl₂ concentration [26]. The resistance of the bacterial isolates was tested by adding HgCl₂ at concentrations of 0.15 ppm and 0.2 ppm; this test was carried out via the agar disk diffusion technique. First, a liquid culture of bacteria was prepared in NB medium with a bacterial cell density of 10⁷ CFU/mL via a hemocytometer obtained from inoculating bacterial isolates into 15 mL of liquid NB medium, followed by incubation at 37°C for 24 h. Furthermore, a HgCl₂ solution with concentrations of 0.15 ppm and 0.2 ppm in sterile distilled water was prepared, and then, as much as 50 µL of each concentration of HgCl₂ was dripped onto the blank disk until it was absorbed. The dried blank disk was placed on the surface of NA on a plate containing bacterial isolates that were further tested three times. The preparation was incubated at 37°C for 2x24 hours. Measurements were then taken of the inhibition zone around the blank disk via a digital caliper with the following provisions:

$$\text{Clear zone diameter (mm)} = \text{Total diameter} - \text{Colony diameter} \dots\dots\dots (1)$$

Statistical analysis

Data analysis for the bacterial sensitivity test to the highest concentration of HgCl₂ was performed via one-way analysis of variance (ANOVA) via IBM SPSS Inc. v.25 software with a confidence level of 95%. Data were considered significant if the p value was ≤ 0.05. Tukey's test was performed to determine the level of difference between isolates.

RESULTS

The results of bacterial isolation from Brantas River water samples via selective media revealed 28 bacterial isolates. The morphological characteristics of the bacterial isolates are presented in Table 1. The results of the shape of the bacterial colonies were mostly round with varying sizes and were classified as small, medium, or large. In addition, some colonies were oval. The entire bacterial colony had a convex elevation, whereas some had a flat elevation. The texture of the colony is also diverse; some colonies are smooth, rough, and contoured, with edges that look complete and wavy. The optical characteristics that appeared in each colony were partly shiny and partly faded. The pigmentation of each colony was white, milky white, and cream, and some colonies appeared transparent.

Table 1. Morphological observations of bacterial colonies isolated from Brantas River selective media by the addition of 0.01 ppm HgCl₂

Code Isolate	Criteria					
	Shape	The Edge	Elevation	Texture	Optical	Pigmentation
RS111	Medium Round	Comprehensive	Convex	Contoured	Shining	White
RS112	Medium Round	Flowing	Convex	Contoured	Shining	White
RS113	Oval	Comprehensive	Flat	Slippery	Fade	Beige
RS114	Medium Round	Comprehensive	Pulvinate	Slippery	Shining	White
RS115	Small round	Flowing	Convex	Rough	Fade	Milky white
RS116	Small round	Flowing	Convex	Contoured	Shining	White
RS117	Oval	Comprehensive	Flat	Contoured	Fade	Beige
RS118	Small round	Flowing	Convex	Slippery	Fade	Transparent
RS119	Big round	Flowing	Convex	Rough	Shining	Milky white
RS1210	Medium Round	Comprehensive	Pulvinate	Contoured	Shining	White

Code Isolate	Criteria					
	Shape	The Edge	Elevation	Texture	Optical	Pigmentation
RS1211	Medium Round	Comprehensive	Flat	Slippery	Fade	Transparent
RS1212	Oval	Flowing	Convex	Contoured	Shining	Milky white
RS1213	Medium Round	Comprehensive	Flat	Slippery	Fade	Milky white
RS1214	Small round	Flowing	Convex	Contoured	Shining	White
RS1215	Small round	Flowing	Convex	Rough	Fade	Milky white
RS1216	Small round	Flowing	Convex	Contoured	Shining	Transparent
RS1217	Oval	Comprehensive	Pulvinate	Slippery	Fade	Transparent
RS1218	Small round	Flowing	Convex	Rough	Fade	White
RS1219	Oval	Flowing	Flat	Slippery	Fade	Beige
RS1220	Oval	Comprehensive	Flat	Slippery	Fade	Transparent
RS1221	Big round	Comprehensive	Convex	Rough	Shining	Milky white
RS1322	Medium Round	Comprehensive	Convex	Contoured	Shining	Milky white
RS1323	Big round	Comprehensive	Convex	Rough	Shining	White
RS1324	Oval	Comprehensive	Flat	Slippery	Shining	Beige
RS1325	Small round	Flowing	Convex	Slippery	Fade	White
RS1326	Small round	Comprehensive	Flat	Slippery	Fade	Transparent
RS1327	Small round	Comprehensive	Convex	Slippery	Fade	White
RS1328	Small round	Comprehensive	Convex	Slippery	Fade	Milky white

Remarks: RS: code sample from the Brantas River, isolate codes with numbers 111-119: isolates taken from replication treatment 1, isolate codes with numbers 1210-1221: isolates taken from replication treatment 2, isolate codes with numbers 1322-1328: isolates taken from replication treatment 3.

The results of bacterial resistance testing with several additional concentrations of HgCl₂ (0.03 ppm, 0.05 ppm, and 0.1 ppm) are shown in Table 2. The addition of 0.03 ppm HgCl₂ resulted in 19 resistant isolates from 28 isolates. The next observation, by increasing the concentration of HgCl₂ to 0.05 ppm, revealed that 13 of the 28 isolates were resistant to 0.05 ppm HgCl₂.

Table 2. Bacterial resistance testing at HgCl₂ concentrations of 0.03, 0.05, and 0.1 ppm

No.	Isolate Code	Concentration		
		0.03 ppm	0.05 ppm	0.1 ppm
1	RS111	+	+	-
2	RS112	+	-	-
3	RS113	+	-	-
4	RS114	+	-	-
5	RS115	+	+	-
6	RS116	-	-	-
7	RS117	+	+	-
8	RS118	+	+	+
9	RS119	-	-	-
10	RS1210	+	+	-
11	RS1211	-	-	-
12	RS1212	-	-	-
13	RS1213	-	-	-

No.	Isolate Code	Concentration		
		0.03 ppm	0.05 ppm	0.1 ppm
14	RS1214	+	+	+
15	RS1215	-	-	-
16	RS1216	-	-	-
17	RS1217	+	+	+
18	RS1218	+	+	-
19	RS1219	+	-	-
20	RS1220	+	+	-
21	RS1221	+	+	+
22	RS1322	+	+	-
23	RS1323	+	+	+
24	RS1324	-	-	-
25	RS1325	+	+	-
26	RS1326	+	-	-
27	RS1327	+	-	-
28	RS1328	-	-	-

Remarks: + (Able to grow), - (Unable to grow), isolate codes with numbers 111-119: isolates taken from replication treatment 1, isolate codes with numbers 1210-1221: isolates taken from replication treatment 2, isolate codes with numbers 1322-1328: isolates taken from replication treatment 3.

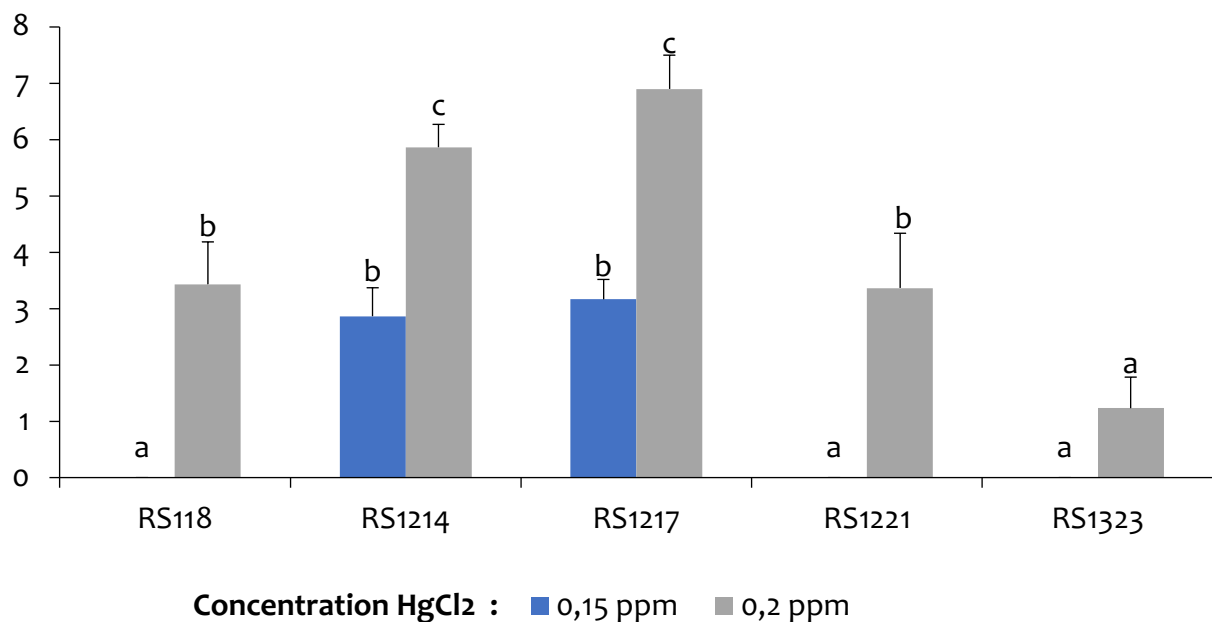


Figure 2. Sensitivity of bacterial isolates to HgCl₂ concentrations of 0.15 ppm and 0.2 ppm. The different notations indicate a significant difference among the isolates ($p < 0.05$). **Remarks:** RS: code sample from the Brantas River, isolate codes with numbers 111-119: isolates taken from replication treatment 1, isolate codes with numbers 1210-1221: isolates taken from replication treatment 2, isolate codes with numbers 1322-1328: isolates taken from replication treatment 3.

The results of the real difference test of the sensitivity of five bacterial isolates to HgCl₂ concentrations of 0.15 ppm and 0.2 ppm showed that the treatment with a HgCl₂ concentration of 0.15 ppm was significantly different from the treatment with a HgCl₂ concentration of 0.2 ppm. Isolates RS118, RS1221, and RS1323, with a HgCl₂ of 0.15 ppm, did not show any growth inhibition; therefore, the three isolates were classified as resistant to a HgCl₂ of 0.15 ppm. In

contrast, in isolates RS1214 and RS1217, there was a clear zone with inhibition zone values of 2.87 mm and 3.17 mm, respectively. These findings indicate that the two isolates were not resistant to 0.15 ppm HgCl_2 . At a HgCl_2 concentration of 0.2 ppm, there was a clear zone in five bacterial isolates; this finding shows that the five isolates were not resistant to a concentration of HgCl_2 (0.2 ppm).

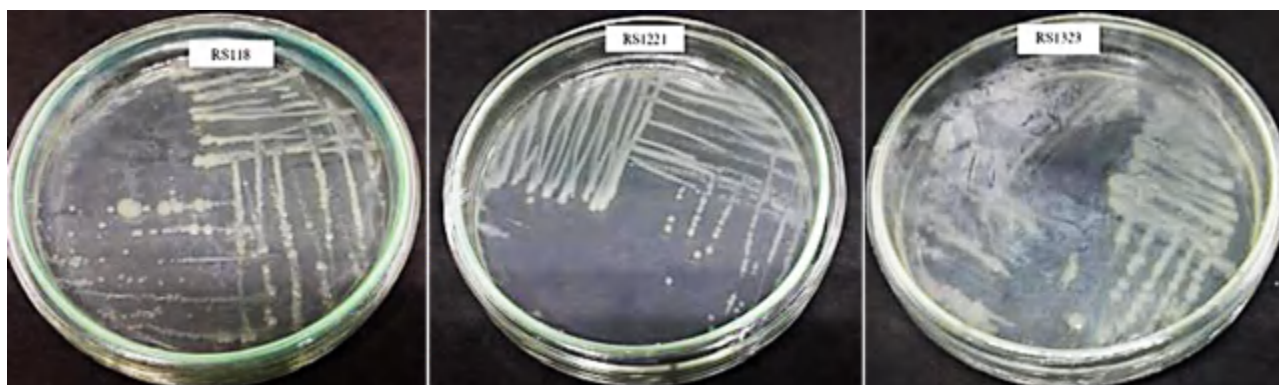


Figure 3. Colonies of bacteria resulting from the purification of superior mercury-resistant isolates with a HgCl_2 concentration of 0.15 ppm, namely, isolates RS118, RS1221, and RS1323

DISCUSSIONS

On the basis of a literature review of previous research results, the resistance of all bacterial isolates tested to HgCl_2 remains very low. The HgCl_2 sensitivity test revealed that the superior isolates could withstand a mercury concentration of only 0.15 ppm. In comparison, at a concentration of 0.2 ppm, no clear zone indicated that the bacterial isolates were not resistant. The variation in bacterial resistance levels observed in this study suggests a complex adaptive response to environmental Hg exposure. This adaptation may be influenced by multiple factors beyond the mere presence of mercury, including the bioavailability of the metal, the presence of other contaminants, and environmental conditions such as pH, temperature, and organic matter content. These findings emphasize the need for a more comprehensive approach to Hg pollution assessment that combines biological indicators with physicochemical parameters and considers the broader ecological context of the river system. Mangesa and Kasmawati reported that bacteria can grow at mercury concentrations of up to 30 ppm in all isolates from gold mining waste collected from Mount Botak and Mount Gogorea [16].

In accordance with the Australian and New Zealand Environment and Conservation Council (ANZECC) & Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ), the concentration of heavy metal mercury in the sediment quality standard is 1.0 ppm [27]. The maximum limit of mercury content for water quality criteria contained in Government Regulation Number 82 of 2001 concerns water quality management and water pollution control, namely, water quality classes (class 1), 0.001 ppm; classes 2 and 3, 0.002 ppm; and class 4, 0.005 ppm [28]. The mercury concentration used in this test was 0.01 - 0.2 ppm above the water quality standards set by the government and below the sediment quality standards (ANZECC and ARMCANZ). Many factors influence the presence of mercury-resistant bacteria in water, including horizontal gene transfer. Horizontal gene transfer allows microbes to adapt more quickly to the environment and contributes to the wider spread of genetic variation. Horizontal gene transfer plays a role in the emergence of new traits that are not obtained through mutation or direct natural selection and accelerates the adaptation of microbial populations by adding genetic variation so that microbes are more adaptive in dealing with various environmental pressures [29].

In addition, the presence of mercury in water can be caused by two factors: first, by industries such as paint factories, paper, electrical equipment, chlorine, and caustic soda; second, by nature itself through the process of rock weathering and volcanic eruptions. High levels of mercury in water are generally caused by industrial waste and byproducts from the use of mercury compounds in agriculture. Mercury can exist in the form of metal, inorganic, and organic

compounds [14,30,31]. Therefore, bacterial resistance to Hg exposure during testing is not a determinant of river Hg pollution. Comprehensive testing of the Hg concentrations in rivers is needed to determine the status of Hg pollution. Bacterial mercury resistance is also influenced by *merA* and *merB*, which are part of the *mer* operon. This operon is found on bacterial plasmids and chromosomes, allowing bacteria to survive in mercury-contaminated environments [6]. Bacteria resistant to mercury have the *merA* and *merB* genes, all of which are broad-spectrum mercury-resistant bacteria. Bacteria with a high level of resistance to mercury can survive in environments containing more than 20 ppm mercury (HgCl_2) [16]. Research results using the isolate *Bacillus cereus* (IHB B 379) revealed that this bacterium could be a high bioaccumulator of HgCl_2 metal stress up to a concentration of 500 ppm [32].

The morphology of the bacterial mercury-resistant cells was identified via microscopic observation with Gram staining. Staining revealed that isolates RS118, RS1221, and RS1323 were gram-positive bacteria with a bacillus shape. Several bacteria resistant to mercury include gram-positive and gram-negative bacteria [33–37]. Several previous studies have also reported that bacteria that are resistant to mercury are mostly bacilli. Research conducted by Assa et al. [34] revealed that five types of bacterial species, namely *Streptococcus* sp., *Pseudomonas* sp., *Bacillus* sp., *Escherichia* sp., and *Klebsiella* sp., were able to survive at concentrations of 5, 10, and 20 ppm. As many as twenty-eight bacterial isolates, from which two strong mercury-resistant strains were identified, were *Pseudomonas aeruginosa* and *Bacillus licheniformis*. Mercury-resistant bacteria have mercury reductase activity and a *mer* operon that converts toxic mercury into nontoxic forms that help them thrive in the presence of mercury [4]. *Bacillus* sp. bacteria can survive up to 40 ppm HgCl_2 and 20 ppm phenyl mercury, indicating that *Bacillus* sp. bacteria are resistant to mercury. *Bacillus* sp. can survive at a HgCl_2 concentration of 40 ppm and produce an inhibition zone of 10 mm [32].

Bacillus is a genus of bacteria that is widely used as a detoxifying agent for heavy metals. *Bacillus cereus*, which is resistant to mercury, has different cell growth and colony morphological characteristics than those that are not resistant to mercury. *Bacillus cereus* (IHB B 379) is a bacterium isolated from the foundation of the gold mining area (PETI) of Danau Biru Singkawang. This bacterium has resistance properties that allow it to grow in media containing 500 ppm HgCl_2 [32]. This study aimed to determine the effects of temperature and HgCl_2 concentration on the growth of *B. cereus* (IHB B 379). The growth test of *B. cereus* was performed via the delusion method, and the number of colonies was calculated via the total plate count (TPC) method. The optimum growth of *Bacillus cereus* (IHB B 379) occurs at a HgCl_2 concentration of 250 ppm [32]. *Bacillus* bacteria isolated from mining areas can remove mercury through the evaporation process so that the mercury content in the soil can be reduced. The bioremediation effect on mercury ranges from 62 to 97.36%, indicating that bacteria are promising candidates for heavy metal bioremediation (especially Hg) mining [13,23,38].

Bacteria that contain only the *merA* gene (*merA*) are called narrow-spectrum mercury-resistant bacteria [39]. This resistance gene is usually influenced by the activity of bacterial responses in the form of adaptation to the environment; therefore, the expressed gene is a resistance gene. Mercury resistance genes (*mer* operons) convert toxic Hg^{2+} into Hg^0 , which has volatile properties such that bacteria can still grow in mercury [40–43]. Previous studies have shown that mercury-resistant bacteria can be identified from five bacterial isolates encoding Sp1, Sp2, Sp3, Sp4, and Sp5 from the river flow surrounding the Batang Toru gold mine in North Sumatra. These bacteria grew at a mercury concentration of 0.1 ppm [44]. Thus, mercury-resistant bacteria could grow at a concentration of 0.1 ppm or more.

Public health implications

The identification of mercury-resistant bacteria in the Brantas River has significant public health implications. The presence of these bacteria suggests potential mercury contamination in the riverine ecosystem, which could pose risks to local populations that depend on rivers for various activities, including agriculture, fisheries, and domestic use. Given that Hg exposure can lead to severe health effects, including neurological disorders, cardiovascular diseases, and developmental issues in children, these findings underscore the need for enhanced environmental

monitoring and public health surveillance in communities along the Brantas River. Furthermore, the discovery of bacteria capable of surviving in mercury-contaminated environments presents potential opportunities for bioremediation strategies that could help mitigate mercury pollution and its associated health risks in affected areas.

Limitations

This study has several limitations. First, the presence of mercury-resistant bacteria alone cannot definitively indicate Hg pollution levels, as bacterial resistance can develop through horizontal gene transfer rather than direct Hg exposure. Second, the study focused solely on bacterial resistance patterns without measuring actual Hg concentrations in river water and sediments, limiting the ability to establish direct correlations between bacterial adaptation and environmental Hg levels. Third, the study was conducted at a single sampling point in the Brantas River, which may not represent the overall condition of the river system. Additionally, the study did not investigate the specific mechanisms of mercury resistance in the identified bacterial strains or characterize the *mer* operon genes responsible for mercury resistance, which would have provided more comprehensive insights into bacterial adaptation processes. Finally, the study did not examine seasonal variations in bacterial populations or their resistance patterns, which could influence the interpretation of the results.

CONCLUSIONS

Bacteria that can grow at concentrations of 0.1 ppm or more are resistant to mercury. Sensitivity testing of 5 bacterial isolates to 0.15 ppm and 0.2 ppm HgCl₂ revealed that at a concentration of 0.15 ppm, there was no inhibition of the growth of bacterial isolates with the codes RS118, RS1221, and RS1323. However, at a concentration of 0.2 ppm HgCl₂, there was an inhibition zone, indicating that the five bacterial isolates were not resistant. Bacterial resistance to Hg exposure at the time of testing is not a determinant of Hg pollution in rivers; various factors cause bacteria to be resistant to Hg, including horizontal gene transfer between bacterial communities. The identification of mercury-resistant bacteria in the Brantas River ecosystem provides valuable insights into potential biological indicators of environmental stress. However, the complexity of bacterial adaptation mechanisms and the various factors influencing Hg resistance underscore the importance of adopting an integrated approach for environmental monitoring. Future studies should incorporate molecular techniques to characterize mercury resistance genes, along with a comprehensive analysis of environmental parameters, to better understand the relationship between bacterial adaptation and mercury contamination in riverine ecosystems.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision: D.H.

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