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Overview of Bacterial Identification for Mercury-resistant bacteria in coastal sediments in North Sulawesi

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Abstract
One of the pollutants whose presence is feared due to its high bodily toxicity. Due to their capacity to bind N_2 from the air and convert ammonium into nitrate, bacteria play a crucial function as decomposers in sediments and promote plant growth. This research aimed to identify microorganisms found in sediments. Mercury-resistant bacteria are present in the environment, however, not all mercury-resistant bacteria must be antibiotic-resistant. The widespread use of antibiotics without realizing it has had a lot of impact on environmental pollution around us. Hospitals, clinics, farms, pharmaceutical factories and various units using drugs/antibiotics are suspected to be the causative factors. Likewise, the residual waste from the use of antibiotics enters the sewer, then is carried by water currents to the waters and sediments. Antibiotics that are not properly degraded can cause pollution to the area where the waste is disposed of, one of which is sediment in the coastal area.
Aim: To determine the overview of Mercury resistant bacteria in the sediments of the coastal area in North Sulawesi.
Method: This research is a descriptive quantitative research method aims to explain the phenomenon that is seen by using numbers that describe the characteristics of the subject under study.
Result: It was found that bacterial in the sediment were resistant to several types of antibiotics.
Conclusion: In Likupang, the microorganisms found were *Staphylococcus-sp* and in Buyat Bay, *Bacillus-sp*. At mercury concentrations of 250 ppm and 500 ppm, mercury-resistant bacteria isolated from sediments on Likupang and Buyat Bay were still able to thrive. At mercury concentrations of 750 ppm and 1000 ppm, *Staphylococcus-sp* and *Bacillus-sp* are still able to proliferate.

Keywords: antibiotics, sediment, environment.

1. Introduction

Sediment is the primary factor influencing coastal morphology (topography and bathymetry). Sediment is created from bits of rock that are the result of weathering. Sediment migration resulting from erosion and deposition causes morphological changes along the coast. Large quantities of pharmaceuticals, classified as emerging pollutants, are utilized in human and veterinary medicine to treat various disorders. Antibiotics in aquatic environments are of particular concern because sustained exposure to low doses may generate antibiotic resistance (Hernández *et al.*, 2007).

In recent years, detections of antimicrobials active chemicals in surface waters have been reported more frequently. In most situations, municipal sewage has been mentioned as a potential input channel. As an input from the agricultural sector is also possible, it should be determined if an input can occur via the pathway of liquid manure application to fields and the subsequent mechanisms of surface run-off/interflow, leaching, and drift(Christian *et al.*, 2003).

Trace amounts of antibacterial agents (antibiotics) have been detected in hospital and municipal wastewaters and in the aquatic environment, according to environmental analytical investigations (W. Giger *et al*., 2003).

Concerns about the potential ecological effects of pharmaceuticals have existed for decades, but only lately have developments in analytical chemistry made it possible to identify these

substances at the concentrations commonly found in the environment. Antibiotics enter the environment by the intentional dumping of excess pharmaceuticals to sewage, the release of antibiotics into sewage through urine and feces, leaching from landfills and discharges from sewage treatment plants or confined animal farming activities (Cordova *et al*., 2007).

2. Material and Methods

This study is a quantitative descriptive investigation. The objective of descriptive quantitative research is to explain observable phenomena using numbers that describe the properties of the issue under study. This study used an experimental laboratory method. The test parameters include the properties of bacteria that thrive at the maximum mercury concentration and the bacteria's ability to decrease mercury.

Sampling was conducted in the coastal regions of Likupang, North Minahasa (point of collection 1°67'59.58"N 125°07'37.0"E) and Buyat Bay (point of collection 0°85'66.72"N 124°70'50.50"E). The collection method adheres to the Indonesian National Standard (SNI). Sediment is collected between 10:00 and 12:00 AM, or when the tide is receding. A sediment grab was used to collect sediment samples. The silt is gathered in a sterile container and placed in a chiller to maintain its condition. In the Laboratory of the Faculty of Mathematics and Natural Sciences (FMIPA) at Unsrat, samples were analyzed. Existing categorization criteria are utilized to separate one type of bacteria from another while identifying bacteria. Bergey's Manual of Determinative Bacteriology includes characteristics of known bacteria as a guide for identifying microorganisms.

Total Plate Count (TPC) was then performed by inoculating mercury-resistant bacteria on Nutrient Agar solid selection media and placing antibiotic disc paper with standard concentrations of numerous types of antibiotics on the surface of the inoculated media using sterile tweezers. The antibiotic disc paper was evenly spaced and then incubated for twenty-four hours. Observe the changes and use a caliper to measure the clear zone.

Bacterial isolation

Bacterial isolation was carried out by serial dilution technique, 1 mL of water sample was diluted with 9 mL of distilled water, for sediment samples taken 1 gram and then diluted with 9 mL of distilled water. The dilution was made 10^{-1} - 10^{-7}. The last dilution was taken as much as 1 mL and then poured into a Petri dish containing nutrient agar medium to which $HgCl_2$ solution had been added with different concentrations of 0.01 ppm, 0.03 ppm, 0.05 ppm, 0.1 ppm and 0.2 ppm. The isolation process was carried out using the pour cup technique. Furthermore, Petri dishes were incubated at 37°C for 2 x 24 hours to obtain mercury-resistant bacterial isolates. From the results of this isolation, one isolate of bacteria that grows at the highest concentration of $HgCl_2$ in water samples and sediment samples will be selected so that 2 bacterial isolates are obtained.

Bacteria identification

Identification was carried out based on observations of cell morphology, gram staining, biochemical tests consisting of oxidase test, catalase, and carbohydrate fermentation with glucose, lactose, maltose, motility test, Indole test, lysine, citrate formation, and catalase.

Data analysis

Data analysis was carried out descriptively with graphs. The data are described according to the observations of each parameter based on the treatment variables.

3. Results and Discussion

Bacteria were isolated from sediment samples taken from the waters of Likupang and Buyat Bay. Results of the analysis of bacterial identification in this study are shown in the table 1.

Table 1: Bacterial Identification Results through Gram Stain, Motility Test and Biochemical Test in Likupang and Buyat Bay.

		Isolate Code	
		T1.4 (Likupang)	B2.3 (Buyat)
Morphological Test			
Gram Stain		+	+
Cell Shape		Coccus	*Bacillus*
Physiological Test and Biochemistry			
Motility		–	–
Indole		+	–
H_2S		+	–
Carbohydrate Fermentation	Gas	+	+
	Glucose	+	+
	Maltose/Lactose	+	+
Lysine		+	–
Citrate		+	+
Catalase		+	+
Identification Results		*Staphylococcus-sp*	*Bacillus-sp*

Bacteria Identification

Bacteria were identified using morphological examination, namely gram staining and biochemical activity testing. Each bacterium has a unique biochemical activity as a result of their distinct enzymatic activities. In biochemical studies, the nature of bacterial metabolism is typically determined by the interaction of metabolites with chemical reagents. In addition, it is observed that certain chemicals can be used as carbon sources and energy sources.

The first step in identifying bacteria is gram staining to determine the type of gram bacteria based on the appearance of bacterial cells. Positive Gram staining was seen in both Likupang and Buyat Bay. In Likupang, the colonies and bacterial cells have a coccus-like look, but in Buyat they are Bacillus-like.

The shapes of bacteria, according to Pratiwi (2008), are spherical (singular: coccus, plural: cocci), rod or cylindrical (singular: bacillus, plural: bacilli), and spiral, which is

curved or circular rod-shaped.

After gram staining, the shape and color of bacterial cells can be determined under a microscope with a 100X magnification. Bacilli-shaped bacteria are cylindrical bacteria with the shape of a short stick or little rod. Basil can be paired with or independent of one another (Dwidjoseputro, 1998). Bacilli exclusively divide along their short axis. The majority of bacilli appear as solitary rods. Bacilli pairs divide to form diplobacilli, while streptobacilli form chains.

Some bacilli resemble cocci and are referred to as coccobacilli (Pratiwi, 2012). The identification of microorganisms was continued by analyzing their biochemical properties.

Gram staining revealed that the bacteria in two locations, Likupang and Buyat Bay, were both gram positive. In gram-positive bacteria, the cell wall is made up of peptidoglycan and special components in the form of *teichoic* and *teichuronic* acids and polysaccharides; in gram-negative bacteria, the cell wall is also made up of peptidoglycan, but the special components are lipoproteins, outer membranes, and lipopolysaccharides. (Tedja 2009). Differences in the bacterial cell wall's constituents. Gram-negative bacteria have a higher tolerance for metals than gram-positive bacteria because their cell walls are capable of binding and immobilizing metal ions, including Hg^{2+}.

Gram-positive bacilli are comprised of spore-forming (bacillus species, clostridium) and non-spore-forming (Listeria, Erysipelothrix, Corynobacterium, Propionibacterium) bacteria (Anonymous 2015).

Based on the results of the performed motility test, neither of the bacteria is motile. Bacterial motility can be induced by a variety of processes, but flagella are the most prevalent. Flagella are found predominantly in bacilli, but sometimes in coccus (Shields and Cathcart 2013).

The indole test was conducted to assess the organism's capacity to breakdown the amino acid tryptophan and create indole. The indole test results for both bacteria were positive in Likupang but negative in Buyat Bay, according to the conducted tests. This indicates that bacteria in Likupang are able to digest amino acid, however bacteria in Buyat Bay are incapable of degrading the amino acid tryptophan because they lack the enzyme tryptophan, which can hydrolyze the amino acid type tryptophan, which has an indole side group, into indole.

TSI agar is a differential medium in tubes used to determine carbohydrate fermentation and H2S generation. Gases from the metabolism of carbohydrates can also be detected. Bacteria can either aerobically (with oxygen) or fermentative (without oxygen) consume carbohydrates (without oxygen). The TSI classifies bacteria according to their ability to ferment lactose, glucose, and sucrose, as well as their ability to produce hydrogen sulfide (H2S) (Lehman 2013).

The TSI test results for both bacteria were K/A (alkaline/acidic), where the slopes of the media were red and the bottom was yellow. In Buyat Bay, bacteria did not produce hydrogen sulfide (H2S) gas; however, bacteria in Likupang formed black precipitate, indicating that only in Buyat Bay did bacteria not make hydrogen sulfide (H_2S) gas.

According to Lehman (2013), the absence of H2S production during the K/A reaction implies that bacteria can exclusively metabolize glucose. Because bacteria cannot utilize lactose or sucrose, peptone (an amino acid) is employed as an aerobic energy source on the slopes of the tube. Utilization of peptone results in the release of ammonia (NH3), which produces an increase in pH, as seen by the color change from yellow to red when the pH indicator phenol red is introduced. At the bottom of the anaerobic tube, bacteria metabolize glucose to produce ATP and pyruvate, which are then transformed into stable acid end products so that the tube's bottom remains acidic.

According to the results of the carbohydrate fermentation test, both bacteria were capable of fermenting glucose and sucrose, as well as maltose and lactose.

The carbohydrate fermentation test tries to identify microorganisms capable of fermenting specific carbohydrates. Patterns of fermentation can be utilized to distinguish between bacterial groups or species. For instance, all families of *Enterobacteriaceae* are categorized as glucose fermenters because they can anaerobically metabolize glucose. Maltose fermentation separates *Proteus vulgaris* (positive) from *Proteus mirabilis* (negative) in this family (Reiner 2013).

Testing catalase using 3% Hydrogen Peroxide (H_2O_2) reagent. Hydrogen peroxide is hazardous to cells due to its ability to deactivate enzymes within cells. Catalase is an enzyme that bacteria utilize to convert hydrogen peroxide into water and oxygen. If bacteria or organisms are catalase-positive, air bubbles will form, and if air bubbles do not form, they are catalase-negative (-). Table 1 indicates that both bacteria from Likupang and Buyat Bay include catalase (+) and air bubbles.

Both bacteria can utilize citrate as a carbon and energy source by metabolizing it. Bacteria from Likupang and Buyat Bay are incapable of degrading into phenylalanine deaminase. Due to the use of Mckonkey agar media for the isolation of enteric gram-negative bacteria and the distinction of lactose-fermenting from non-lactose-fermenting gram-negative bacteria, neither bacterium grew on Mckonkey agar media. This medium is typically used to distinguish bacteria based on their capacity to ferment carbohydrates other than lactose.

The variation in biochemical reactions between the two isolates revealed that the strains of the two isolates were distinct. According to Hartsock (2015), two bacterial cells belong to the same species if their genomes are highly similar. For instance, two bacteria with a 95% degree of similarity are regarded to be of the same species; a 5% difference between the same species could comprise a number of distinct strains. Because strains of the same species have distinct genes, there are changes in bacterial characteristics, such as differences in metabolic abilities such as the capacity to degrade specific carbohydrates.

The table 4.1 above shows that sediments isolate code T1.4 (Likupang) is *Staphylococcus-sp* and B2.3 (Buyat) is *Bacillus-sp*. Bacterial identification in this coastal area shows two identification results for this bacterium. This study only examined bacteria isolated to the genus level, so identification down to the species is needed for more accurate results. As in Bacillus spp whose habitat is almost everywhere, so it is difficult to determine whether the isolated bacteria really came from.

Mercury resistance test

Following the identification of bacteria, a Total Plate Count test is performed to identify mercury-resistant bacteria. Table 2 displays the total plate count (TPC) rate for

mercury-resistant bacteria isolated from sediment in Likupang and Buyat Bay.

Colonies of *Staphylococcus-sp* and *Bacillus-sp* bacteria were grown in LB broth media using a needle that contained mercury, then transferred to the media so that it was slanted using a needle as a culture preparation (antibiotics and mercury resistance), incubated at 37°C for 24 hours, and then stored at 40°C. Re-inoculation of coli in LB broth media with $HgCl_2$at doses of 250 ppm, 500 ppm, 750 ppm, and 1000 ppm was performed. The number of growing colonies was observed.

Table 2: Total Plate Count (TPC) rate for mercury-resistant bacteria.

No	$HgCl_2$ Concentration (ppm)	T1.4 (Likupang)			B2.3 (Buyat)		
		Test results	Growth description	Rate (CFU/ml)	Test results	Growth description	Rate (CFU/ml)
1.	250	+++++++	Growth is very much seen from the turbidity	2.2×10^1	+++++++	Growth is very much seen from the turbidity	3.4×10^2
2.	500	++++	It's growing a lot, but it's still cloudy from the 200ppm	1.7×10^1	++++	It's growing a lot, but it's still cloudy from the 200ppm	2.3×10^2
3.	750	+++	Still growing	1.4×10^1	+++	Still growing	2.4×10^1
4.	1000	+	Still growing	2.5×10^1	+	Still growing	3.9×10^1

In the test for mercury resistance, bacterial growth in LB broth was conducted at four concentrations: 250 ppm, 500 ppm, 750 ppm, and 1000 ppm. The objective is to determine whether or not bacteria can grow in the four stated concentrations.

This test determined that the isolation of mercury-resistant bacteria at a concentration of 250 ppm HgCl2 revealed that the bacteria grew well and in great numbers in the isolates, and that all colonies were red. At a concentration of 500 ppm $HgCl_2$, it was discovered that the bacteria grew in modest numbers and appeared red to clear in all isolates. At a concentration of 750 ppm $HgCl_2$, it was determined that the bacteria developed in tiny numbers in all isolates and that all colonies were reddish-clear. At a dosage of 1000 ppm $HgCl_2$, all isolates continued to exhibit bacterial growth (Table 2). Scratch inoculation was performed on LB broth using the scratch method. Bacteria of the species Staphylococcus and Bacillus were cultured in LB broth with four amounts of mercury. Based on the findings of this test, the bacteria developed most rapidly at a concentration of 250 ppm, as opposed to 500 ppm, 750 ppm, and 1000 ppm.

This demonstrates that there are varied levels of mercury resistance among these bacteria. The fall in the population of one bacterial isolate is indicative of growth inhibition caused by the antimicrobial substance excretion of other bacterial isolates. The outcome of this competition is determined by each microorganism's rate of nutrition intake, metabolic rate, and growth rate.

According to Manampiring and Keppel (2011), a bacterium is considered mercury-resistant if it can survive at a mercury concentration of 1000 ppm. The bacteria generated are mercury-resistant bacteria. Bacterial resistance to inorganic mercury and organic mercury results from a detoxifying mechanism that consists of a succession of attempts by a bacterial cell to become resistant to mercury.

The discovery of Bacillus subtilis as a mercury-resistant bacteria in water is supported by research by Arinda and Shovitri (2012), which states that Bacillus subtilis and Bacillus cereus isolated from Kali Mas Surabaya are members of a group of bacteria with high resistance to mercury (Bacteria Highly Resistant to Mercury or BHRM), where Kali Mas Surabaya has a mercury concentration of 6.38 ppm.

Sholikah and Kuswytasari (2012) found that bacteria of the genus Bacillus are prevalent in nature and can be isolated from a variety of habitats, including mercury-contaminated areas. This viewpoint is reinforced by the findings of Buthelezi et al. (2009), who isolated several species of bacteria from residential wastewater treatment plants (WWTP), including Bacillus subtilis. Pratiwi (2012) discovered the bacterium Bacillus subtilis in the mining area's soil, which had 0.01 ppm of mercury.

Bacillus is a genus of bacteria that includes numerous mercury-resistant species that have been the subject of extensive research. Shovitri et al. (2010) successfully isolated Bacillus from Kali Mas Surabaya water samples that could grow at a concentration of 10 ppm HgCl2. Manampiring and Keppel (2010) recovered microorganisms from the sediments of the Manado Tondano River, one of which was a Bacillus cereus strain that could grow at 0.02% HgCl2. Badjoeri and Zarkasyi (2010) discovered that Bacillus megaterium bacteria isolated from Cisadane River water were able to adapt to a 10-ppm mercury content.

4. Conclusion

The bacteria identified were *Staphylococcus-sp* in Likupang and Bacillus-sp in Buyat Bay. For bacterial isolates.

Bacteria from sediments on Likupang and Buyat Bay were still able to grow at a mercury concentration of 250 ppm and 500 ppm, though at 750 ppm and 1000 ppm the mercury-resistant bacteria that were isolated were Staphylococcus-sp and Bacillus-sp is still growing.

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