Heavy metal (As, Cd, Cr, Hg, Pb) analysis and identification of heavy metal resistant bacteria in sediments from Manado Bay

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Abstract. Environmental pollution from heavy metals is becoming a growing concern due to the adverse effects it is causing throughout the world. This study aims to analyze heavy metal concentrations and identify heavy metal resistant bacteria in the bay of Manado. Sediment samples were collected from five bays in Manado. The concentrations of heavy metals As, Cd, Cr, Hg and Pb were analyzed using ICP-OES, and Hg using CV-AFS. Bacteria from the sediment were grown in nutrient broth media containing heavy metals As, Cd, Cr, Hg and Pb respectively. Microbiology and 16SrRNA gene analysis were used to identify the bacteria that grown on media containing varying concentrations of heavy metals. The results showed that the sediments from the five bays in Manado contained heavy metals with an average concentration of As <1mg/kg, Cd 1.8mg/kg, Cr 6.2mg/kg, Hg <0.07mg/kg, and Pb 11.2mg/kg. The results of microbiological and molecular analysis showed that 5 species of heavy metal resistant bacteria were Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus arlettae, Acinobacter sp., and Brevibacterium sp. The five bacteria found to be resistant to heavy metals can be used to detoxify As, Cd, Cr, Hg, and Pb.

1. Introduction
Heavy metal pollution in rivers and sediments has been a serious concern for a long time due to the negative impact on health. The increase in heavy metal pollution has had a real impact on the life of invertebrates, fish and humans [1]. The maintenance of water quality and sediment is a major problem in fast-growing cities as hygiene structures do not grow with population growth and urbanization [2]. Heavy metals are found in water, are persistent, bioaccumulated, and toxic to the environment [3,4].
The abundance of heavy metals in the aquatic environment comes from natural sources and as a result of human activities. Therefore, it is necessary to take action to control and determine the amount of metal content in the aquatic environment. A large number of toxic heavy metal deposits are discharged into the environment due to anthropogenic activities [5,6], as well as through natural processes that also contribute to metal contamination in the aquatic environment [7,8].

Based on a toxicological point of view, heavy metals such as Hg, Cd, Pb, Cr, and others are non-essential, with no known benefits in the body or that can even be toxic. This heavy metal can cause health effects for humans depending on where the heavy metal is bound in the body. The toxic power that is owned will work as a barrier to the work of the enzyme, so that the body's metabolic processes are cut off [9]. Furthermore, this heavy metal will act as a cause of allergies, mutagens, teratogens or carcinogens for humans. The entry route can be through the skin, respiration and digestion [10] (Kristianingrum, 2007).

So far, the methods used to determine the levels of heavy metals are atomic absorption spectroscopy (AAS), chromatography and UV-Vis spectroscopy. However, the AAS method cannot be used for simultaneous analysis even though it has a low detection limit, while the UV-Vis chromatography and photometric techniques are used to add complexing agents, or derivatization so that the technique is relatively more complicated. The Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) method has the ability to measure analytes simultaneously, with high sensitivity, with low analyte detection limits to ppb units and can be done easily and quickly. This technique is widely used for the analysis of heavy metals found in aquatic environments, as demonstrated by [11, 12]. Cold vapor atomic flame spectrophotometry (CVAFS) is the preferred method for the analysis of mercury levels, in terms of sensitivity, selectivity and analytical productivity.

Heavy metals in the environment can cause a decrease in the microbial population both in quantity and species diversity. However, there are often certain types of microbes that are resistant or tolerant of the presence of heavy metals. Several microbes have developed efficient systems for metal detoxification. The mechanisms can be classified into 5 categories: intracellular absorption, export, reduction, extracellular absorption and extracellular detoxification. Almost all bacterial resistance gene mechanisms are encoded in plasmids and transposons, allowing for the spontaneous transfer of genes or mutations that cause bacteria to be metal-resistant [13].

Several bacteria have been found from aquatic environments that are resistant to heavy metals, including Bacillus sp. [14], Gemella sp. and Micrococcus sp. [15]. Pseudomonas aeruginosa and Proteus mirabilis were found to be resistant to mercury [16]. Stenotrophomonas sp. and Pseudomonas sp. isolated from Buyat beach exhibited resistance to arsenic [17].

Manado is the capital city of North Sulawesi Province, where 5 rivers flow into, where along the river there are community gold mining activities, agriculture, plantations, livestock, as well as urban residential communities. All of them can discharge waste into the river which can cause an increase in the concentration of heavy metals which are harmful to freshwater and marine organisms, even in humans who consume them. From the above background, it is necessary to analyze heavy metals and heavy metal resistant bacteria in sediments in the Manado bay.

2. Materials and methods
2.1 Research samples
Sediment samples from 5 bays or estuaries of Tondano river (A), Sario river (B), Talawaan river (C), Bahu river (D) and Kandou hospital waste (E), which passed through Manado City, were each taken at 3 points with a depth of 10 cm from the sediment surface. The samples were collected in sterile bottles, the pH of the sediment was measured, then the samples were stored in an ice box filled with ice gel, then transported to the PS Farmas laboratory, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University.
2.2 Heavy metal analysis
A total of 500 g of sediment samples from each sampling location were prepared and stored in sterilized tubes, sent to PT. Indonesian WLN Manado branch. There, the determination of the levels of heavy metals As, Cd, Cr and Pb using ICP-OES analysis techniques, and determination of Hg levels with the CV-AFS analysis technique.

2.3 Identification of heavy metal resistant bacteria
10 g of sediment samples were dissolved in 0.9% NaCl solution until the volume reached 100 mL, then filtered using Whatman No.1 filter paper. The filtrate was sequentially diluted in 0.9% NaCl solution. For the isolation and quantification of metal tolerant bacteria, a $10^{-4}$ dilution of each sample was added to nutrient broth (NB) (Merck) which contains one of the following metals: $K_2Cr_2O_7$ (Merck), Arsenic (Merck), PbCl$_2$ (Sigma), HgCl$_2$ (Sigma), with concentrations of 25, 50, 100, 200, 300, 400 mg/L, then incubated at 37°C for 24 hours. The growing bacterial colonies were observed. Each colony that grew in the culture with the highest concentration of heavy metals, and looked different from one another, was isolated and inoculated on nutrient agar (NA) with a T-shaped streak (3 quadrants), then incubated for 24 hours. The growing bacterial colonies were then observed. A pure culture is made by taking the bacterial isolate with a loop needle, then inoculated on agar slant in a zigzag pattern, then incubated for 1 x 24 hours at 37°C.

2.4 Microbiological identification of heavy metal resistant bacteria
Heavy metal resistant bacteria were identified microbiologically by morphological, physiological and biochemical tests consisting of indole test, citrate test, catalase test, carbohydrate fermentation test, and lysine decarboxylase test.

2.5 Identification of heavy metal resistant bacteria using the 16S rRNA gene
Isolation of genomic DNA was conducted using the Genomic DNA Mini Kit (Geneaid) method. DNA was stored at 2-8°C. Amplification was conducted using PCR combi block machine (whatman biometra Germany). The primary PCR process is the universal primer pair bact F1 (forward) and uniB1 (reverse). The mold used for amplifying this gene was the bacterial genomic DNA which had been isolated. Amplification by PCR technique was conducted with variations in reagent composition and modified PCR reaction conditions. PCR Reagent composition: ddH$_2$O 20uL, UniBI primer 30pmol/uL 1.5uL, BactFl primer 30pmol/uL 1.5uL, 2x MyTag HS Red Mix 25uL, 2uL Templates. PCR Reaction Conditions, those are Initial denaturation of 95°C 180", Denaturation of 95°C 30", Annealing of 72°C 30", extension of 72°C 90", and final extension 60". Molecular species identification was conducted by using nucleotide sequences from 16S rRNA gene sequencing through the Basic Local Alignment Search Tool (BLAST) method through NCBI online media, then to see the kinship of each isolate, online alignment was carried out through the website http://expasy.org/tools/.

3. Results and discussion
3.1 Heavy metal analysis
The results of the examination of heavy metal levels from five sediment sampling locations showed that almost all of the sediment samples examined did not meet the requirements for Class II waters in PP no. 82 of 2001. The results of heavy metal analysis in sediments are shown in Table 1.

| Heavy metal | Heavy metal concentration (mg / L) |
|-------------|----------------------------------|
|             | A  | B  | C  | D  | E  |
| As          | 0.75 | 0.68 | 0.57 | 0.77 | 0.65 |
| Cd          | 1.85 | 1.68 | 1.82 | 2.06 | 1.77 |
| Cr          | 7.3  | 8.4  | 5.9  | 5.9  | 3.6  |
| Hg          | 0.03 | 0.02 | 0.05 | 0.03 | 0.13 |
| Pb          | 13.6 | 8.6  | 14.3 | 11.6 | 7.8  |
As illustrated in Table 1, the average examination of heavy metal levels in five bays in Manado revealed that arsenic concentrations remained within the standard limit of < 1 mg/L. The results of this study showed an increase in arsenic levels when compared to the study conducted by Lasut et al. and Maabuat et al. [18, 19], who obtained results with concentrations of 0.0012 and 0.0002 ppm, respectively. However, arsenic levels still meet the requirements stipulated in Government Regulation Number 82 of 2001 concerning Water Quality Management and Water Pollution Control, namely 1 mg/L for class II water, which is designated for water recreation infrastructure/facilities, freshwater fish farming, livestock, water to irrigate crops, and or other uses which require the same water quality as the said use.

In the aquatic environment, arsenic is found in two forms, namely as pentavalent arsenate, As (V), and trivalent arsenite, As (III). The uptake in sediment depends on the form of arsenic. Under anaerobic conditions, microbial activity can form arsenic into gaseous methyl arsenic and enter the atmosphere [20]. Arsenic is known as a carcinogenic compound. Arsenic poisoning cannot be seen immediately, so the most likely action is prevention [21].

Cadmium concentration at five locations ranged from 1.68 - 2.06 mg/L. Thus the cadmium level exceeds the threshold set in Government Regulation Number 82 Year 2001, namely 0.01 mg/L. Cadmium has a very unique effect on children because it helps brain development. But on the other hand, it has a negative impact on adult humans which can increase the risk of breast, cardiovascular, lung and heart cancer. Other effects include kidney failure, rheumatism, arthritis and bone damage [22]. Cadmium can undergo biotransformation and bioaccumulation in living organisms. Cadmium will accumulate in the human body, and can only leave the body after 20-30 years [23].

The chromium concentration in the five samples ranged from 3.6 to 8.4 mg/L. Thus, the chromium concentration at the five estuary locations in Manado has exceeded the threshold set in Government Regulation Number 82 Year 2001, namely 0.05 mg/L. Chromium in water comes from nature in very small amounts, such as the process of rock weathering and run-off from land; it can increase in large numbers due to human activities such as industrial activities, household waste and other activities through waste that enters the water [24].

The concentration of mercury in the sample, on average, exceeds the threshold set in Government Regulation Number 82 of 2001, which is 0.002 mg/L only. The highest concentration is in the sample from location E. The high concentration of mercury is likely influenced by hospital waste, where the use of a thermometer temperature measuring device generally contains mercury metal. High concentrations of mercury in the biosphere will accumulate in the environment causing poisoning to various organisms, especially humans and animals, so that it can have an impact on humans in the form of physiological disorders, nervous system disorders, growth disorders and kidney disorders. But in the human body there are bacteria that are resistant to mercury. Although exposed to mercury directly for a long time, such as in seafood which is often consumed by humans such as fish, it does not have a negative impact or cause symptoms of mercury poisoning [25, 26].

The lead concentration in the five samples had exceeded the allowable threshold, which ranged from 7.8 to 14.3 mg/L, exceeding the threshold set in Government Regulation Number 82 of 2001, namely 0.03 mg/L. Lead is widely used as a base material or catalyst in the process of making electronic goods. Ceramic furniture that contains a mixture of white and black lead, such as plates, bowls, and others, is a source of lead pollution that can be carried by rainwater into the aquatic environment. The impact of lead in the body is that it can cause damage to the kidneys, liver, central nervous system and reproductive system. Lead can also increase blood pressure, cause disturbances in the respiratory and digestive tracts, and can inhibit the formation of hemoglobin in the blood, which can cause anemia [27].

The growth of bacteria on NB media containing each heavy metal is shown in Table 2. The concentrations for each of the heavy metals were 25, 50, 100, 200, and 300 and 400 ppm. All bacterial isolates grew at a concentration of 50 mg/L on the five heavy metals. This shows that all isolates are resistant to all heavy metals. According to Chojnacka that bacteria isolated from environments contaminated with heavy metals are bacteria that have high resistance to heavy metals around them [28].
Table 2. Bacterial growth on NB media supplemented with various concentrations of heavy metals

| Heavy metals | 50 mg/L | 100 mg/L | 200 mg/L | 300 mg/L |
|--------------|---------|----------|----------|----------|
| As           | ++      | +        | -        | -        |
| Cd           | ++      | ++       | ++       | +        |
| Cr           | ++      | ++       | +        | +        |
| Hg           | +       | -        | -        | -        |
| Pb           | ++      | ++       | +        | +        |

Microbes that live in metal-rich environments tend to be more resistant to heavy metals than those that live in non-metal-rich environments [29]. This resistance is through an adaptation mechanism in bacteria to metals, namely the presence of RND (resistance, nodulation, cell division) protein which regulates metal transport through the cell membrane [30] (Nithya, 2011). This is due to differences in chromosomal, transposon, and plasmids that regulate the resistance system in each bacteria [31].

3.2 Results of identification of heavy metal resistant bacteria

The morphological test results (Table 3) showed that of the ten isolates, there were seven isolates in the form of bacilli and three coccuses, while the results of Gram staining showed that there were seven Gram positive isolates and 3 Gram negative isolates. The biochemical test results showed that all isolates were negative for the indole test, only 2 isolates tested positive for H$_2$S, a small proportion could form gas, most were positive for lysine, four isolates were positive for citrate and all isolates were positive for catalase test. The results of identification based on Bergey's Manual of Systematical Bacteriology, 2004, show that isolates Cd2, Cr1, and Hg2 belong to the genus Pseudomonas, isolates Cd3, Cr2, Hg1, Cd1, As1, Pb1, and As2 are of the genus Bacillus. Microbiological identification is only a preliminary test in determining bacterial species, therefore for accurate species determination, 16SrRNA gene analysis was carried out. The results of the 16SrRNA gene analysis of ten heavy metal resistant bacterial isolates can be seen in Table 4.

Table 3. Results of the morphological, physiological and biochemical analysis of bacteria

| No. | Isolate code | Cell shape | Gram | Motility | Indol | H$_2$S Gas | Carbohydrate fermentation | Lysin | Citrate | Catalase |
|-----|--------------|------------|------|----------|-------|-------------|---------------------------|-------|---------|----------|
| 1.  | Cd2          | coccus     | -    | -        |       | -           | -                         | +     | +       | +        |
| 2.  | Cd3          | basil      | +    | -        | -     | -           | -                         | +     | -       | +        |
| 3.  | Cr2          | coccus     | +    | +        | -     | -           | -                         | -     | +       | +        |
| 4.  | Hg1          | basil      | +    | -        | -     | +           | +                         | -     | +       | +        |
| 5.  | Cd1          | coccus     | +    | -        | -     | -           | -                         | -     | -       | +        |
| 6.  | Cr1          | basil      | -    | -        | -     | -           | -                         | -     | +       | +        |
| 7.  | As1          | basil      | +    | +        | -     | -           | +                         | -     | -       | +        |
| 8.  | Pb1          | basil      | +    | +        | -     | +           | +                         | -     | +       | +        |
| 9.  | Hg2          | basil      | -    | +        | -     | -           | +                         | -     | -       | +        |
| 10. | As2          | basil      | +    | -        | -     | -           | +                         | -     | +       | +        |

The results of molecular identification using the 16S rRNA gene showed that the arsenic resistant bacterial isolates As1 and As2 were similar to *Bacillus cereus* strain BXC15 and *B. cereus* strain WHX 1, respectively. Ghosh et al. reported the presence of *Bacillus* sp from the riverbed of Brahmaputra in India, which can be used to bio-transform arsenic [32]. Cadmium resistant isolates, Cd2, Cd3 and Cd1, each have similarities with *Pseudomonas aeruginosa* strain PAC6, *B. cereus* strain WHX 1 and *B. cereus* strain MD152, respectively. Sinha and Mukharjee reported that *P. aeruginosa* KUCd1, which was resistant to Cd, could be used to detoxify Cd in the environment [33].
The isolates that were resistant to chromium, Cr1 and Cr2 each had similarities with *P. aeruginosa* strain PAC6 and *Staphylococcus arlettae* strain IHB B. Munawaroh et al. reported that *P. aeruginosa* can reduce the toxic Cr(VI) compound to the less toxic Cr(III) as much as 94.73% within 48 hours [34]. Sagar et al. reported that *S. arlettae* was able to reduce 98% Cr (VI) to Cr (III) within 24 hours [35].

**Table 4.** The results of identification of heavy metal resistant bacteria using the 16SrRNA gene

| Isolate Codes | Species | Maximum Identity | Query Cover |
|---------------|---------|------------------|-------------|
| Cd2           | *Pseudomonas aeruginosa* strain PAC6 | 99.3% | 100% |
| Cd3           | *Bacillus cereus* Strain WHX 1 | 100% | 100% |
| Cr2           | *Staphylococcus arlettae* strain IHB B | 100% | 99% |
| Hg1           | *Bacillus cereus* strain PJA4.2 | 100% | 100% |
| Cd1           | *Bacillus cereus* Strain MD 152 | 99.81% | 100% |
| Cr1           | *Pseudomonas aeruginosa* strain PAC6 | 99.93% | 100% |
| As1           | *Bacillus cereus* strain BXC15 | 100% | 100% |
| Pb1           | *Brevibacterium sp.* strain MN3-3 | 99.80% | 100% |
| Hg2           | *Acinetobacter sp.* strain f19 | 99.85% | 100% |
| As2           | *Bacillus cereus* strain WHX 1 | 100% | 100% |

Mercury resistant bacterial isolates, Hg1 dan Hg2, each has similarities with *B. cereus* strain PJA 4.2 and *Acinetobacter sp.* strain f19, respectively. Fatimawali et al. succeeded in isolating *Bacillus sp.* from community mining waste in North Sulawesi [16]. The genus *Bacillus* was most abundant in sediments contaminated with mercury, indicating that this genus was resistant to environments with high mercury concentrations [36]. Pushkar et al. stated that the genera *Bacillus, Enterobacter, Klebsiella* and *Acinetobacter* can be used for bioremediation of mercury from river water [37]. Pb-resistant bacterial isolate (Pb1 isolate) has similarities with *Brevibacterium sp.* strain MN3-3. Ojoawo et al. stated that the species *Brevibacterium* sp. can be used for bioremediation of Cu, Zn, and Mg but is still resistant to Pb [38]. According to the data presented, the ten bacterial isolates isolated from Manado Bay have the potential to be used for bioremediation of heavy metals in the environment.

**4. Conclusion**

Along with industrialization and anthropogenic activities in urban environments, water bodies have been subjected to heavy metal pollution. The results showed that the sediments obtained from Manado Bay, North Sulawesi, Indonesia, were contaminated with heavy metals As, Cd, Cr, Hg and Pb. The sediments examined in this study contain bacteria that are resistant to these heavy metals, namely *B. cereus, P. Aeruginosa, S. arlettae, Acinetobacter sp.*, and *Brevibacterium sp.* The five species of bacteria have the potential to be used for detoxification of heavy metals As, Cd, Cr, Hg and Pb in the environment.

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