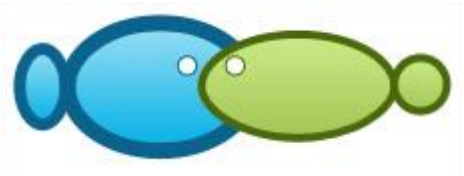

Study on the survival of *Eucheuma denticulatum* cultured in a controlled container with added mercury chloride ($HgCl_2$)

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Abstract. The purpose of the study was to examine the survival of red alga *Eucheuma denticulatum* cultivated in a controlled container with the addition of $HgCl_2$ compounds at concentrations of 0 (control), 1, 2.5, and 5 ppm $HgCl_2$. The research was conducted using an experimental method with four treatments, each with three replications. After the acclimatization process, the macroalga was continued to be cultivated for 28 days (4 weeks) in the Laboratory of Aquaculture Technology of the Faculty of Fisheries and Marine Science, Sam Ratulangi University. Analysis of mercury content in algae was carried out using the mercury analyzer method. Analysis of tissue morphology and elements contained in alga using Scanning Electron Microscopy (SEM) and Energy-Dispersive Spectroscopy (EDS). Isolation of mercury-resistant bacteria was also carried out with several types of tests, namely physiological and biochemical tests and identification using Gram Stain. The results of the study showed that in the first week of the algae cultivation process, there was a disturbance in the algae metabolism process, so the color of the treated algae changed to pale compared to the control, which could grow with increased weight and thallus growth. Mercury content in the second week at aquaria, with concentrations of 1, 2.5, and 5 ppm, was 54.94 ppm, 202.45 ppm, and 155.52 ppm, respectively, while the control did not change since the first week, namely 16.27 ppm. SEM analysis at 5,000x magnification in the first week of adding five ppm of $HgCl_2$ showed differences in the morphological structure of the surface of algae tissue, which was irregular and tended to be damaged, as compared to the control. The results of the analysis of minerals contained in algae with five ppm $HgCl_2$ treatment were carbon, oxygen, sodium, magnesium, and chlorine. The aluminum element was only present in the control. Absorption and accumulation of chlorine occurred at 5 ppm-$HgCl_2$ treatment. Chlorine content increased to 20.70%, while at control, the content was 11,75%. In *E. denticulatum*, there were *Bacillus* spp., which are resistant to mercury and still grow up to 14 CFU g^{-1} at a concentration of 1000 ppm. *Bacillus* spp. can absorb mercury (Hg^{2+}), thus reducing it into a relatively non-toxic form

Key Words: macroalgae, mercury analysis, SEM, EDS, mercury-resistant bacteria.

Introduction. *Eucheuma denticulatum* is one of the red algae used as a raw material in producing carrageenan (Nurmilla & Aprillia 2021) and is widely used as a food additive (Herawati 2018), cosmetic (Tumbelaka et al 2019) and pharmaceutical (Naseri et al 2020). This species can adapt to polluted marine environments, has good economic value, and is commonly cultivated in the marine waters of North Sulawesi (Yuniarsih et al 2014).

E. denticulatum is one of the macroalgae found a lot in Indonesian waters. Macroalgae are differentiated into three kinds: red algae (Rhodophyta), green algae (Chlorophyta), and brown algae (Phaeophyta) (Kepel et al 2012). Macroalgae are dispersed in the marine waters along the Minahasa Peninsula, North Sulawesi (Kepel et al 2020). They have ecological functions and industrial benefits. *Eucheuma* sp. is a red algae that has the potential to be utilized in aquaculture and industry (Novianty et al 2022). Human activities such as mining may result in environmental pollution. In the case of Totok Bay waters, gold mining at the upstream river supplied high waste of gold processing, including sediment and metal that polluted the environment, such as mercury (Hg). Mercury entering the marine ecosystem has a toxic property that, in turn, disrupts

the physiological process of marine organisms, including algae (Mantiri et al 2019). Several studies have been carried out in these waters to determine the concentration of heavy metals, especially in macroalgae and sediments where the algae grow. The researchers' concerns arise if the metal enters the food chain in waters; it could be transported to the human body through the food chain, and it is dangerous for human health. Mantiri et al (2018) reported that metal was detected in algae in the waters of Totok and Blongko Bay. Another research by Nasprianto et al (2019) also found that in Totok Bay, mercury concentration in sediment was 2.6 ppm, and in macroalgae was 0.74 ppm.

Research on the ability of *E. denticulatum* to survive in mercury chloride ($HgCl_2$) contaminated water in controlled containers is important to be conducted. This research is crucial to determine how far the alga can survive in polluted environmental conditions. The objective of the study was to evaluate the survival of *E. denticulatum* cultivated in a controlled container with the addition of $HgCl_2$ as treatment with different concentrations. The purpose of the research was to evaluate the potential of *E. denticulatum* as an environmental bioremediation agent.

Material and Method

Preparation of culture container. *E. denticulatum* taken from Arakan waters was transported to the Aquaculture Technology Laboratory of Sam Ratulangi University. After being weighed, the alga was placed into four aquaria with different treatments. Water quality including pH, salinity, temperature, and dissolved oxygen was measured daily, while weighing and measuring the algae were carried out every seven days. The growth of algae was measured by weighing the initial wet weight and the final wet weight

The containers used for cultivating macroalgae were 4 glass aquaria measuring 40x40x40 cm each. The thickness of the glass was 5 mm. Sea water and sediment were obtained from Likupang waters in front of the Marine Field Station of the Faculty of Fisheries and Marine Science, Sam Ratulangi University. Before being used in the experiment, the water was filtered using a pump and then sterilized in an autoclave at 121^{o}C for 30 minutes, while the sediment was cleaned and then sterilized in an oven at 100^{o}C for 30 minutes. Each aquarium was equipped with an aerator, a thermometer, a rope to tie the macroalgae, and a 3000-lux light. The aquaria were also marked for water height control (Alamsjah et al 2010).

Sample collection. Sample of red macroalgae was gathered from Arakan Village, South Minahasa, placed in a cool box containing seawater, and then transported to the Laboratory of Aquaculture Technology of the Faculty of Fisheries and Marine Science, Sam Ratulangi University. The macroalga obtained was *E. denticulatum.* The macroalga was then identified morphologically using the identification guide of Trono (1997). Before starting the cultivation process, the macroalga was acclimatized for three days to adapt to the new environment. Macroalga, water, and sediment were first analyzed for mercury metal content.

Culture of red macroalgae in the controlled container. After the acclimatization process, the macroalga was continued to be cultivated for 28 days (4 weeks) in the Laboratory of Aquaculture Technology of the Faculty of Fisheries and Marine Science, Sam Ratulangi University. The research used four treatments consisting of 0 (control), 1, 2.5, and 5 ppm, each with three replications (Figure 1). In each aquarium, there were 100 g macroalgae tied at a rope. The culture used Walne media, as much as 1.5 ml $L^{-1,}$ as a nutrient for macroalgae.

Observation was done daily to notice the color change of the macroalga and to control the water temperature and the water volume. Measurement of water quality and collection of macroalga samples were conducted every week at the same time. Samples of macroalga taken were dried using a spinner, weighed using an analytical balance, and measured their length with a ruler (cm).

Further analysis. For further analysis, macroalga samples were taken at week 1, 2, 3, and 4. Analysis of mercury content was carried out on the macroalgae thallus using the Mercury Analyzer method. Identification of bacterial species in the thallus and analysis of mercury-resistant bacteria in the thallus were carried out on the control and treatment with 5 ppm $HgCl_2$. Analysis of cell surface was done using Scanning Electron Microscopy (SEM) with a resolution of 1024x768 pixels, magnification of 5,000 times, and working distance (WD) of 11 mm, and mineral analysis in macroalgae was conducted using Energy-dispersive spectroscopy (EDS). The analysis was conducted at the Integrated Research and Testing Laboratory, Gajah Mada University, Jogjakarta.

Result and Discussion

Growth and color changes of macroalgae in cultivation containers. In the control treatment, the alga increased in weight by 3.5 g in week 1, and in week 2, it increased by 0.5 g, then it decreased by 6.2 g in week 3. The alga decreased in weeks 2 and 3 because of the adaptation process to the new environment. However, in week 4, there was an addition of 1.7 g. In 1 ppm $HgCl_2$ treatment, there was a drastic weight reduction of 26.3 g in week 1, the decrease occurred until week 4, namely 32.4 g. At treatment 2.5 ppm $HgCl_2$, there was a decrease in weight as much as 17.6 g in the 1st week, then continued to decrease until the 4th week of 32.7 g. Treatment of 5 ppm $HgCl_2$ also resulted in a decrease in weight as much as 10.7 g in week 1, while in weeks 3 and 4, there was a decrease in weight of 76 g, meaning there was no growth (Figure 1). In the control treatment, thallus growth was indicated by an increase in thallus branches (Figure 2).

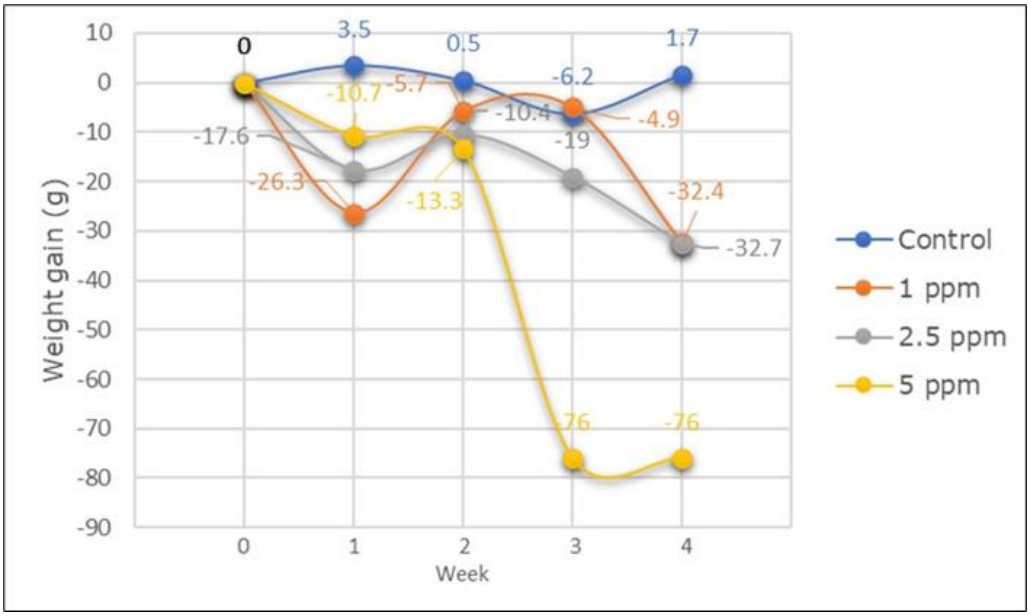

Figure 1. Weight gain of the alga measured each week.

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Figure 2. Growth of thallus in control treatment at day 7.

It was concluded that *E. denticulatum* cultivated with control treatment displayed an increase in weight indicated by the growth of new thallus (Figure 2), while in all treatments added with the $HgCl_2$, the weight of algae decreased due to the presence of toxic mercury in the form of $HgCl_2$. Mercury exposed to algae can inhibit physiological processes such as photosynthesis and cell division. A similar result had been reported by Simanjuntak et al (2016), where microalgae *Botryococcus braunii* exposed to 2 ppm $HgCl_2$ showed a significant decrease in the number of cells on the eighth day, indicated by the number of cells 1.9 x 10^4 cells mL^{-1} as compared to the control.

The color of algae at the time it was taken from Arakan Village was reddish brown. The color seemed to change after adding the $HgCl_2$ compound as a treatment. The color changed from brownish to pale, but in week 2, the color changed to green, especially in the alga treated with $HgCl_2$. It was assumed that the cells of the alga grew again until week 4. In red algae, there are pigments chlorophyll a and d, carotenoids, and phycoerythrin (Mantiri et al 2019). When algae cells are damaged due to the addition of mercury beyond the threshold established by the *International Organization for Standardization* (ISO), the chlorophyll will be denatured gradually, but the carotenoid pigments remain. Thus, the brownish color is still there. The toxicity of mercury inhibits enzymes involved in chlorophyll biosynthesis, such as the ALA-synthase enzyme. The decrease in chlorophyll production has a direct influence on the green color degradation. Mercury can inhibit the synthesis of photosynthetic pigments such as chlorophyll and fucoxanthin. Fucoxanthin gives a specific brown color to macroalgae. When the production of fucoxanthin decreases, the color of the algae can change to paler or green due to the dominance of the remaining chlorophyll pigment. In red algae *Gracilaria* sp., mercury exposure caused a color change from reddish brown to pale green or even white (bleaching) (Dwiyanti & Muahiddah 2023).

Table 1

Observation of growth and color change of algae in cultivation aquaria

Days	*Control*	*1 ppm*	*2.5 ppm*	*5 ppm*
0				
1				
7				
14				
28				No growth

Monitoring water quality during the cultivation process of Eucheuma denticultum. Suitable seawater quality is an important factor in the cultivation process of algae. In this study, the water quality parameters monitored were water temperature, salinity, pH and dissolved oxygen (DO). Water chemical parameters ideal for grass growth are phosphate in the range of 0.0057-0.0185 mg L^{-1}; pH 6.8-9.6 (Rukka et al 2022), salinity 22-32 ppt (Suniada et al 2014), and optimum salinity for seaweed growth is 28-34 ppt (Astriana et al 2019). According to Pusvariauwaty et al (2015), the appropriate DO for seaweed growth is greater than five ppm. Water quality during this research is still good for the cultivation process, except in containers treated with $HgCl_2$, where the pH value decreased compared to the control (GR No. 22 2021). Algae exposed to mercury can cause oxidative stress that damages cell membranes, consequently triggering the release of acidic compounds from the algal cytoplasm into the surrounding medium, lowering pH levels.

Table 2

Monitoring of water quality during the cultivation process of *Eucheuma denticulatum*

Parameters	*Control*	*1 ppm*	*2.5 ppm*	*5 ppm*
Temperature (^{0}C)	27.43	27.46	28	27.79
Salinity (ppt)	33.07	32.64	32.75	32.54
Potential of hydrogen pH	7.20	6.85	6.71	6.74
Dissolved oxygen DO (mg L^{-1})	12.33	10.53	9.39	9.1

Mercury content of Eucheuma denticulatum. The mercury content in algae obtained from the waters around Arakan Village was found to be 16.27 µg g^{-1} (control). At one ppm $HgCl_2$ treatment, the mercury content of algae in the 1st week was 109.62 mg Kg^{-1} and in the 2nd week was 54.94 mg Kg^{-1}. At 2.5 ppm $HgCl_2$ treatment, the mercury content at week 1 was 129.29 mg $Kg^{-1,}$ and week 2 was 202.45 mg Kg^{-1}. Meanwhile, at five ppm $HgCl_2$ treatment, the mercury content was 160.58 mg Kg^{-1} at week 1 and 155.52 mg Kg^{-1} at week 2 (Figure 3). These results proved that *E. denticulatum* can absorb the mercury present in water. The increase in mercury concentration at weeks 1 and 2 occurred due to the bioaccumulation mechanism. Bioaccumulation is an absorption process of chemical compounds, such as heavy metals from an environment that is faster than the ability of algae to remove those chemicals so that the concentration of mercury in algae increases. Previous research by Hosea et al (2019) found that at week 3, lead (Pb) as much as 1.49 mg Kg^{-1} was detected in *Kappaphycus alvarezii* in nature.

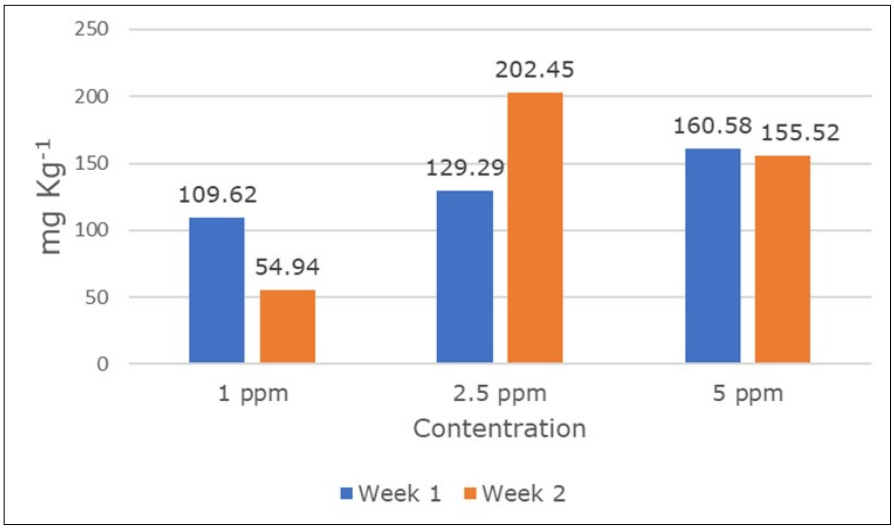
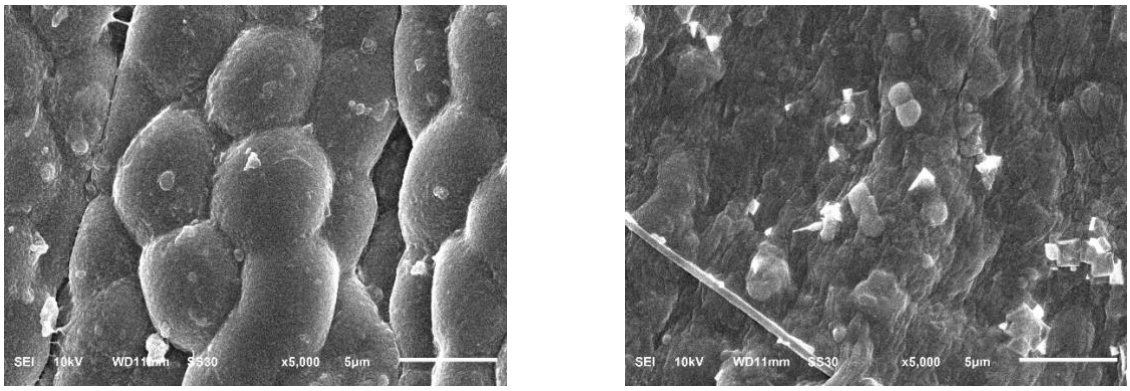


Figure 3. Mercury content of the cultivated *E. denticulatum*.

Analysis of surface morphology of macroalgae. The result of the Scanning Electron Microscope (SEM) is a type of electron microscope that uses an electron beam to scan the surface of a sample. SEM is based on transferring (or rastering) an electron beam across a sample and detecting the electrons emitted from the surface. The incoming electrons are either backscattered from the atomic surface species or diffuse into the sample material. SEM is used primarily to characterize the surface topography of materials (Figure 4).



M-1 P0 **M-1 P5**

Magnification 5,000x (a) Magnification 5,000x (b)

Figure 4. Results of cell surface analysis of *E. denticulatum* algae without treatment and treatment at 5,000x magnification (M-1 P0: alga cultivated at the first week without the addition of $HgCl_2$; M-1 P5: alga cultivated at the first week with the addition of 5 ppm $HgCl_2$ as treatment).

Figure 4a displayed clearly the structure of alga cells in the control, which is round or oval and close to each other. These cells arrange in colonies and tissues. The surface of the cells appears smooth with several small structures attached, which are probably particles, debris, or other microorganisms. A scale of 5 µm indicates that each cell has a few micrometers in size. SEM allows to observe of microstructure of cells such as cell walls, pores, and surface patterns. The surface texture looks fairly uniform, with a few bumps or particles in some areas. This texture can indicate characteristics of the algal cell wall, such as the presence of polysaccharides or a protective layer.

Compared to Figure 4a, Figure 4b displayed the surface of cells looks rougher, uneven, and apparently there was damages or deformation in the cell walls. This damage occurred due to mercury toxicity which reacts with cell wall components, such as proteins, lipids or polysaccharides. The picture clearly shows small particles or structures attached to the surface of the cells. This small structure is likely an interaction between Hg^{2+} and organic molecules in the cell wall. The cell surface appears to be more irregular with bulge and depressions. This can indicate environmental stress experienced by algae due to the addition of mercury. There is a long structure like fibers at the bottom left of the image. This is an organic component released from algal cell walls due to mercury exposure.

Hg^{2+} from $HgCl_2$ can damage the structure of membranes and cell walls through reactions with thiol groups (-SH) on proteins or enzymes, and oxidative stress due to the formation of reactive oxygen species (ROS). Algae are able to endure, probably due to resistance mechanisms occur, such as the deposition of mercury in cell walls or the binding of Hg^{2+} with certain compounds to reduce toxicity. The image above shows significant changes on the surface of the algae after treated with $HgCl_2$. These changes reflect the effects of mercury toxicity, which induces structural damage to the cell wall. Research by Tumembouw et al (2022) showed that there are changes in the shape of the cell structure of the *Kappaphycus alvarezii* cultivated with different organochlorine treatments. This showed that toxic materials in waters can change the surface structure of algae cells.

Energy dispersive X-Ray spectroscopy (EDS). EDS spectrum describes x-rays of various macro and micro elements in the form of an energy spectrum which is important in identifying the concentration of elements such as sodium, magnesium, silicon, phosphorus, sulfur, chloride, potassium, calcium, manganese, iron and zinc. The results of the EDS analysis of *E. denticulatum* were shown in Figure 5 while the elements were presented in Table 3.

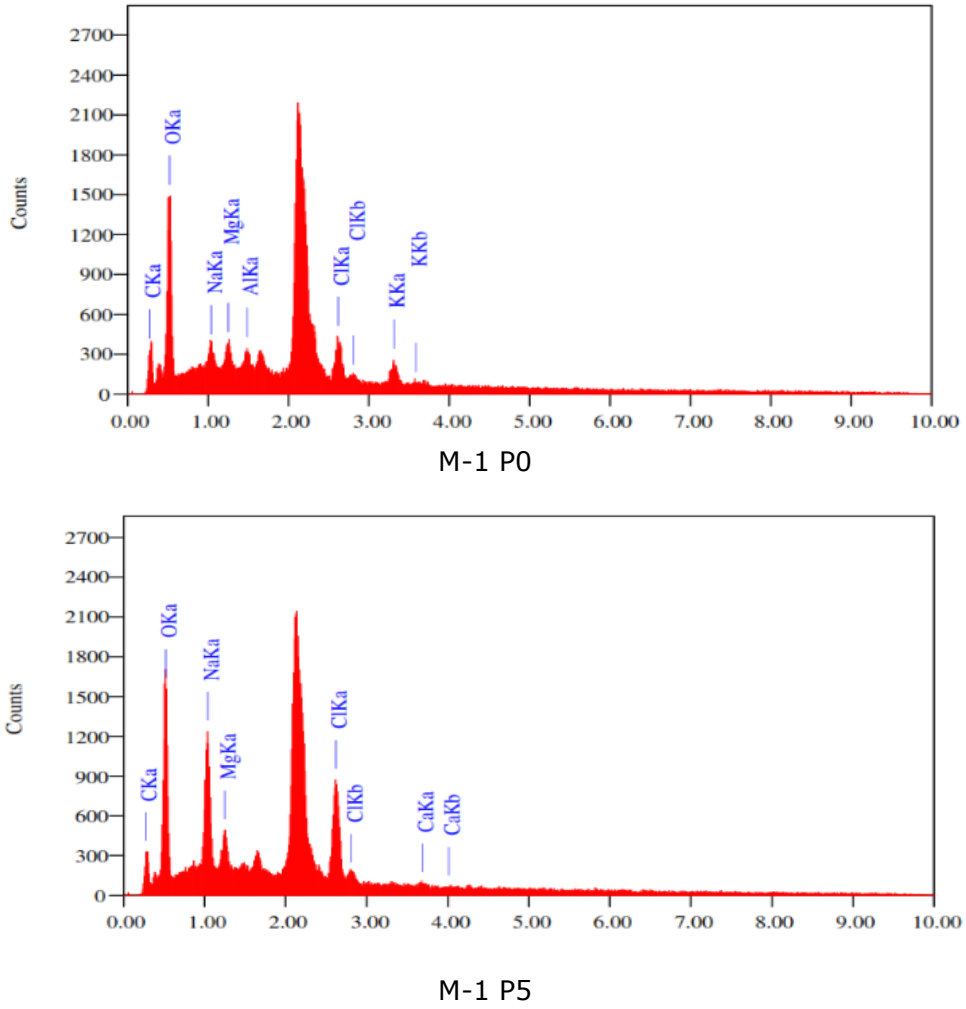


Figure 5. EDS analysis of *Eucheuma denticulatum* cultivated in a controlled container (M-1 P0: alga cultivated at the first week without the addition of $HgCl_2$; M-1 P5 = alga cultivated at the first week with the addition of 5 ppm $HgCl_2$).

Table 3

Analysis of elements in *Eucheuma denticulatum*

Elements characteristic	*Elements concentration*	
	M-1 P0	*M-1 P5*
	Mass (%)	*Mass (%)*
Carbon (C)	15.28	15.97
Oxygen (O)	54.02	44.33
Sodium (Na)	3.13	12.96
Magnesium (Mg)	3.67	4.07
Aluminium (Al)	2.82	-
Chlorin (Cl)	11.75	20.79
Potassium (K)	9.32	-
Calcium (Ca)	-	1.88
Total	100%	100%

Figure 5 and Table 3 showed a mineral composition found in *E. denticulatum* cultured in the first week without an $HgCl_2$ addition by a mass order from the highest to the lowest, which is O>C>Cl>K>Mg>Na>Al. The calcium is none-detected, while on the algae cultivated in the first week with 5 ppm $HgCl_2$ addition, the mineral component from the highest to the lowest is O>Cl>C>Na>K>Mg>K. On the other algae, the mineral composition is relative have no similarities, and it depends on the treatment. The mineral composition showed that the analysis results of EDS of the algae *Ulva lactuca* cell wall C>O>Mg>S>Si>Ca>Na>Al>K>Cl>Se (Reka et al (2017). On brown algae *Turbinaria decurrens* and *T. ornata* contain elements of biomineral compounds dominated by C of 60% and 52.8%, and O 29.7% and 29.3% (Kepel et al 2025). The result in Table 3 showed that the carbon element and oxygen relatively did not change much between the control and the treatment. This is because algae can absorb and store carbon in unfavorable aquatic environmental conditions. Li and Yao (2024) determined that algae may be adaptive to various environmental conditions, including their growth abilities in waste liquid. Mercury cannot be detected with this tool. Chlorine was detected to increase 2 times higher in the 5 ppm treatment. Chlorine is an element that, when heated, can produce several compounds, including mercury chloride. EDS analysis was carried out by heating using the tool used. Therefore, the results obtained by the treatment of adding five ppm $HgCl_2$ were around 2 times higher than those obtained in the control. The biosorption of metal ions relies on the unique surface features of the biomass, the concentration of these ions, and the physicochemical parameters of the solution. In recent years, microalgae have garnered great interest for their capacity to remove metals (temperature, pH, etc.) (Al-Hussieny et al 2014).

Mercury-resistant bacteria in Eucheuma denticulatum. Mercury-resistant bacteria are bacteria that can survive in environments contaminated with mercury, a heavy metal that is toxic to most organisms. This resistance usually occurs through genetic mechanisms, in which the bacteria have genes that allow them to neutralize, convert, or remove mercury from their cells (Barkay et al 2003). The bacterial colonies growing on algae treated with 0 and 5 ppm $HgCl_2$ for mercury-resistant bacteria were presented in Table 4.

Table 4

Total Hg-resistant bacteria in *Eucheuma denticulatum* treated with 0 and 5 ppm $HgCl_2$

No.	*Concentration Hg (ppm)*	*Test result of Hg-resistant bacteria*	
		0 ppm $HgCl_2$	*5 ppm $HgCl_2$*
1	Control (bacteria colony)	$1.6x10^2$	$2.4x10^2$
2	250	$3.9x10^1$	$7.3x10^1$
3	500	$2.4x10^1$	$3.1x10^1$
4	1000	$1.3x10^1$	$1.4x10^1$

Calculation of TPC in agar medium showed bacterial growth of 160 colonies g^{-1} (in control) and 240 colonies g^{-1} (in 5 ppm treatment). After adding a Hg concentration of 1,000 ppm, the bacteria still grew as much as 13 colonies g^{-1} (control) and 14 colonies (5 ppm). At 500 ppm Hg, the growth of bacteria was 24 colonies g^{-1} (control) and 31 colonies g^{-1}, while at the lowest concentration of 250 ppm Hg, Hg-resistant bacteria grew the most, that was 39 colonies g^{-1} (control) and 73 colonies g^{-1} (5 ppm). The existence of mercury-resistant bacteria is important in bioremediation. This organism can be used to reduce contaminants in the environment because it can help clean soil or water contaminated with mercury (Kotwal et al 2018). Research by Van Gobel et al (2021) shows that there were mercury-resistant bacteria in the brown algae *Padina australis*, which lives naturally in Kima Bajo waters. These bacteria also can resist mercury up to concentrations of 1,000 ppm.

Identification of the type of bacteria. Bacteria exhibit unique biochemical activities due to their varying enzymatic functions. Biochemical studies regarding the metabolic characteristics of bacteria are typically determined by the interaction between metabolites and the chemical reagents used. In this research, the identification of Hg-resistant bacteria at concentrations of 250, 500, and 1,000 ppm, along with controls through Gram stain and biochemical tests of algae cultivated under the control treatment (0 ppm $HgCl_2$ treatment), is presented in Table 5 below.

Table 5

Identification of bacteria in alga control treatment

No.	Isolate code	Morphological test		Physiological and biochemical test									Identification results
							Carbohydrate fermentation						
		Cell shape	Gram stain	Motility	Indole	H_2S	Gas	Glucose	Sucrose/ Lactose	Lysine	Citrate	Catalase	
1	Control	bacillus	+	+	-	-	-	+	-	+	-	+	*Bacillus* sp.
2	250 ppm	bacillus	+	-	-	-	+	+	-	-	-	+	*Bacillus* sp.
3	500 ppm	bacillus	+	+	-	-	-	+	-	+	-	+	*Bacillus* sp.
4	1000 ppm	bacillus	+	+	-	+	+	+	-	+	-	+	*Bacillus* sp.

Morphological tests of the four isolates showed that all the bacteria were bacilli and gram-positive. Physiological and biochemical motility tests on 250 ppm isolate showed the bacteria did not move and no visible growth in the area around the Ose needle puncture. Meanwhile, the bacterial isolates in the control, 500 and 1,000 ppm, were motile, meaning that the growth spread in the area around the Ose puncture. The results of the H_2S test showed that the 1,000 ppm isolate of bacteria was positive, meaning the bacteria were able to produce hydrogen sulfide. Bacillus spp. found in this research can survive in environments contaminated with mercury. The ability of *Bacillus* spp. to absorb heavy metal mercury into cells resulted in the accumulation of heavy metal mercury into the cells of this bacteria. The ability of this bacteria has the potential as a bioremediation agent to reduce mercury contamination (Uno & Thalib 2020).

Conclusions. *Basillus* spp. are found in red algae *Eucheuma denticulatum* and can absorb mercury chloride ($HgCl_2$), thus relatively reducing the toxicity of mercury in an environment. Identification of mercury-resistant bacteria within the algae is important and valuable to the bioremediation and environmental aspects. The research results can provide insight into the management of *E. denticulatum* cultivation in polluted marine environments and find out the interactions between marine organisms and heavy metals in an environment.

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