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RESEARCH ARTICLE

Euglena sp. as Mercury Phycoremediation Agent in FWS-CW System: Growth and Productivity, Photosynthetic Pigments, SOD Activity, and Equilibrium Kinetic Models

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Abstract Society now faces a problem with environmental pollution, primarily due to industrial pollution. Mercury is a poisonous pollutant with a widespread distribution that settles in ecosystems. Numerous traditional methods have been used to clean mercury contamination. Bioremediation is one potential and eco-friendly method of reducing toxicants by using organisms. Phycoremediation uses algae, such as *Euglena* sp., in its process. This research aimed to analyze the growth, productivity, photosynthetic pigments, and Superoxide dismutase activity of *Euglena* sp. and analyze the kinetic model of mercury content in *Euglena* sp. using Pseudo-First-Order and Pseudo-Second-Order equations in the Free Water Surface-Constructed Wetlands (FWS-CW) system. This research shows that *Euglena* sp. chlorophyll a, chlorophyll b, carotenoid, and total chlorophyll content decrease at the lowest level at a concentration of 15 ppm. SOD activity of *Euglena* sp. increased with the increase of mercury stress. However, it was insignificant for the concentrations of 5 ppm, 10 ppm, and 20 ppm. The growth and productivity of *Euglena* sp. decrease with the increase of mercury stress. These experiences happen because *Euglena* sp. carries out a detoxification process. The mercury phycoremediation process by *Euglena* sp. is more suitable with the Pseudo-Second-Order kinetic model with an R^2 value of 0.43. These results indicate that *Euglena* sp. can potentially be a phycoremediation agent of mercury with a maximum mercury concentration of 15 ppm.

Keywords: Chlorophyll, *Euglena* sp., Kinetic models, Mercury, Superoxide dismutase.

Introduction

Environmental pollution has become a problem for society in many nations, particularly developing countries, including Indonesia. Industrial pollution is primarily to blame for this environmental pollution [1]. Industrial anthropogenic activities such as smelting, coaling, burning, and mining can release heavy metal residue. Lead (Pb), Arsenic (As), Cadmium (Cd), Silver (Ag), Iron (Fe), Zinc (Zn), Chromium (Cr), Nickel (Ni), Copper (Cu), Platinum (Pt), Palladium (Pd), and Mercury (Hg) are the most prevalent and persistent pollutants in the environment [2;3].

Mercury (Hg) is a toxic and non-essential heavy metal that is silver in color, volatile, and odourless [4;5]. Mercury can be found in nature in three primary forms: elemental, organic, and inorganic. Among others, the mercury type that is the most common in the environment is inorganic mercury. Inorganic mercury can change form to organic mercury through the methylation process in anaerobic aquatic environments. This organic mercury is hazardous compared to other forms of mercury. This type of mercury can penetrate the cells of aquatic organisms [6].

Mercury has adverse effects on the environment and organisms. At high concentrations, combined with prolonged exposure, mercury can negatively influence the neurological development of the foetus, the

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endocrine, and the respiratory systems. In algae, mercury stress disrupts the cell division process, inhibiting growth. Mercury can also cause oxidative stress caused by increased Reactive Oxygen Species (ROS) [5]. This experience triggers cellular damage [7]. Excess mercury in algal cells can also cause degradation of photosynthetic pigments and abnormal enlargement of vacuole size [8]. Mercury exposure in *Aquarius palifolius* causes changes in nitrate reductase activity by 9.5%–13.5% and a decrease in leaf number by 50%–65% [9].Therefore, mercury exposure to the environment must be reduced to reduce its adverse effects.

Numerous traditional methods have been used to clean mercury contamination, a severe issue constantly worsening. However, this practice also has adverse effects, including high energy and costs, the release of hazardous byproducts, etc [10; 3] One of the potential methods to reduce toxicant exposure to the environment is phycoremediation [11]. Phycoremediation is a kind of bioremediation that uses macroalgae or microalgae in its process. This process does not require much energy, tends to be more cost-effective, reduces sludge formation; and algae have a faster growth rate than higher plants, can recycle nutrients, and have high biomass production [3]. Many studies also state that microalgae can grow in various stressful environmental conditions [12].

Euglena sp. is a microalgae that can be used in phycoremediation. *Euglena* sp. is a species of unicellular, eukaryotic algae with microscopic flagella and no cell wall that can live in contaminated aquatics [13;14]. *Euglena* sp. has a higher tolerance to heavy metals than other microalgae, so it can be used in the phycoremediation process. Microalgae-supported wastewater treatment is cost-effective, has low energy consumption, and is environmentally friendly, with high biomass yields for biofuel production [15].

In removing mercury, the mercury will be absorbed into the *Euglena* sp. cell wall and accumulated in the chloroplast, mitochondria, or cytoplasm for detoxification [16]. This process will affect growth and productivity. *Euglena* sp. has different mechanisms of resistance and removal of heavy metals [17]. When exposed to stress, *Euglena* sp. can stimulate ROS production [18]. As much as the level of stress increases, the level of ROS in the cell also increases. *Euglena* sp. will produce antioxidant enzymes to prevent the damaging effect of excess ROS production [7]. The first expressed antioxidant enzyme in microalgae was Superoxide dismutase (SOD). SOD has a function to break the toxic Superoxide ions inside the cell [19].

In addition to synthesizing antioxidant enzymes, the photosynthetic pigment is also one algae parameter sensitive to environmental alteration. Several kinds of photosynthetic pigment include chlorophyll a, chlorophyll b, and carotenoid. Chlorophyll is a photosynthetic pigment with a phyrol ring structure that catches light during photosynthesis [20]. Carotenoid is a photosynthetic pigment with antioxidant function [21]. The photosynthetic pigment is significantly affected by environmental stress. Chlorophyll content decreases in the heavy metal stress because of inhibition of its synthesis by metal stress. Carotenoid content is higher than chlorophyll in stress conditions because it has an antioxidant function that protects the photosynthetic apparatus.

This research used the Free Water Surface-Constructed Wetlands (FWS-CW) system. It is a system that builds with the surface flow and the height of the water shallow [22]. This system can reduce toxicants in aquatic environments through the sorption process of plants or algae. This research aimed to evaluate the kinetics model of this bioremediation and examine the growth, productivity, photosynthetic pigments, and SOD activity of *Euglena* sp. that live under mercury stress in the FWS-CW system. The kinetic model processes data in liquid waste treatment. It helps determine the variables and adsorption mechanisms involved [23]. The process can be evaluated using the Pseudo-First Order (PFO) and Pseudo-Second Order (PSO) models. The PFO model suggests that the adsorbate concentration exceeds the number of active sites on the adsorbent surface. The PSO model shows that the adsorption capacity of the adsorbate is proportional to the number of active sites on the adsorbent [23; 24;25].

Materials and Methods

The materials used in this research were *Euglena* sp. culture (Strain IDN-28), Cramer Myers medium, $HgCl_2$, filter paper, pyrogallol, ddH_2O, phosphate buffer 50 mM pH 7.4, Tris-Cl buffer 50 mM pH 8.2, EDTA buffer 1 mM pH 8.0, 70% alcohol, and distilled water.

Optimization of cultures condition

Euglena sp. cultures were grown with Cramer Myers medium in bottle culture. When the quantity of cultures was sufficient, they were moved into the FWS-CW reactor to be treated. The treatments in this

study consisted of giving $HgCl_2$ stress with concentrations of 0 ppm, 5 ppm, 10 ppm, and 20 ppm. The treatments were carried out for 14 days.

Cell Counting Measurement

The growth parameter of *Euglena* sp. was collected with the number of cells for every $HgCl_2$ treatment. The number of *Euglena* sp. cells were measured by counting cells using a hemocytometer. One ml of samples was taken and then placed into a hemocytometer. The hemocytometer was then observed using the Opti Lab application on the computer. The cell count was a cell in the five-medium grid inside the hemocytometer. The results of the measurement of the cell are then counted using the following equation:

$$\text{Cell density (cell/mL)} = \text{Count results} \times 5 \times 10^4 \text{cell/mL} \quad (1)$$

Biomass Measurement

The productivity parameter of *Euglena* sp. was collected with the biomass for every $HgCl_2$ treatment. Biomass of *Euglena* sp. was done by measuring dry weight. First, the filter paper was weighed with analytical scales. 5 mL of the sample was taken and placed on the filter paper. Filtrates were dried for 24 hours, and filter paper containing *Euglena* sp. biomass was weighed. The biomass measurement is calculated with the following equation:

$$\text{Biomass} = \text{End weight of filter paper} - \frac{\text{Early weight of filter paper}}{\text{Amount of sample}} \quad (2)$$

Chlorophyll Content

The *Euglena* sp. Samples were taken for 15 mL. The samples were then centrifuged at 3500 g for 15 minutes. The results were then extracted with 80% of acetone. Extracts were then taken for 2 mL and then measured spectrophotometrically with the wavelength of 663 nm, 645 nm, and 470 nm. The results are then counted with the following equations:

$$\text{Chlorophyll a} = 12.21A_{663} - 2.81A_{645}$$
$$\text{Chlorophyll b} = 20.13A_{645} - 5.03A_{663}$$
$$\text{Carotenoid} = \frac{(1000A_{470} - 3.27\ \text{Chlorophyll a} - 104\ \text{Chlorophyll b})}{229}$$
$$\text{Total Chlorophyll} = \text{Chlorophyll a} + \text{Chlorophyll b}$$

Superoxide Dismutase Assay

SOD activity was measured with the pyrogallol method. 15 mL of *Euglena* sp. samples were centrifuged at 3500 g for 15 minutes, and the supernatant was removed. The pellet was then added to sodium phosphate buffer solution and centrifuged at 10000 rpm for 15 minutes, 4℃. The results were then measured spectrophotometrically with other solutions needed, with the wavelength 325 nm for 5 minutes. The results of spectrophotometry methods were then counted using the following equation:

$$SOD = \frac{\frac{(dAb - dAs)}{dAb} \times 100\%}{50\%} \times 1.018 \times \frac{1}{8} \quad (3)$$

Equilibrium Kinetic Models

Equilibrum kinetic models measured with Mercury Analyser. Sample of mercury (90 mL) dissolved in water from each treatment was taken and placed in a plastic bottle. These samples were taken on days 3, 7, and 14. After collection, the samples were tested for mercury content using a Mercury Analyzer. The data obtained were used to calculate the reaction kinetics model using formulas 4 and 5.

Data Analysis

The data of Superoxide dismutase and photosynthetic pigments were processed using Microsoft Excel. The results were then tested with ANOVA and DMRT tests with a level of 95% and significance (α=0.05).

Results and Discussion

Cell Density

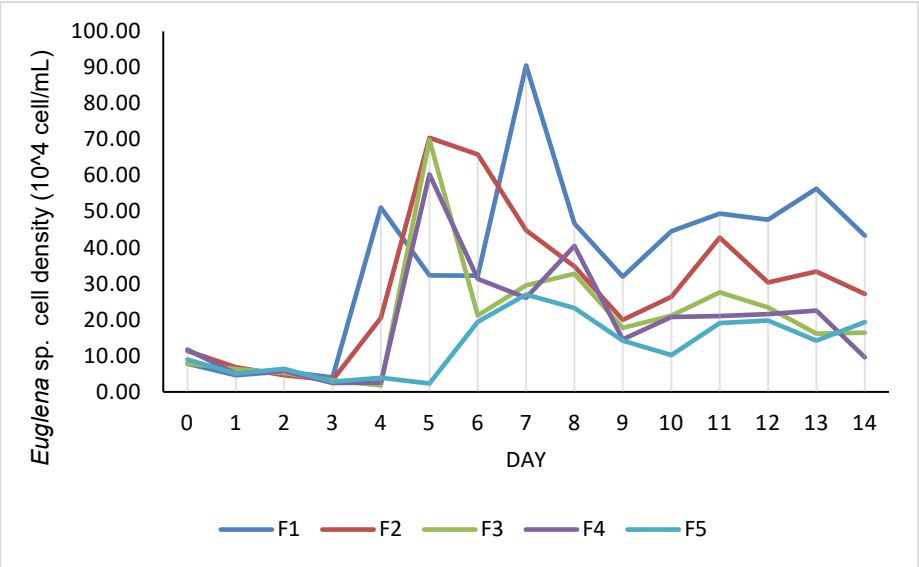

Figure 1. *Euglena* sp. cell density in various concentrations of $HgCl_2$ treatment for 14 days [26]
F1: $HgCl_2$ treatment concentration 0 ppm (control)
F2: $HgCl_2$ treatment concentration 5 ppm
F3: $HgCl_2$ treatment concentration 10 ppm
F4: $HgCl_2$ treatment concentration 15 ppm
F5: $HgCl_2$ treatment concentration 20 ppm

Cell density can be used to determine the growth phase of microalgae [27]. The cell density curve of *Euglena* sp. treated with $HgCl_2$ can be known as the life cycle of *Euglena* sp. from each treatment. The results show *Euglena* sp. can carry out its life cycle, although it lives under $HgCl_2$ stress [28]. The curve also shows an up-and-down cycle that represents the life cycle of algae. The life cycle of algae consists of a lag phase, exponential phase, nutrition deficiency phase, stationary phase, and death phase [29; 15].

The results show the highest density of *Euglena* sp. in each treatment during 14 days, which shows the ability of *Euglena* sp. to live in every treatment to gain the maximal growth of its cycle during 14 days (Figure 1). From the highest cell density, it can be known that $HgCl_2$ (control) treatments have the most density among others, whereas the 20 ppm treatments have the least density. It shows that the *Euglena* sp. that lives in control treatment has the most optimal condition for growth because there is no stress inside it [30].

Contrary to that, the 20 ppm treatments have the highest stress level among others. The stress of mercury inhibited the cell division of *Euglena sp.* got inhibited. The inhibition of cell division can be caused by cellular damage from the excess production of ROS [31]. The inhibition of cell division decreased *Euglena* sp.'s growth, so the cell density also decreased.

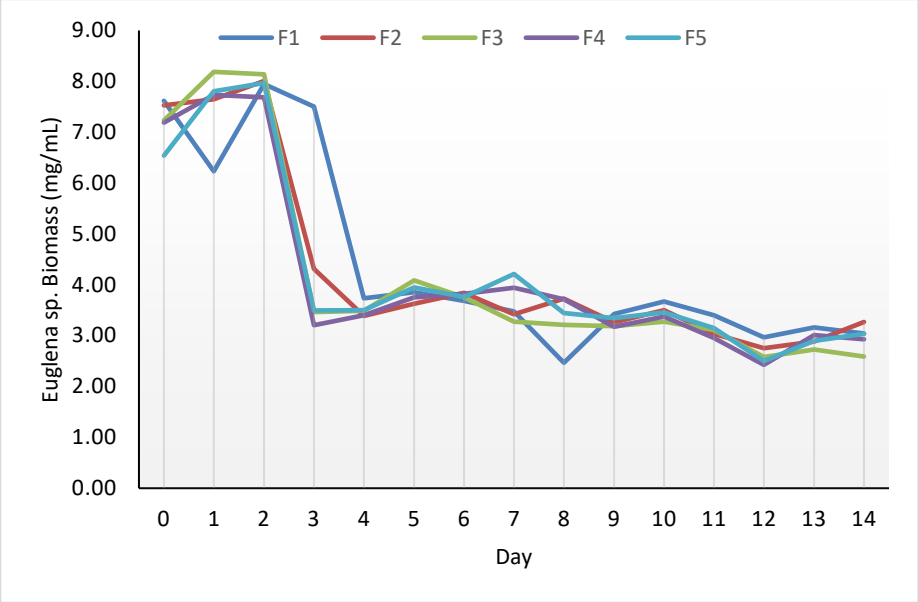

Figure 2. *Euglena* sp. biomass in various concentrations of $HgCl_2$ treatment for 14 days [26]
F1: $HgCl_2$ treatment concentration 0 ppm (control)
F2: $HgCl_2$ treatment concentration 5 ppm
F3: $HgCl_2$ treatment concentration 10 ppm
F4: $HgCl_2$ treatment concentration 15 ppm
F5: $HgCl_2$ treatment concentration 20 ppm

The biomass of *Euglena* sp. is influenced by the process of photosynthesis and various factors that determine the growth of algae. Productivity can be known from the biomass value or dry weight, which is the net result of photosynthesis [30]. Based on the curve of *Euglena* sp. biomass treated with several concentrations of $HgCl_2$, it can be known that the biomass tends to decrease. Results show that although *Euglena* sp.'s biomass tends to decrease, *Euglena* sp. can still produce biomass in mercury stress. The results of the biomass of the 14th-day show there is no significance between all treatments (Figure 2). The *Euglena* sp. treated with mercury can maintain biomass production [32]. In the 5 ppm treatment, the biomass was higher than in the 0 ppm treatment, but in 10 ppm, 15 ppm, and 20 ppm treatments, the biomass was lower than in the 0 ppm control (Figure 2). It shows that a small concentration of stress can stimulate biomass production, but the biomass tends to decrease at a higher concentration of stress [7]. The production of lipids inside algae biomass causes the increase of biomass in small concentrations of stress. The stress can stimulate algae to produce more lipids to reduce the cellular damage by ROS [33]. At the same time, the decrease in biomass production in higher stress concentrations was caused by the damage of chloroplast in excess stress concentrations. The damage to chloroplasts can decrease the photosynthetic activity, so the biomass due to photosynthesis tends to decrease [34].

Photosynthetic Pigments

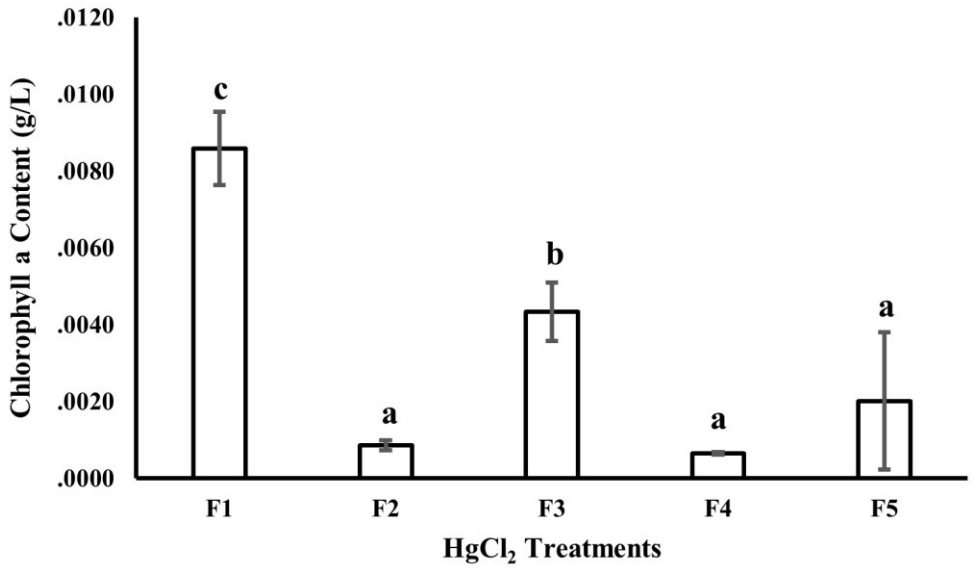

Figure 3. Chlorophyll a content of *Euglena sp.* treated with several concentrations of $HgCl_2$ [26]
F1: $HgCl_2$ treatment concentration 0 ppm (control)
F2: $HgCl_2$ treatment concentration 5 ppm
F3: $HgCl_2$ treatment concentration 10 ppm
F4: $HgCl_2$ treatment concentration 15 ppm
F5: $HgCl_2$ treatment concentration 20 ppm

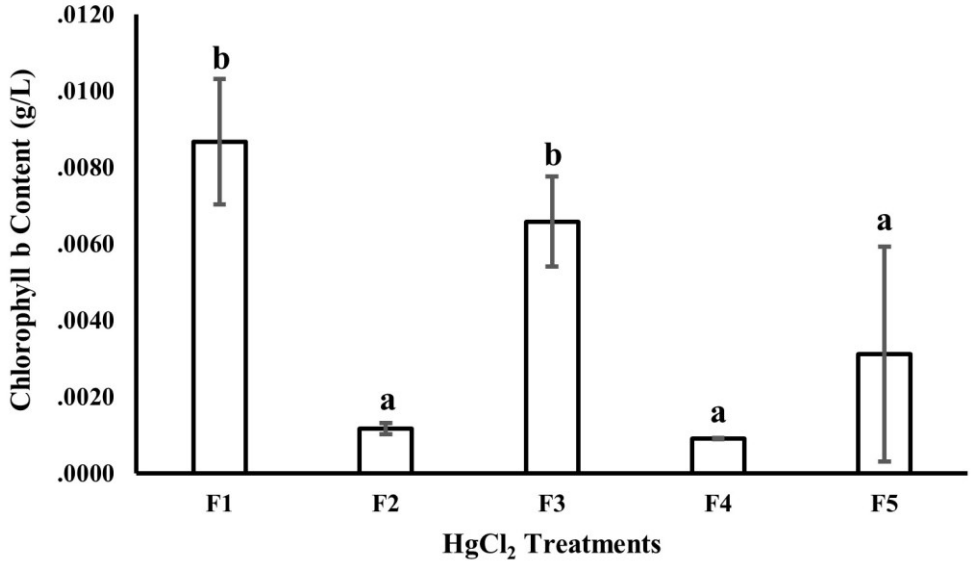

Figure 4. Chlorophyll b content of *Euglena sp.* treated with several concentrations of $HgCl_2$ [26]
F1: $HgCl_2$ treatment concentration 0 ppm (control)
F2: $HgCl_2$ treatment concentration 5 ppm
F3: $HgCl_2$ treatment concentration 10 ppm
F4: $HgCl_2$ treatment concentration 15 ppm
F5: $HgCl_2$ treatment concentration 20 ppm

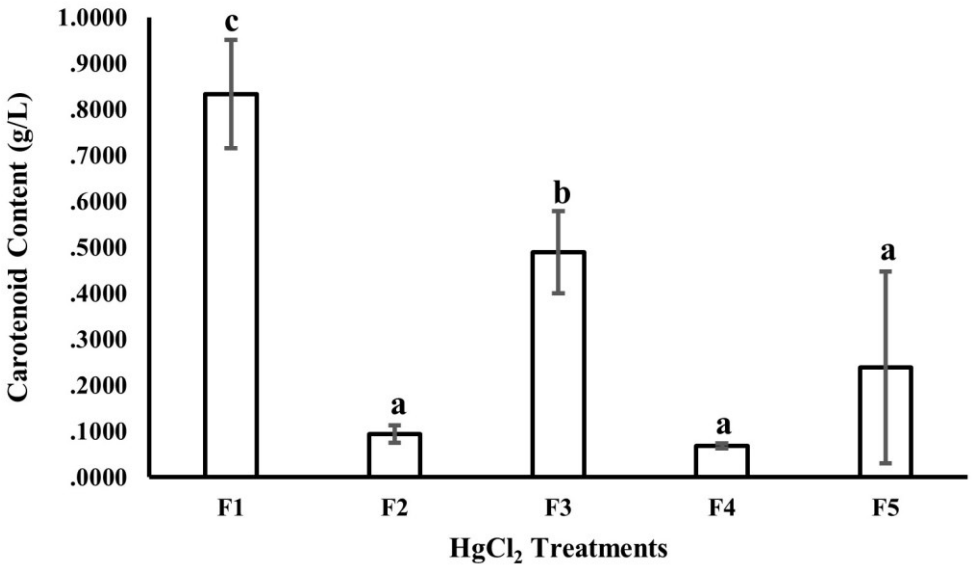

Figure 5. Carotenoid content of *Euglena sp.* treated with several concentrations of $HgCl_2$ [26]
F1: $HgCl_2$ treatment concentration 0 ppm (control)
F2: $HgCl_2$ treatment concentration 5 ppm
F3: $HgCl_2$ treatment concentration 10 ppm
F4: $HgCl_2$ treatment concentration 15 ppm
F5: $HgCl_2$ treatment concentration 20 ppm

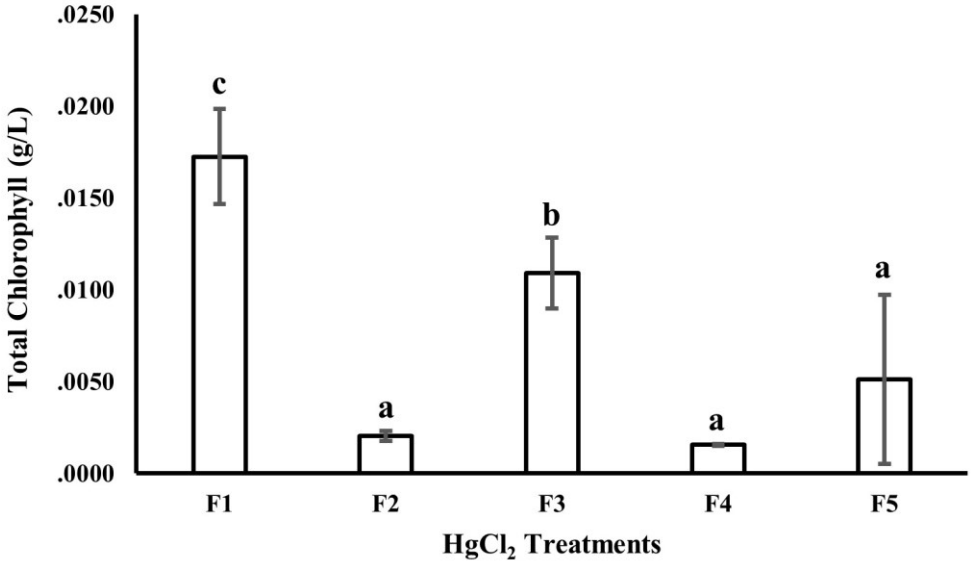

Figure 6. Total Chlorophyll content of *Euglena sp.* treated with several concentrations of $HgCl_2$ [26]
F1: $HgCl_2$ treatment concentration 0 ppm (control)
F2: $HgCl_2$ treatment concentration 5 ppm
F3: $HgCl_2$ treatment concentration 10 ppm
F4: $HgCl_2$ treatment concentration 15 ppm
F5: $HgCl_2$ treatment concentration 20 ppm

Photosynthesis is an essential process for autotrophic photosynthetic organisms such as microalgae. The primary photosynthetic pigments consist of chlorophyll a, b, and carotene, which appear in varying amounts. Chlorophyll is found in the thylakoids and is bound to the chlorophyll-binding protein complex in chloroplasts. Chlorophyll synthesis is induced by light, and all the enzymes needed for the chlorophyll synthesis process are found in chloroplasts [35].

Chlorophyll is needed in photosynthesis, so if the chlorophyll content is high, then photosynthesis is more efficient. Photosynthesis and chlorophyll are markers for algae, cyanobacteria, and plants when stress occurs because the photosynthesis and chlorophyll systems are sensitive to environmental stress. When stress happens in these organisms, the chlorophyll content and photosynthesis rate will decrease.

Figures 3, 4, 5, 6, and 7 show *Euglena* sp.'s photosynthetic pigment that lives in several concentrations of $HgCl_2$ treatments. The chlorophyll a and b contents in *Euglena* sp. tend to decrease in the higher concentrations of $HgCl_2$ stress. The significant results of the chlorophyll content in the 0 pm treatments with the 5 ppm, 10 ppm, 15 ppm, and 20 ppm treatments show that $HgCl_2$ stress significantly decreased the chlorophyll content of *Euglena* sp (Figure 3, 4, 6) The damage of chloroplast can cause a significant difference in chlorophyll content. The inhibition of its biosynthesis can cause a decrease in chlorophyll content because of the stress. In the center of the chlorophyll structure, there were Mg ions. When exposed to heavy metal stress, the Mg ions are substituted by metal ions such as Ni, Cu, Zn, Cd, Hg, and Pb. The incapability of its structure damaged it, and chlorophyll could not exist [36]. The insignificant difference between 0 and 10 ppm treatments shows *Euglena* sp. can be adapted by $HgCl_2$ stress in 10 ppm stress. It can also be caused by the acute stress of 10 ppm treatments, which makes the stress very high in the early days of treatments. So, on the 14th day, when the chlorophyll data was taken, the stress was not as significant as in the early days of treatments [34]. It can make an insignificant difference in the chlorophyll b content of 0 ppm and 10 ppm (Figure 4).

The total chlorophyll content in *Euglena sp.* tends to decrease in the higher concentrations of $HgCl_2$ stress. It is show that $HgCl_2$ stress (Figure 6) significantly decreased the chlorophyll content of *Euglena* sp. The damage to chlorophyll structure causes a significant difference between control and mercury treatments. Heavy metal stress can substitute for Mg ions inside the chlorophyll structure. The structure was incapable and decreased chlorophyll content [36]. The carotenoid contents in *Euglena* sp. tend to decrease in the higher concentrations of $HgCl_2$ stress (Figure 5). The results show significant differences between 0 ppm treatments and other treatments. It shows that mercury significantly decreased carotenoid content. The stress of excess antioxidant capacity of carotenoids can cause the decrease of carotenoids. It can cause the carotenoid structure to be damaged by toxic ion pressure [34; 37]. The contents of the carotenoid are higher than those of total chlorophyll. The carotene is one of the antioxidant agents in algae, so it was synthesized in higher concentrations in stress conditions [21].

Superoxide Dismutase

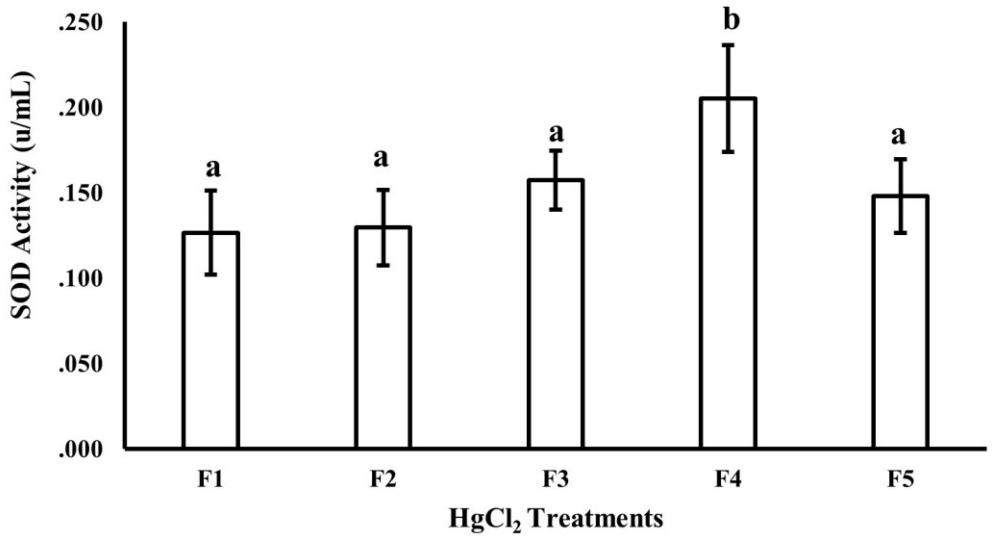

Figure 7. SOD activity of *Euglena* sp. in various concentrations of $HgCl_2$ treatment for 14 days [26]
F1: $HgCl_2$ treatment concentration 0 ppm (control)
F2: $HgCl_2$ treatment concentration 5 ppm
F3: $HgCl_2$ treatment concentration 10 ppm
F4: $HgCl_2$ treatment concentration 15 ppm
F5: $HgCl_2$ treatment concentration 20 ppm

SOD, as the primary defence enzyme, plays a role in the elimination process of the highly toxic anion of superoxide, then transforms into a less toxic form of H_2O_2 [18]. SOD, in the process, does not work alone but collaborates with enzymes others to reduce free radicals and prevent further harmful effects. SOD works together with catalase and peroxidase to remove H_2O_2 and then converts H_2O_2 to H_2O and O_2 [19]. As the first enzyme expressed reduces the effects of stress, SOD activity is influenced by stress that occurs in organisms, mainly algae. In microalgae, the greater the stress, the more ROS are produced, so that SOD activity also increases to prevent damage to cellular cells in microalgae [38].

Based on the results of the SOD activity of *Euglena* sp. in $HgCl_2$ stress, it can be known that the activity of SOD tends to increase in the high concentration of stress. The SOD activity of *Euglena* sp. increased from $HgCl_2$ 0 ppm, 5 ppm, 10 ppm, and 15 ppm. Then in the 20 ppm concentrations, the activity of SOD was down (Figure 7). It can be caused by excess mercury stress, which can be a factor that causes damage to the *Euglena* sp. cell. The damage to the cell can cause the reduction of the synthesis of the coding gene that stimulates the production of SOD. So, it can cause the production of SOD to decrease [39].

The higher concentration of stress caused the increase of SOD activity. The high stress level can cause the free radicals inside the cell to increase. In order to prevent the excess of radical stress, algae were synthesized as antioxidant enzymes. The first enzyme produced was SOD, so the level of stress influences the activity of SOD. The higher stress can cause the SOD activity to increase [38;21].

Kinetic Model

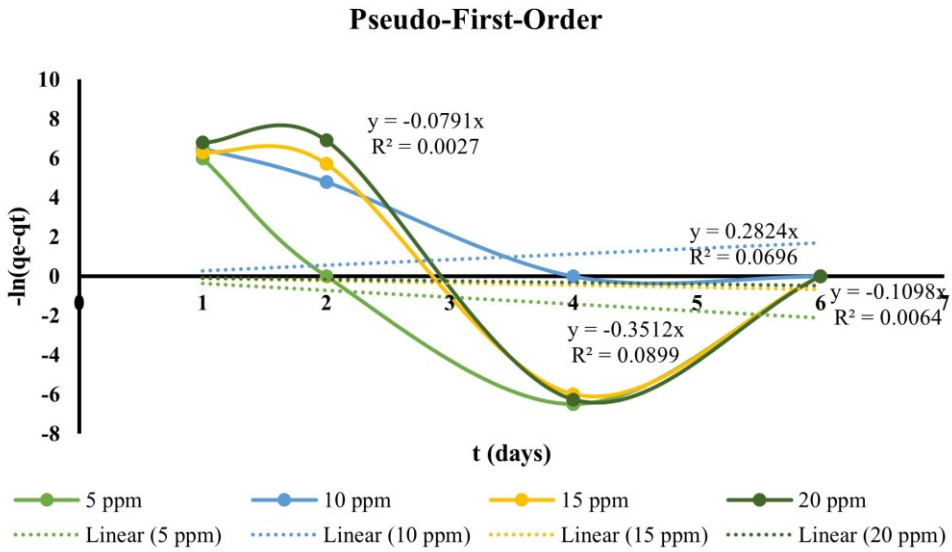

Figure 8. Pseudo-First-Order kinetic model [40]

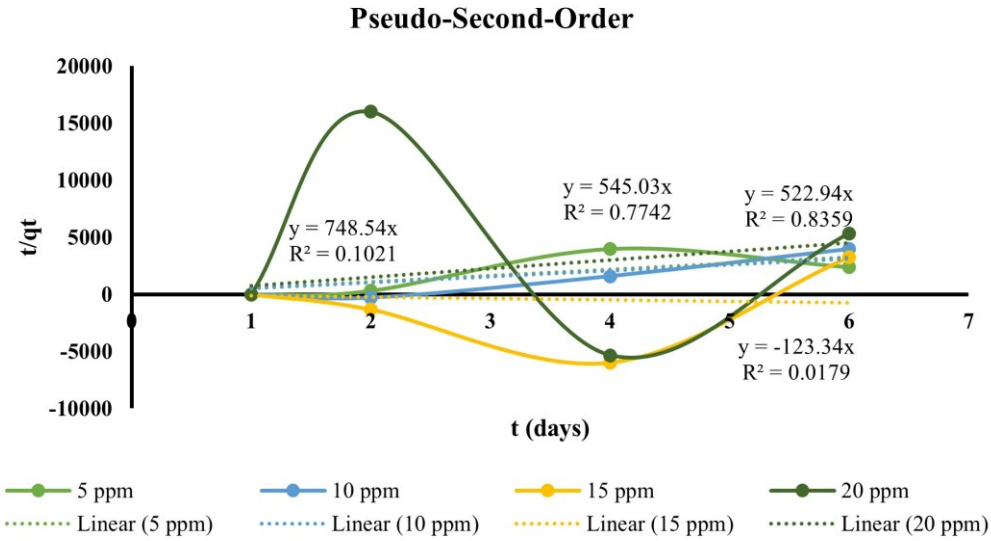

Figure 9. Pseudo-Second-Order kinetic model [40]

In applying bioremediation technology, an understanding of kinetic models is very necessary. Kinetic models test the influence of several parameters on the reaction rate and the time required to reach the equilibrium point. This model will describe the biosorption process where heavy metal ions are absorbed [3].

Pseudo-First-Order models and Pseudo-Second-Order models have been commonly used to provide information about the biosorption process. The Pseudo-First-Order model, also known as the Lagergren model, shows physisorption events. This model assumes that metal ions will be adsorbed into one adsorption site. The rate of site adsorption's occupation is proportional to the number of unoccupied sites. Pseudo-Second-Order models show chemisorption events involving valence forces. In this event, ion exchange occurs between the adsorbent compound and the heavy metal that will be removed. The rate of site adsorption's occupation is proportional to the square of the number of unoccupied sites [41]. The kinetic models are expressed by the linear equations (4) and (5):

Pseudo-First-Order.

$$\ln(q_e - q_t) = \ln q_e - k_1 t$$

q_e (mg/g): the amount of metal ions adsorbed per unit mass of the adsorbent at the equilibrium point.
q_t (mg/g): the amount of metal ions adsorbed per unit mass of the adsorbent at time.
k_1 (min^{-1}): Pseudo-First-Order rate constant [41].

Pseudo-Second-Order

$$\frac{t}{q_t} = \left(\frac{1}{k_2 q_e^2}\right) + \left(\frac{t}{q_e}\right)$$

q_e (mg/g): adsorption capacity at the equilibrium point.
q_t (mg/g): adsorption capacity at time.
k_2 (min^{-1}): Pseudo-Second-Order rate constant [41].

Based on the two kinetic models in Figures 8 and 9 above, the R^2 value of the Pseudo-First-Order kinetic model (Figure 8.) is 0.042. Meanwhile, the R^2 value of the Pseudo-Second-Order kinetic model (Figure 9.) is 0.43. The R^2 value of the Pseudo-Second-Order kinetic model is higher than the R^2 value of the Pseudo-First-Order kinetic model. Therefore, the phycoremediation process in this study is more in line with the Pseudo-Second-Order kinetic model.

Conclusions

This research investigated the potential of *Euglena* sp. to remove mercury from contaminated water in the Free Water Surface Constructed Wetland system. $HgCl_2$ stress decreased the growth rate of *Euglena* sp. at the lowest level at a concentration of 15 ppm. This stunted growth also results in low biomass production. Stress caused by $HgCl_2$ causes a decrease in the content of chlorophyll a, chlorophyll b, carotenoid, and total chlorophyll of *Euglena* sp. at the lowest level at a concentration of 15 ppm. The Superoxide dismutase (SOD) activity in *Euglena* sp. increased due to $HgCl_2$ stress. However, this increase was insignificant in $HgCl_2$ concentrations of 5 ppm, 10 ppm, and 20 ppm. This mercury detoxification process causes a decrease in *Euglena* sp. growth and biomass. These results indicate that *Euglena* sp. is tolerant to mercury and can be used as a mercury remediation agent until the mercury concentration is 15 ppm. Moreover, this phycoremediation process fits the Pseudo-Second-Order kinetic model.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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