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Cadmium effects on growth and photosynthetic pigment content of *Chaetoceros gracilis*

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ABSTRACT

Cadmium wastewater released by human activities will eventually enter the aquatic environment. Living organism that lives around could potentially exposed by these cadmium, including *C. gracilis* which play a critical role as a primary producer. Cadmium can reduce the growth rate and pigment content in *C. gracilis*. Therefore, toxicity testing becomes important to do considering that *C. gracilis* has many benefits such as shrimp feed and have active compounds for antibacterial and biodiesel. Toxicity test was performed in 96-hours with a series of concentration 0, 0.56, 1.0, 1.8, 3.2, 5.6 mg/L. IC_{50} cadmium results in concentrated cell density and chlorophyll-a *C. gracilis* were 1.62 mg/L and 3.4 mg/L, while carotenoids were not in accordance with the contribution of cadmium. NOEC and cadmium LOEC to *C. gracilis* cell density were 0.56 mg/L and 1.0 mg/L.

***Keywords*:** Carotenoids, Cell density, *Chaetoceros gracilis*, Chlorophyll-a, Toxicity test

1. INTRODUCTION

Factories or industries that dispose waste directly into the waters without being processed cause the chemicals contained in the waste to pollute the environment, especially heavy metals

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[1-3]. The remainder of the material or waste that is not treated first if released into the can reduce the quality of waters, especially the wealth of biota. Many industries use cadmium for electroplating and galvanization because cadmium has a non-corrosive feature [4]. Cadmium is also widely used in mining [5], alloy manufacturing [6], color pigments in paints [7], ceramics [8], plastics and its stabilizers [9], cathodes for Ni-Cd in batteries [10], photographic materials [11], rubber [12], soap [13], fireworks [14], textile printing [15] and pigments for glass and dental enamel [16]. Cadmium is found in basic sediments and suspended particles in natural waters [17]. Heavy metals such as cadmium can settle on the bottom of the water and have a residence time of up to thousands of years [18]. Bioaccumulation and biomagnification in several ways, namely through the skin, respiratory tract and food canal [19]. Cadmium has been classified as one of the dangerous heavy metals. Cadmium is a heavy metal compound with the second highest level of toxicity. The sequence of toxicity in aquatic organisms is $Hg^{2+} > Cd^{2+} > Ag^{+} > Ni^{2+} > Pb^{2+} > As^{2+} > Cr^{2+} > Sn^{2+} > Zn^{2+}$ [20]. Cadmium has harmful effects on aquatic organisms in many ways that affect the properties of many biological molecules, such as blocking and reducing thiol sites in proteins. In addition, cadmium can be accumulated through the food chain, which is a serious threat to human health [21]. Plankton community, both phyto and zooplankton, holds a pivotal role in aquatic ecosytem [22'23]. For instance, *Chaetoceros gracilis* is one type of diatom which plays an important role in the feed of marine biota. In addition, *C. gracilis* has chlorophyll and carotenoid pigments which can act as antioxidants. Therefore, *C. gracilis* can be developed in the food and pharmaceutical industries.

The use of *C. gracilis* as animal feed for shrimp larvae has begun to be used because of its good nutritional content (carbohydrate, fat, protein) [24]. The survival rate of shrimp larvae fed *C. gracilis* was high, namely 79.3% in *P. vannamei*, 84.8% in *P. stylirostris* [25], and 89% in M. ensis [26]. In addition, *C. gracilis* has antimicrobial compounds that can inhibit the growth of some pathogenic bacteria such as *Vibrio harveyi* and *Staphylococcus aureus* [27]. *C. gracilis* has the ability to grow quickly so that it becomes one of the diatoms that is suitable for cultivation and further research. Besides having fast growth, *C. gracilis* also has a lipid content high enough so that it can become one of the diatom for biofuels [28].

The increased concentration of cadmium in *C. gracilis* can affect its metabolic process, especially on chloroplasts so that cell growth is inhibited and photosynthesis is disrupted [29]. Cadmium can inhibit the biosynthesis of aminolevulentic acid and protochlorophyllide reductase so that the chlorophyll pigment is reduced [30]. In addition, cadmium can also affect carotenoid pigments that act as antioxidants [31]. If carotenoids cannot inhibit ROS (reactive oxygen species), the growth of *C. gracilis* will be disrupted and the carotenoid content can be reduced. The reduced carotenoid content also impacts on its function as a photoprotection, which protects *C. gracilis* from excessive light exposure so that it damages photosynthetic pigments [32]. Therefore, it is important to do a toxicity test in order to know the acceptable limit of cadmium concentration and its effect on the pigment content of *Chaetoceros gracilis*. Chlorophyll pigments play an important role for photosynthesis and growth, while carotenoids play a role in photoprotection and antioxidant activity.

2. MATERIALS AND METHODS

This research was done at the Laboratory of Marine Chemistry and Ecotoxicology, Oceanography Research Center, Indonesian Institute of Sciences, North Jakarta, on February

2019 until April 2019. This research uses the static toxicity test method and the *C. gracilis* as a test organism. The author observes the growth curve and the definitive test. Observation of the growth curve was carried out to determine the growth phase of *C. gracilis* definitive test was carried out to determine the effect of cadmium heavy metal on *C. gracilis* based on LOEC and NOEC values. Then the analysis of chlorophyll-*a* pigments and carotenoids was carried out to see the effects of exposure to cadmium on photosynthetic pigments.

2. 1. Materials

C. gracilis culture were obtained from Mariculture Laboratorium, Research Center for Oceanography, Indonesian Institute of Sciences. *C. gracilis* were cultured with sterilized seawater with Walne's media. All seawater that used in test were filtered with 0.45 μm Sartorius filter papers and sterilize by 15 min autoclaved 1.5 Pa pressure at 121 °C.

2. 2. Washing and Sterilizing Procedure

Equipment made from glass or plastic base is soaked with teepol for 15 minutes then rinsed with tap water. Then the equipment is washed with 10% nitric acid (HNO_3) by soaking it for 15 minutes to remove heavy metal leftovers, then rinsed with distilled water 3 times. Then the glassware is washed with acetone to remove the remaining organic material, then rinsed with distilled water 3 times. The glassware that has been washed is covered with aluminum foil, then sterilized by using an autoclave at 121 °C 1.5 Pa pressure in 15 minutes. Finally the glassware is dried using an oven for 1 hour with a temperature of 110 °C.

2. 3. Definitive Test

Cadmium solution stock (1000 mg/L) was prepared using cadmium chloride monohydrate ($CdCl_2 \cdot H_2O$) diluted in aquadest. Range of cadmium concentration that used in this test are 0, 0.56, 1.00, 1.8, 3.2 and 5.6 mg/L. Each concentration (1000 mL) was prepared with cadmium solution stock diluted with autoclaved natural seawater with Walne's non EDTA Media. Water quality test was measured with water quality checker using 700 mL cadmium solution. 100 mL solution filled into Erlenmeyer flask with 3 replicate for each concentration, then *C. gracilis* was added as much as 1 mL with a cell density of 1×10^6 cell/mL to obtain an initial density of 10^4 cell/mL. The Erlenmeyer is covered with aluminum foil to prevent contamination.

Every day the Erlenmeyer solution is stirred twice to stay homogeneous and the Erlenmeyer position is randomized so that each Erlenmeyer gets evenly illuminated. Culture density was observed for 1, 24, 48, 72, and 96 hours. The test was stopped (termination) by taking the test solution from each Erlenmeyer as much as 0.9 mL and lugol as much as 0.1 mL as preservatives in a 2 mL sample vial. The density of *C. gracilis* was counted using a haemocytometer under a microscope.

2. 4. Chlorophyll-*a* and Carotenoid Analysis

The *C. gracilis* culture that had been tested for 96 hours of toxicity was filtered using Sartorius filter paper 0.45 μm and millipore glass. Filtering was carried out for culture at each concentration and control. Each filter paper is used for one replicate. Filter paper that already contains *C. gracilis* is folded and wrapped in aluminum foil and labeled before storing it in the refrigerator until the analysis is carried out.

Analysis of chlorophyll-a pigments and carotenoids was carried out by extracting *C. gracilis* with 7 mL concentrated acetone. Then the sample was centrifuged at a speed of 3000 rpm for 20 minutes so that the pigment filtrate separated.

The solution obtained is maintained in a light proof so that the pigment is not damaged. Absorbance test was performed with a UV-Vis spectrophotometer with wavelengths of 664 nm, 647 nm, 630 nm, 480 nm, and 510 nm to determine the concentration of chlorophyll-*a* and carotenoids.

2. 5. Data Analysis

Calculation of the percentage of inhibition the development of the number of cells when compared to the control is calculated by the following formula [33]:

$$I\% = \frac{C - T}{C}\ x\ 100\%$$

Information:

I% : Percentage of growth inhibition
C : Average number of cells in the control solution
T : Average number of cells in cadmium solution

Data obtained from this study were analyzed using the ICPIN (Inhibition Concentration Program) program version 2.0 to calculate IC50 values for 96 hours with a confidence level of 95%. Then the analysis was carried out with the TOXSTAT program to see LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration).

Before calculating NOEC and LOEC, the data must be transformed in log10 because *C. gracilis* growth has a logarithmic pattern, then the data must be tested for normality using the Shapirowilks test and homogeneity using the Bartlett's test.

Then spectrophotometric analysis at a wavelength of 750 nm, 630 nm, 644 nm, 647 nm, 480 nm, and 510 nm was carried out to determine the chlorophyll-*a* and carotenoid levels of *C. gracilis*. The content of chlorophyll-*a* and carotenoids is calculated by the following formula [34]:

$$Chlorophyll - a\ (Chl\ a)\left(\frac{mg}{L}\right) = 11{,}85E664 - 1{,}54E647 - 0{,}08E630$$

$$Total\ Carotenoids\ \left(\frac{mg}{L}\right) =\ 7{,}6\ (E480 - 1{,}49E510)$$

3. RESULTS AND DISCUSSION

3. 1. *Chaetoceros gracilis* Growth Curve

Observation of the growth of *C. gracilis* culture was carried out for eleven days and a growth curve with an exponential pattern was obtained followed by the death phase at the end of the observation (Figure 1).

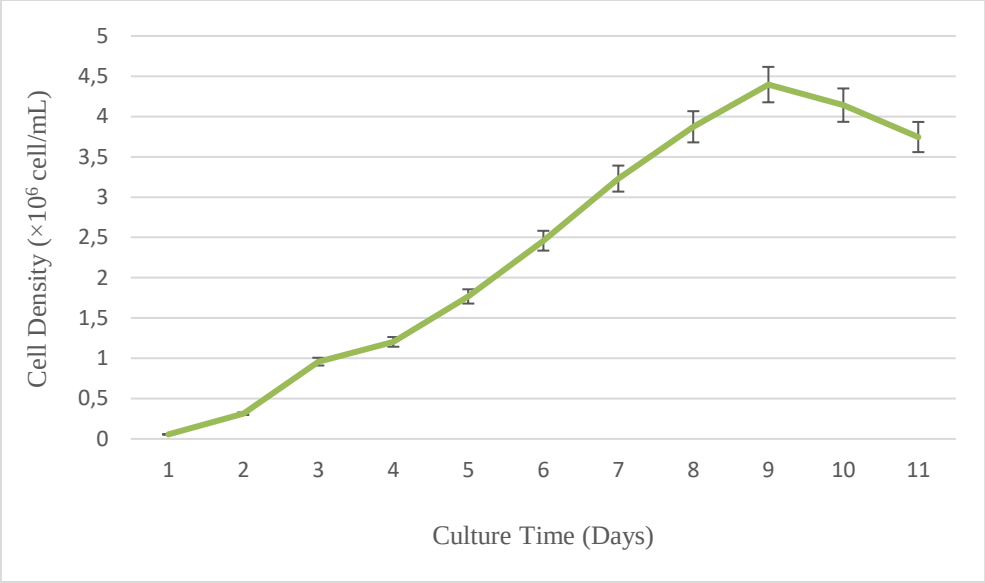

Figure 1. Growth Curve of *Chaetoceros gracilis*

With the growth curve, the exact time for toxicity testing can be determined. Growth of C. gracilis begins with the lag phase, which is the phase when the population increases in very small amounts. This phase is the adaptation phase of *C. gracilis* to the medium. If on the first day a toxicity test is carried out, *C. gracilis* will experience a decrease in cell numbers very quickly because it is still very sensitive to toxicity. Then proceed with the exponential phase (log), which is the phase when the cell divides with a constant growth rate until growth in this phase reaches its maximum.

Then there is the stationary phase which is the phase where growth begins to decrease compared to the logarithmic phase. In this phase the rate of reproduction or cell division is the same as the death rate in the sense that the addition and reduction of plankton is relatively the same so that the density of plankton tends to remain. If during the stationary phase the test is carried out, the growth rate cannot be observed precisely because growth has begun to stagnate and will decline. The last phase is the death or declination phase, where there is a decrease in the number/density of plankton and in this phase the mortality rate is faster than the rate of reproduction [35].

The growth phase of *C. gracilis* can determine the right time to carry out a toxicity test and the most appropriate phase is the exponential phase when the cell divides constantly. The exponential phase is characterized by a rapid growth rate, a constant rate of cell division, constant metabolic activity, and a state of balanced growth between food intake and increase in diatoms. Diatoms in the exponential phase have a high capacity for photosynthesis [36], so that if the toxicity test is carried out at the exponential phase it can be concluded that the decrease in growth rate is caused by toxicity.

Based on the growth curve above, the right day for a toxicity test is the fourth or fifth day, when the exponential phase occured. This culture was carried out 2 times to ensure that on the fourth day the number of *C. gracilis* cells had reached a density of $1x10^6$ cell/mL in order to meet the toxicity test requirements.

3. 2. Definitive Test

The definitive test starts with a cell density of 10^4 cell/mL. Cell density was calculated for 24, 48, 72, and 96 hours. Water quality test result shows the condition is good for the toxicity test. The result indicated that only cadmium had an effect on the growth of *C. gracilis* because the value of water quality at each concentration was comparatively same (Table 1). *C. gracilis* response to cadmium can be seen in Figure 2. Based on data obtained *C. gracilis* growth decreases with increasing cadmium concentration. According to Yunianandа [37], the length of exposure time is also directly proportional to cadmium concentration, which is the longer the exposure, the lower the growth rate. This shows a negative relationship between concentration and the number of cells, where the number of cells will decrease if the concentration increases.

Table 1. Water Quality Test

Parameter	Concentration (mg/L)					
	0	0.56	1.0	1.8	3.2	5.6
Temperature (°C)	24.61	24.65	24.61	24.62	24.57	24.63
pH	8.06	8.22	7.88	8.02	8.17	8.04
Salinity (ppt)	31.5	31.2	31.1	31.4	31.5	31.2
Dissolved Oxygen (mg/L)	6.25	6.25	6.34	6.26	6.23	6.23

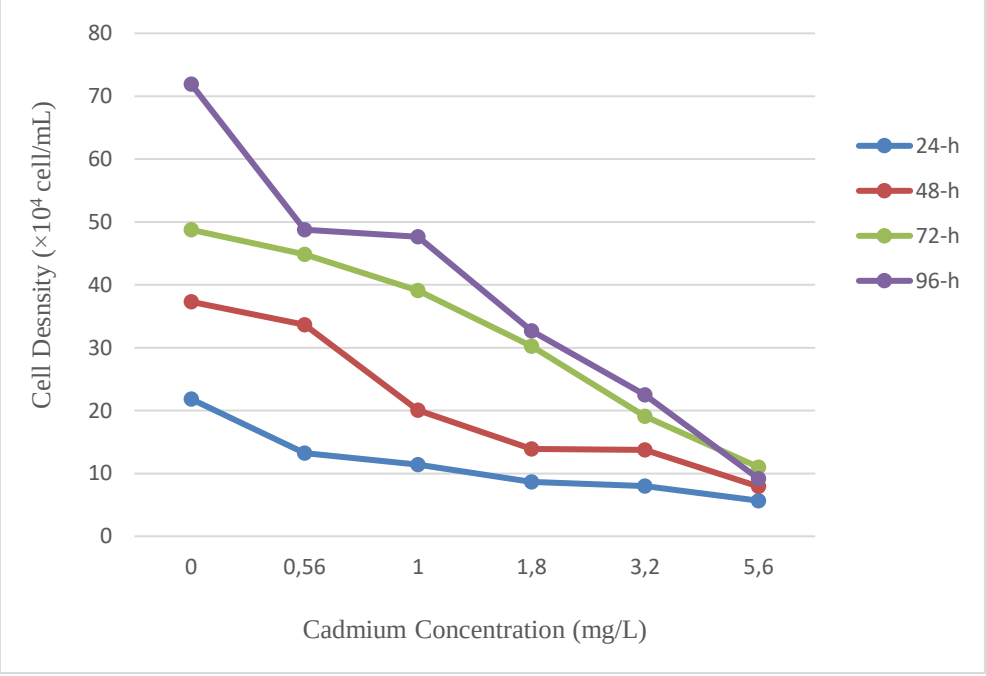

Figure 2. *Chaetoceros gracilis* Cell Density Against Cadmium

In addition to cell density, the percentage of cadmium inhibition of *C. gracilis* were also calculated and can be seen in Table 2. Calculations were carried out on the number of cells at 96 hours because it was the end point of the toxicity test. The results showed that the higher the cadmium concentration the greater the percentage of inhibition. This shows that cadmium can bind to cell proteins causing toxic effects on cells or organisms [30].

Based on the Table 2 it can be seen that the inhibition ranges from 32.21 to 87.25%. Inhibition of as much as 50% is between concentrations of 1.0 and 1.8 mg/L which indicates that the IC_{50} value is in the range of that concentration. Cadmium IC_{50} against *C. gracilis* in this study was 1.62 mg/L. The NOEC and LOEC value were 0.56 and 1.0 mg/L.

Table 2. Inhibition Percentation of Cadmium Against *C. gracilis.*

Toxicant	Concentration (mg/L)	Cell Density ($\times 10^4$ cell/mL)	I (%) *
Cadmium (Cd)	Control	71,92	-
	0,56	48,75	32,21
	1,0	47,67	33,72
	1,8	32,67	54,57
	3,2	22,5	68,71
	5,6	9,17	87,25

*) I (%) = Inhibition Percentage

Cadmium heavy metal has been shown to significantly inhibit the growth of *C. gracilis*. Absorption of heavy metals in the culture of *C. gracilis* occurs in two stages [30]: 1) The initial stage consists of rapid passive absorption and 2) followed by slow active absorption. At the cellular level, passive absorption begins with the interaction of heavy metals and cell walls. Cell walls contain extracellular enzymes that function in the absorption of the elements needed by cells. On active absorption, the heavy metal is transported through the cell membrane to the cytoplasm. A compound can enter the cell membrane if the compound is lipophilic.

This is because the cell membrane is formed by two layers of lipids (lipid bilayer). Lipophilic compounds will dissolve in the lipid layer so that they can bind to cell proteins. In order to cross the cell membrane, a facilitated diffusion process occurs in heavy metal ions. In the diffusion process heavy metal ions are facilitated by the permease enzyme which is a cell membrane protein that can bind to heavy metals so that it can pass through the lipid membrane [30].

When heavy metal ions are already in the cell membrane, enzymes and cell organelles in the cytoplasm become the main purpose of these ions. The organelles that are most sensitive to heavy metals are chloroplasts. Chloroplasts are organelles that act as sites in photosynthesis, which function to receive water and carbon dioxide and convert to carbohydrates and oxygen with the help of sunlight [38]. Chloroplasts that have been exposed to heavy metals can be

damaged so that the photosynthesis process is disrupted. Chloroplast cannot receive sunlight properly so it cannot convert water and carbon dioxide. If the photosynthesis process is disrupted, then phytoplankton cannot receive food and the oxygen content produced decreases [39].

3. 3. Chlorophyll-*a* and Carotenoid Analysis

The amount of chlorophyll-a and carotenoid pigments can be seen in Table 3. Chlorophyll-*a* levels in *C. gracilis* exposed to cadmium decreased compared to controls, but the decrease in levels was not linear. However, the IC_{50} cadmium value of chlorophyll-*a* levels can be calculated because the response indicates that there is a decrease of more than 50% compared to the control. The IC_{50} value of cadmium against chlorophyll-*a* is 3.4 mg/L.

Table 3. Chlorophyll-*a* and Carotenoid Content in *C. gracilis.*

Sampel	Klorofil-*a* (µg/mL)	Karotenoid (µg/mL)
Kontrol	0,585457	0,327707
0,56	0,319547	0,34405
1	0,18532	0,372513
1,8	0,26919	0,390793
3,2	0,47215	0,263727
5,6	0,114163	0,409073

The decrease in chlorophyll-*a* levels by cadmium occurs because cadmium can also cause chlorosis. Chlorosis caused by cadmium heavy metals can go through two pathways, namely the direct inhibition of the 5-aminolevulinate dehydratase enzyme which plays a role in chlorophyll synthesis, and through the replacement of iron (Fe) and magnesium (Mg) metals involved in chlorophyll synthesis. The inhibition of these enzymes causes chlorophyll synthesis to not occur optimally and the amount of chlorophyll production decreases [28].

The IC50 cadmium value of chlorophyll-a is higher than the effect on *C. gracilis* cell growth. This is caused by the process of cadmium entering the cell membrane which is protected by two lipid layers. When cadmium has entered the cell membrane, many cell organelles in the cytoplasm will be influenced by cadmium such as mitochondria, vacuoles, ribosomes, chloroplasts, and other organelles. The concentration of cadmium that enters the cytoplasm will be localized into some of these cell organelles so that a large concentration is needed to influence the chloroplast which has an impact on chlorophyll levels [37-40].

Meanwhile, the number of carotenoids did not experience a large decrease compared to controls. These results indicate that carotenoids which act as antioxidants can inhibit the interference of cadmium heavy metal ions. Carotenoids are the most effective antioxidants for removing superoxide free radicals among nine main antioxidants (ascorbate, glutathione,

polyamine, α-tocopherol, carotenoids, catalase, ascorbate peroxidase, superoxide dismutase, and glutathione-S-transferase) [29].

4. CONCLUSIONS

Cadmium has been shown to have a significant effect on the growth of *C. gracilis*. The IC_{50} value of 96 hours of cadmium to the growth of *C. gracilis* was 1.62 mg/L while the chlorophyll-*a* content was 3.4 mg/L. The cadmium NOEC and LOEC values for growth were 0.56 and 1.0 mg/L. Cadmium has an impact on the decrease in the amount of chlorophyll-*a*, whereas in carotenoids there is no significant change in cadmium exposure.

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