Biochemical Responses and DNA Damage of *Chlorella pyrenoidosa* H. Chick upon Exposure to Combined Cu and Cd at Environmentally Realistic Levels

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ABSTRACT

Monitoring aquatic ecosystems is necessary to prevent or reduce the impact of metal pollutants on ecosystems and human health. Biological responses, or biomarkers, can provide quick and direct evidence of exposure to environmental stressors. This study evaluated the sensitivity of biomarkers of *Chlorella pyrenoidosa* after short-term exposure to combined copper and cadmium at environmentally realistic concentrations with the following biological endpoints: growth; alkaline phosphatase activity; chlorophylls, pheophytin-a, carbohydrate, and protein content; and DNA damage. *C. pyrenoidosa* was exposed to three combinations of copper and cadmium for 120 min, or for 48 h to assess its DNA damage. To assess the sensitivity of the biological responses to the combined metals, integrated biomarker response (IBR) analysis was also performed. The results demonstrated that exposure to combined Cu and Cd caused an inhibition of growth and activity of alkaline phosphatase, a decrease of chlorophyll-a and -b and protein, and an increase of pheophytin-a and tail factors DNA. The IBR analysis affirmed that the inhibition of alkaline phosphatase activity and the decrease of protein level were responsive biomarkers for exposure to the combined metals.

1. INTRODUCTION

Copper (Cu) and cadmium (Cd) represent metals that generally pollute aquatic ecosystems and can cause toxic effects on aquatic organisms. Copper is an essential metal for organisms at low concentrations and is needed, for example, to stabilize protein structures and catalyze enzymatic reactions (Torres et al., 2008). Increased concentrations of copper in aquatic ecosystems mainly come from anthropogenic activities, such as copper mine drainage and copper-based pesticides (Qian et al., 2009). On the other hand, cadmium is a non-essential metal for organisms and is toxic at low concentrations. The main sources of increased cadmium concentrations in aquatic ecosystems include electroplating, smelting, alloy manufacturing, and batteries (Cheng et al., 2016). Aquatic organisms can absorb metals, which will be transferred through food chains, leading to biomagnification. These metals can cause toxic effects on aquatic organisms, which in turn can disrupt the balance of the ecosystem. Ultimately, these metals can threaten human health (Lecoeur et al., 2004; Qian et al., 2009; Cheng et al., 2016). The toxic effects of metal combinations in organisms can be stronger than the effects of the individual metals (Zeb et al., 2016; Nugroho et al., 2017).

Monitoring aquatic ecosystems is necessary to prevent or reduce the impact of metal pollutants on ecosystems and on human health. Biological markers or biomarkers, which are biological responses at lower levels of biological organization, can provide quick and direct evidence of exposure to environmental stressors (short-term response) (Adams et al., 2001). Biomarkers also integrate the effects of chemical mixtures on organisms in

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ecosystems (Bartell, 2006). In the development of biomarkers for environmental monitoring, it is important to evaluate the effectiveness of their responses in quantifying the environmental condition. The sensitivity of the responses to environmental stressors is a critical component of the evaluation process. The response should be sensitive to an environmental stressor at lower concentrations and should occur within a short time period after an exposure (Bartell, 2006).

Microalgae, primary producers in the food chains of aquatic ecosystems, are known to have high sensitivity to the presence and concentrations of metals. The use of microalgal growth as a parameter of metal presence is considered more useful and consistent than biomass and total cell count (Campanella et al., 2000; Lin et al., 2005; Jiang et al., 2016). The toxicity of metals in microalgae can also be studied by measuring inhibition of enzyme activities in the microalgae. Inhibition of alkaline phosphatase enzyme activity is often used as a parameter to determine the presence and concentration of metals in aquatic ecosystems. Alkaline phosphatase resides on the microalgal cell wall, so metals will readily interact with the enzyme (Awasthi, 2012; Jiang et al., 2016). Microalgal interaction with Cu and Cd may also interfere with protein and carbohydrate metabolism, inhibit the biosynthesis of chlorophylls, and increase the concentration of pheophytin-a (Campanella et al., 2000; Tripathi and Gaur, 2006; Perales-Vela et al., 2007; Arunakumara and Xuecheng, 2008; Ngo et al., 2009; Nugroho and Frank, 2011; Miazek et al., 2015).

Cu and Cd play a role in increased reactive oxygen species (ROS) production (Qian et al., 2009). These species cause DNA damage through the oxidation process. Genotoxicity of DNA damage can be analyzed with a comet assay by determining the number of denatured DNA fragments that migrate out of the cell nucleus during electrophoresis. The test has high sensitivity to detect DNA damage in single cells (Erbes et al., 1997; Desai et al., 2006; Cadet and Wagner, 2013).

Previous research has generally considered the biological responses of microalgae to a single metal, and only rarely have the combined effects of two or more been assessed. This study evaluated the sensitivity of biological responses of *Chlorella pyrenoidosa* to short-term exposure to combinations of copper and cadmium at environmentally realistic levels with biological endpoints: growth, alkaline phosphatase activity, DNA damage, and chlorophylls, carbohydrate, and protein content. The rapid and sensitive responses of the endpoints were assessed by integrated biomarker response analysis. The results identify promising biomarkers for biomonitoring of aquatic metal pollution.

2. METHODOLOGY

2.1 Chemical materials and glassware

Chemicals were of analytical grade (Merck, Jakarta, Indonesia). Walne's medium (Walne, 1970) was used as a microalga growth medium and was sterilized before use. To test the effects of combined Cu and Cd, stock solutions were prepared by dissolving 0.01 g $CuSO_4 \cdot 5H_2O$ and 0.01 g of $CdCl_2$ separately with bidistilled water in a 100 mL volumetric flask, resulting in Cu and Cd concentrations of 0.40 and 0.55 mmol/L.

All glassware was washed twice with 50% HNO_3 solution (65%, 225 g/L), aquadest, and bidistilled water, and then sterilized in an autoclave at 121 ^oC for 15 min.

2.2 Test organism and culture conditions

Chlorella pyrenoidosa was obtained from the Research Centre for Brackish Water Aquaculture (Jepara, Indonesia). In the laboratory, the culture was maintained in Walne's medium in 1-L Erlenmeyer (Pyrex) flasks following the method of Nugroho and Frank (2011). Cultures in the exponential phase were used for all tests, and the initial density for each test was 5-6 × 10^6 cells/mL.

2.3 Experimental design

For testing the combined effects of Cu and Cd, into each of three flasks containing 600 mL *C. pyrenoidosa* culture (5-6 × 10^6 cells/mL) was added Cu and Cd from a stock solution to establish different concentrations of Cu and Cd, respectively, i.e., 0.3 and 0.09, 1.5 and 0.9, and 15 and 9 µmol/L. Three flasks containing 600 mL of microalgal culture were used as controls. The concentrations of metals represent the range which might typically be found in aquatic ecosystems that receive effluent from industry and agricultural land use. The range was from the water quality standard (0.3 µmol/L Cu and 0.09 µmol/L Cd) to 15 µmol/L Cu and 9 µmol/L Cd (Rajaganapathy et al., 2011; Tjahjono and Suwarno, 2018; Elfidasari et al., 2019).

At 0, 15, 30, 60, 90, and 120 min after the addition of Cu and Cd, 2-mL aliquots were transferred

into 2-mL microtubes to calculate the growth of microalgae. Furthermore, 2-mL aliquots were taken from each Erlenmeyer flask and transferred into 15-mL polypropylene (PP)_tubes (Biologix, Jakarta, Indonesia) for the analysis of alkaline phosphatase activity. At the same sampling times, a 90-mL aliquot was divided into two 50-mL PP tubes of known weights. The first tube was used for protein and carbohydrate analyses, and the second for chlorophyll analysis. For DNA damage analysis, 2 mL was transferred from each Erlenmeyer flask into 2-mL microtubes at 2, 12, 24, and 48 h after addition of the metals.

2.4 Growth

Microalgal growth was followed by calculating cell density using a hemocytometer (Grigoryev, 2014). Specific growth rate was calculated according to Gani et al. (2016):

$$\mu = \frac{\ln N_2 - \ln N_1}{T_2 - T_1}$$

Where; μ = specific growth rate

N_2 and N_1 = cell density at time t_2 and t_1

The growth inhibition of microalgae was expressed as an inhibition percentage (PI) as PI (%) = $(1-(N/N_o) \times 100)$, where N and N_o are cell density (cells per mL) in the culture with a combination treatment of metals and in the control culture, respectively (Qian et al., 2009).

2.5 Determining chlorophyll-a, chlorophyll-b, and pheophytin-a

An aliquot (45 mL) of culture was centrifuged at 6,000 g at 4 °C for 10 min; the supernatant was removed, and 30 mL of distilled water was added and the suspension centrifuged again at 6,000 g at 4 °C for 10 min. The supernatant was again removed, and the pellet was weighed to obtain the wet weight. The pellet was used for chlorophyll and pheophytin analyses.

Chlorophyll-a and chlorophyll-b were determined using the method of Warren (2008). The pellet in a 2-mL microtube (Eppendorf) was ground with a pestle and then mixed with 1 mL of methanol and centrifuged at 16,873 g at 4 °C for 2 min. The supernatant was transferred to another microtube and the pellet was re-extracted by adding 1 mL of methanol, and again centrifuged at 16,873 g at 4 °C for 2 min. The supernatant obtained was mixed with the previous supernatant, and 200 µL was put into a microplate (Iwaki, Jakarta, Indonesia), and absorbance values were recorded using a microplate reader (SH-1000 Corona Electric, Jakarta, Indonesia) at wavelengths of 652 and 665 nm. The absorbance value reading was converted to 1-cm pathlength microplate using the following formula:

$$A_{652,\ 1\ cm} = (A_{652,\ microplate} - blank)/pathlength$$

$$A_{665,\ 1\ cm} = (A_{665,\ microplate} - blank)/pathlength$$

The analysis was completed by calculating the concentration of chlorophylls through the following formula:

$$\text{Chlorophyll-a } (\mu g/mL) = -8.0962\ A_{652,\ 1\ cm} + 16.5169\ A_{665,\ 1\ cm}$$

$$\text{Chlorophyll-b } (\mu g/mL) = 27.4405\ A_{652,\ 1\ cm} - 12.1688\ A_{665,\ 1\ cm}$$

Pheophytin-a content was determined using samples whose absorbance value had been read through the chlorophyll analysis. The samples were mixed with 10 µL 0.1 M HCl, allowed to stand for 90 sec, and their absorbance value was read using a microplate reader at a wavelength of 665 nm. Pheophytin-a concentration was calculated with the formula of Lorenzen in Sartory (1982):

$$\text{Pheophytin-a } (mg/L) = (A_{665b} - 3.0\ (A_{665b} - A_{665a}))12.5 \times 1.5 \times 0.973$$

Where; A_{665b} = absorbance value before acid is added

A_{665a} = absorbance value after acid is added

Chlorophyll and pheophytin-a contents were expressed as µg/g wet weight (ww).

2.6 Determining proteins and carbohydrates

Forty-five mL of culture in a 50-mL PP tube of known weight was centrifuged at 6,000 g at 4 °C for 10 min. The supernatant was discarded, and the pellet was washed with 40 mL phosphate-buffered saline (PBS) and 40 mL bidistilled water through resuspension and centrifugation. The wet weight of the pellet was calculated by subtracting the weight of the PP tube from the weight of the tube containing the algae. For analysis of protein and carbohydrate, 40 mL liquid nitrogen was transferred into the tube, and then the pellet was ground using a pestle to break the cells. The addition of liquid nitrogen and grinding were conducted twice, and 2 mL of protease inhibitor cocktail was then immediately added to the tube. The

sample was then centrifuged at 6,000 g at 4 °C for 10 min. The supernatant was transferred to a 2-mL microtube.

Protein content was determined with a dye-binding assay (Kruger, 1994), using a bovine serum albumin standard curve, and expressed as µg/g ww. Carbohydrate was measured using the phenol-sulfuric acid assay (Masuko et al., 2005). The carbohydrate content was determined using a glycogen standard curve and expressed as µg/g ww.

2.7 Determining alkaline phosphatase activity

An aliquot of 2 mL culture in a 15-mL PP tube was mixed with 1 mL 0.5 M Tris-HCl buffer pH 8 and 2 mL 0.3 mM p-nitrophenyl phosphate, incubated for 4 h at 37 °C and then mixed with 0.1 mL 0.1 M NaOH. Subsequently, the sample was filtered with Whatman paper number 42 (Whatman, Jakarta, Indonesia) of known weight and used for alkaline phosphatase activity analysis following the modified method of Ihlenfeldt and Gibson (1975) and Yuan et al. (2017), with a 4-nitrophenol standard curve. Activity was expressed as nmol/min/g ww.

2.8 Analysis of DNA damage

DNA damage was measured with an alkaline comet assay following the method of Hazlina et al. (2019). Tail factors were used to assess the extent of DNA damage, by measuring the comet's tail length (tail factor) of about 100 cells using TriTek Comet Index 2.0 under a fluorescence microscope. Tail factors were calculated according to Ivancsits et al. (2002).

2.9 Data analysis

The variability of alkaline phosphatase, chlorophylls, pheophytin-a, proteins, and carbohydrates was tested with one-way analysis of variance (ANOVA) with exposure time as an independent variable, followed by Dunnett's test ($p<0.05$) if a significant difference was obtained.

To assess the stress of combined Cu and Cd on the microalgae, all biomarkers except DNA damage were combined into a stress index termed the integrated biomarker response (IBR). The IBR index was determined following Bertrand et al. (2016) and Iturburu et al. (2018) for each combined concentration. The IBR index was calculated based on the S values of all biomarkers. The S value shows the gradient value of a biomarker on combined metal exposure, in which the highest S value follows the highest biological effect. The IBR analysis is presented in a star plot.

3. RESULTS AND DISCUSSION

3.1 Growth

During exposure to combined Cu and Cd, growth was decreased, especially in the first 15 min (Figure 1). However, the decrease compared to the 0 min value was significant only at the two highest concentrations of combined Cu and Cd ($p<0.05$). The specific growth rate (µ) showed negative values and tended to decrease with increasing combined Cu and Cd concentrations (Table 1). At the end of the exposure, the growth inhibition value (PI) increased with increasing combined Cu and Cd concentrations, i.e., 16% (0.3 µmol/L Cu+0.09 µmol/L Cd), 32% (1.5 µmol/L Cu+0.9 µmol/L Cd), and 37% (15 µmol/L Cu+9 µmol/L Cd).

The results indicated that the inhibitory metals' effect on microalgal growth depends on metal concentrations (Zulfikar, 2018). This growth inhibition could reflect the physiological and biochemical status of the microalgae. The inhibited growth may be caused by the effect of the metals on photosynthesis by blocking electron transport in photosystem II (PSII) or inhibition of chlorophyll biosynthesis, resulting in reduced cell mitotic rate and affecting microalgal growth (Cid et al., 1995; Pawlik-Skowrońska and Skowroński, 2001; Tripathi and Gaur, 2006; Arunakumara and Xuecheng, 2008). Qian et al. (2009) demonstrated that upon individual exposures to Cu (1.5 µmol/L) and Cd (1.0 µmol/L) in *C. vulgaris* for 48 h, the PI was 16% and about 8%, respectively. When *C. vulgaris* was exposed to combined Cu and Cd at the same concentrations for 48 h, the PI increased to 60%, reflecting synergistic growth inhibition (Qian et al., 2009). The current study also demonstrated a synergistic effect since exposure to 1.5 µmol/L Cu+0.9 µmol/L Cd for 120 min, the PI was 32%.

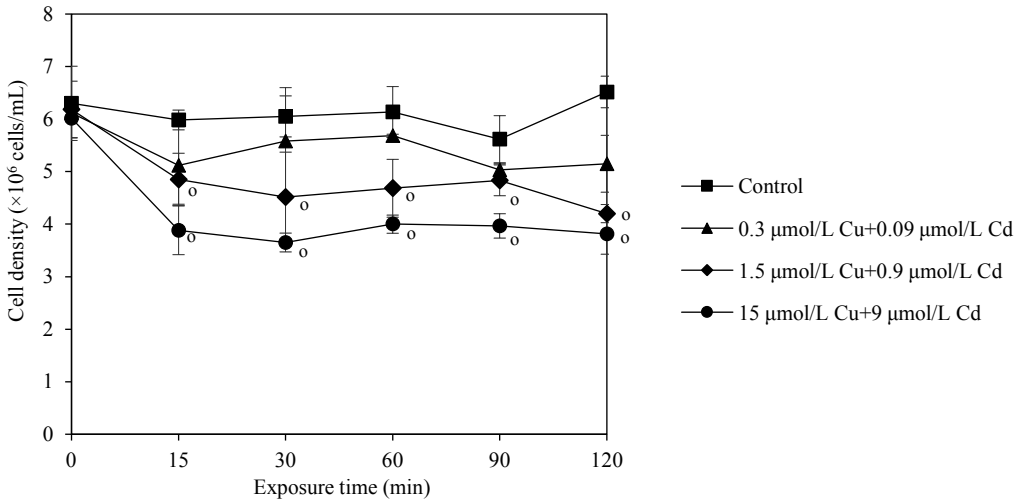

Figure 1. Growth of *C. pyrenoidosa* during exposure to Cu and Cd in combination. Data are expressed as mean ± standard deviation (n=3). A significant difference in comparison to control in each exposure is indicated by °.

Table 1. Specific growth rates of *C. pyrenoidosa* during exposure to combinations of Cu and Cd

Combination concentration of Cu and Cd (µmol/L)	Specific growth (rate/h)
0	$0.02^{a} \pm 0.01$
0.3+0.09	$-0.09^{b} \pm 0.02$
1.5+0.9	$-0.19^{c} \pm 0.01$
15+9	$-0.23^{c} \pm 0.02$

Data are expressed as mean ± standard deviation (n=3). Identical letters indicate no significant difference (p>0.05).

3.2 Chlorophyll-a, chlorophyll-b, and pheophytin-a

Administration of combined Cu and Cd to the microalgae caused a decrease in chlorophyll-a and chlorophyll-b (Figures 2 and 3). For chlorophyll-a, a significant decrease occurred at 120 min for the combination of 0.3 µmol/L Cu+0.09 µmol/L Cd (Figure 2(b)), and at 60 min for the combinations of 1.5 µmol/L Cu+0.9 µmol/L Cd (Figure 2(c)) and 15 µmol/L Cu+9 µmol/L Cd (Figure 2(d)). At the end of the exposure, the decrease in chlorophyll-a in all three combinations ranged from 20% to 30%. For chlorophyll-b, the combination of the lowest Cu and Cd concentration resulted in a significant decrease of chlorophyll-b at 15 min (Figure 3(b)). On the other hand, in the combinations of 1.5 µmol/L Cu+0.9 µmol/L Cd and 15 µmol/L Cu+9 µmol/L Cd, a significant decrease began at the 60 min (Figure 3(c) and (d)). The decrease in chlorophyll-b continued until the end of the exposure, eventually ranging from 20% to 25%.

These findings indicated that the effects were associated with increased concentrations of Cu and Cd and the duration of exposure (Zulfikar, 2018). Qian et al. (2009) also reported effects of Cu and Cd on the chlorophyll-a and chlorophyll-b of *C. vulgaris* at a similar concentration as used in this study, namely 1.5 µmol/L Cu and 1.0 µmol/L Cd, for 48 h. Upon individual exposures to Cu and Cd, the chlorophyll-a decreased by about 27 and 16%, and the chlorophyll-b also decreased by about 20 and 10%, respectively. Exposure to the combined Cu and Cd decreased the chlorophyll-a and chlorophyll-b by about 77 and 50%, indicating a strong synergistic effect. Combined Cu and Cd inhibited the expression of psbA, the gene that encodes the D1 protein of PSII. The inhibition decreased in PSII activity and the rate of electron transfer, thereby reducing the content of chlorophylls (Qian et al., 2009). Such a combination (1.5 µmol/L Cu+1.0 µmol/L Cd) also synergistically enhanced the induction of reactive oxygen species (ROS) formation in *C. vulgaris*, which caused damage to the photosynthetic apparatus, resulting in the decrease of chlorophyll content and producing a synergistic inhibition of algal growth (Qian et al., 2009). In comparison with the study of Qian et al. (2009), the results of this study did not lead to synergistic effects.

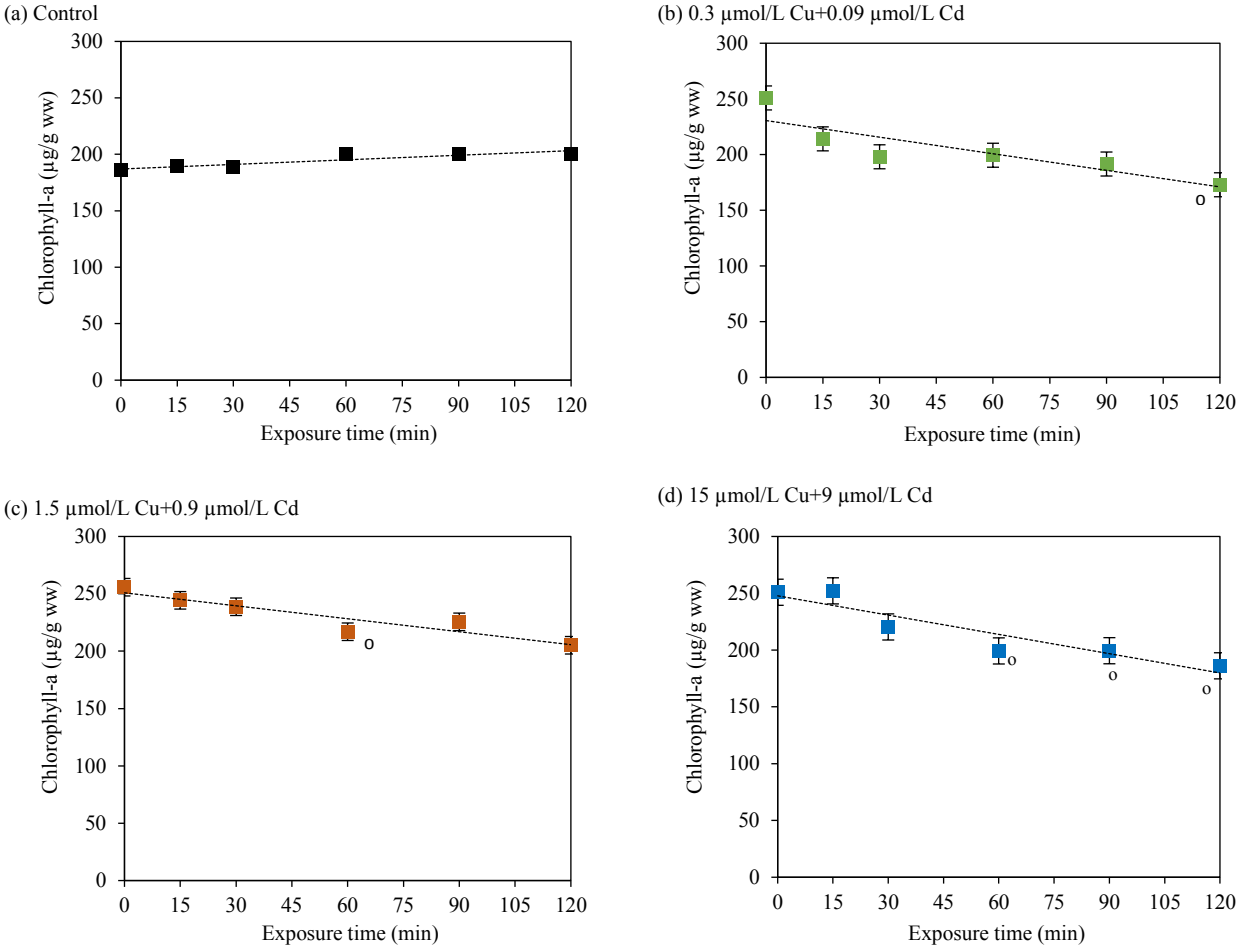

Figure 2. Chlorophyll-a content (µg/g ww) in *C. pyrenoidosa* after exposure to the combinations of (a) 0 µmol/L Cu+0 µmol/L Cd (control), (b) 0.3 µmol/L Cu+0.09 µmol/L Cd, (c) 1.5 µmol/L Cu+0.9 µmol/L Cd, and (d) 15 µmol/L Cu+9 µmol/L Cd. Data are expressed as mean ± standard deviation (n=3). A significant difference in comparison to control is indicated by º.

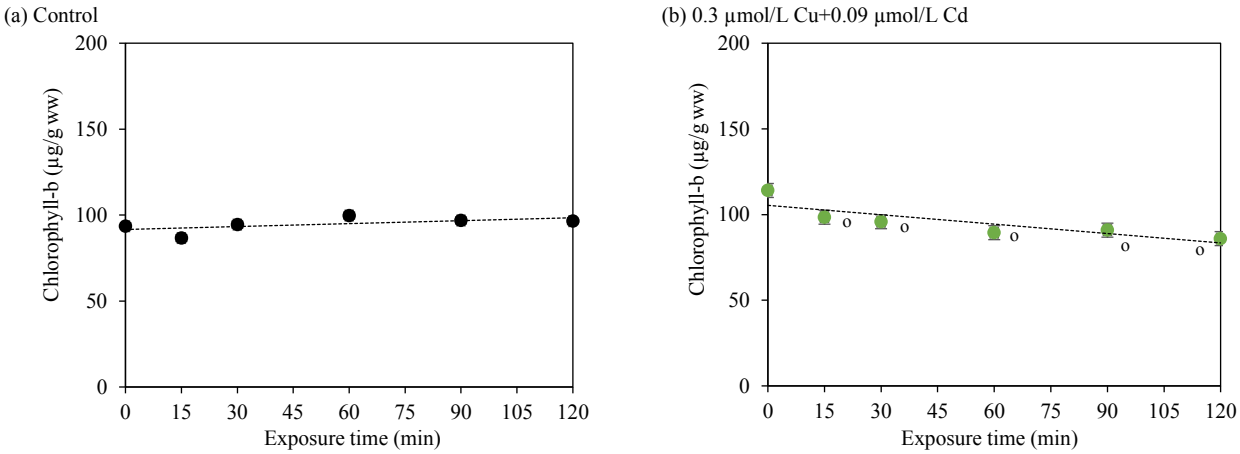

Figure 3. Chlorophyll-b content (µg/g ww) in *C. pyrenoidosa* after exposure to the combinations of (a) 0 µmol/L Cu+0 µmol/L Cd (control), (b) 0.3 µmol/L Cu+0.09 µmol/L Cd, (c) 1.5 µmol/L Cu+0.9 µmol/L Cd, and (d) 15 µmol/L Cu+9 µmol/L Cd. Data are expressed as mean ± standard deviation (n=3). A significant difference in comparison to control is indicated by º.

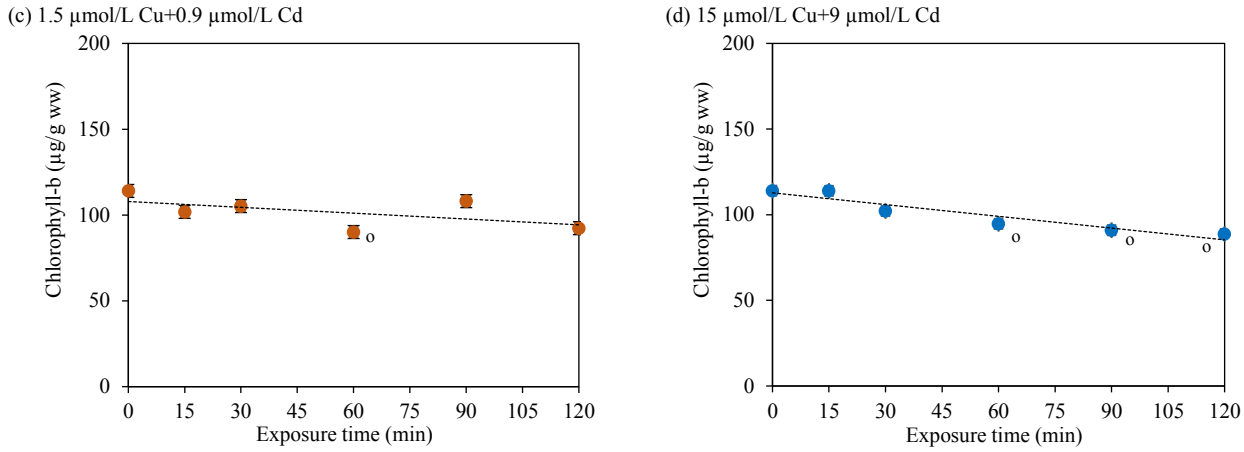

Figure 3. Chlorophyll-b content (µg/g ww) in *C. pyrenoidosa* after exposure to the combinations of (a) 0 µmol/L Cu+0 µmol/L Cd (control), (b) 0.3 µmol/L Cu+0.09 µmol/L Cd, (c) 1.5 µmol/L Cu+0.9 µmol/L Cd, and (d) 15 µmol/L Cu+9 µmol/L Cd. Data are expressed as mean ± standard deviation (n=3). A significant difference in comparison to control is indicated by º (cont.).

Pheophytin-a content tended to increase during the exposure (Figure 4). For all metal combinations, these elevations were significant at 15 min after the exposure (Zulfikar, 2018). At the end of the exposure, the content of pheophytin-a at the combinations of 0.3 µmol/L Cu+0.09 µmol/L Cd (Figure 4(b)), 1.5 µmol/L Cu+0.9 µmol/L Cd (Figure 4(c)), and 15 µmol/L Cu+9 µmol/L Cd (Figure 4(d)) increased by 28%, 53%, and 45%, respectively, compared to the content of pheophytin-a at 0 min.

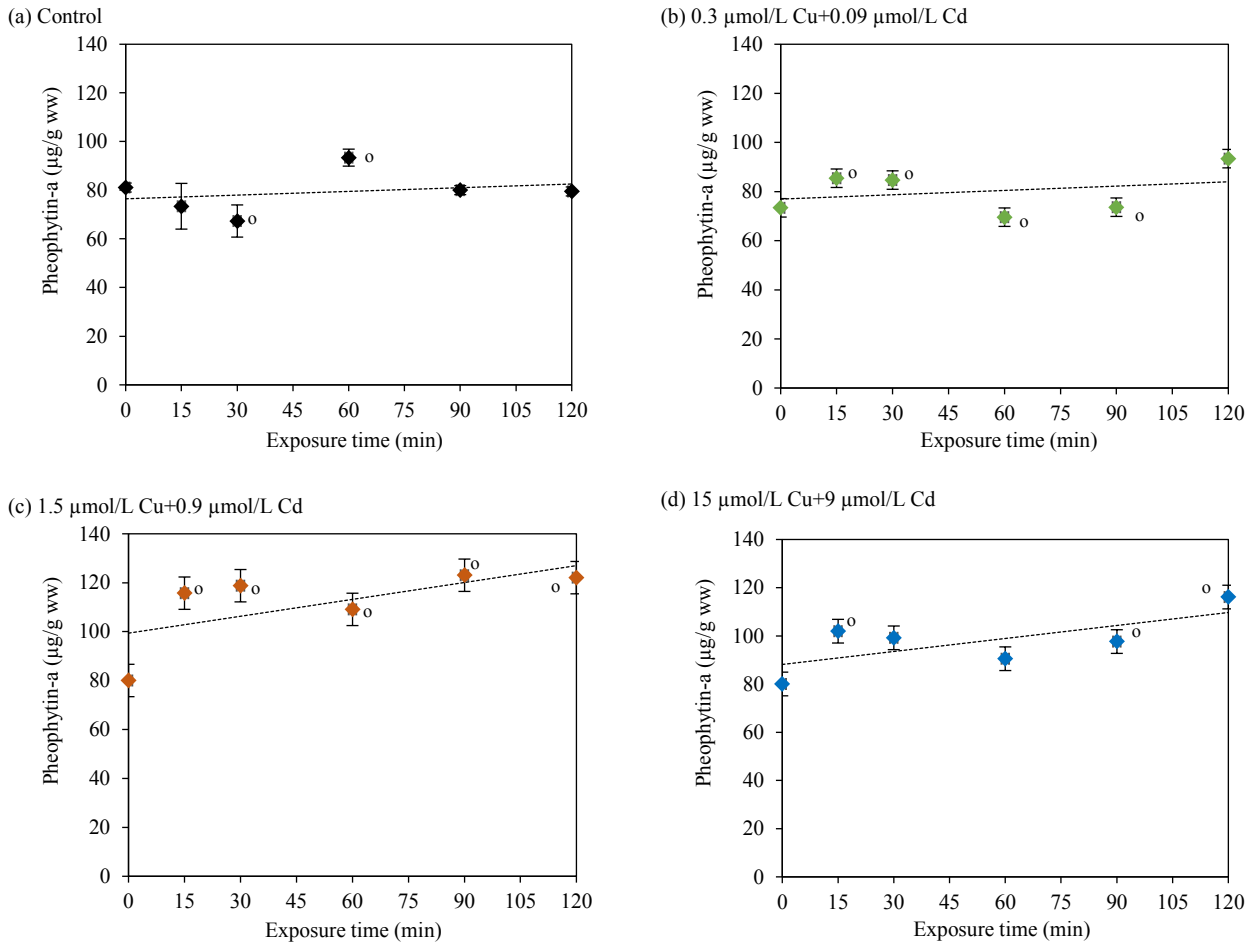

Figure 4. Pheophytin-a content (µg/g ww) in *C. pyrenoidosa* after exposure to the combinations of (a) 0 µmol/L Cu+0 µmol/L Cd (control), (b) 0.3 µmol/L Cu+0.09 µmol/L Cd, (c) 1.5 µmol/L Cu+0.9 µmol/L Cd, and (d) 15 µmol/L Cu+9 µmol/L Cd. Data are expressed as mean ± standard deviation (n=3). A significant difference in comparison to control is indicated by º.

In this study, the decrease of chlorophyll-a content was accompanied by a significant increase of pheophytin-a content starting from the lowest combination of Cu and Cd concentration at 15 min after the exposure (Figure 4(b), (c), and (d)). The increased pheophytin-a level is due to the degradation of chlorophyll-a by the replacement of Mg on the porphyrin ring with free metal (Küpper et al., 1998). In *Parachlorella kessleri*, administration of Cu alone (5.9 µmol/L) increased the content of pheophytin-a (44%, $p<0.05$) at 96 h after the exposure (Nugroho and Frank, 2011). For Cd, the concentration of 0.07 µmol/L administered to *P. kessleri* for 120 h significantly increased the pheophytin-a content (50%) (Ngo et al., 2009). Shehata et al. (1999) reported that exposure of a mixture of metals, including Cu (1.6 µmol/L) and Cd (0.4 µmol/L), to natural phytoplankton assemblages (*Oscillatoria mougeotii* and *Scenedesmus quadricauda*) for 10 days resulted in an inverse relationship between chlorophyll-a and pheophytin-a. This current study also showed a similar pattern, but the decreased chlorophyll-a and increased pheophytin-a occurred at a shorter exposure period. The reverse relationship was also confirmed by the ratio of chlorophyll-a and pheophytin-a. From control to the highest combined concentration, the ratio tended to decrease, i.e., 2.5, 1.9, 1.7, and 1.6. This ratio reportedly serves as a good indicator of the physiological state of microalgae (Bačkor and Váczi, 2002).

3.3 Carbohydrates and proteins

After combined metal exposure, carbohydrate content in all combinations of Cu and Cd tended to decrease (Figure 5(b), (c), and (d)), but the carbohydrate levels were not significantly different from that at 0 min ($p>0.05$). At the end of the exposure, the carbohydrate decrease ranged from 12% to 22%, and the highest decrease occurred at the highest combined concentration (Zulfikar, 2018).

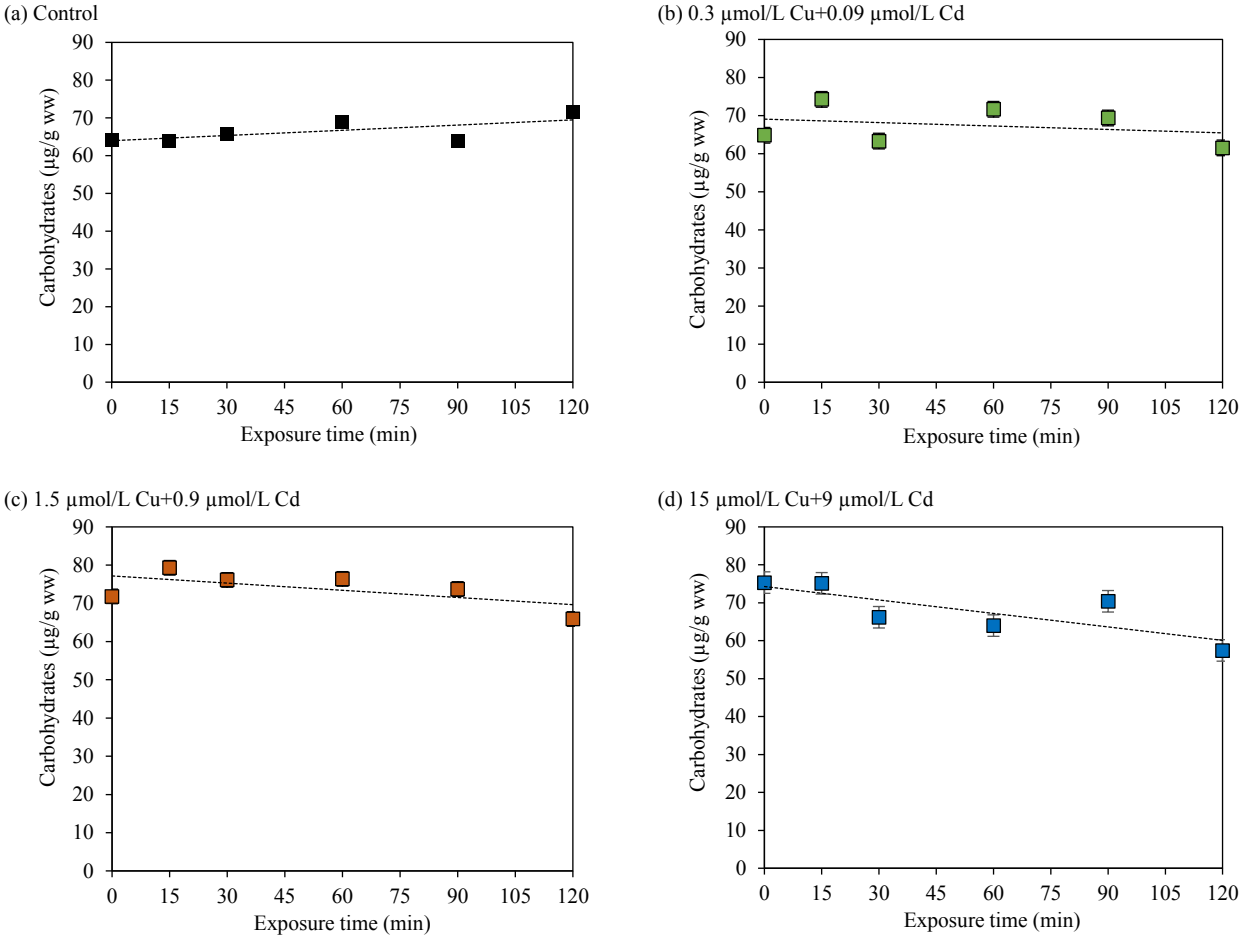

Figure 5. Carbohydrate content (µg/g ww) in *C. pyrenoidosa* after exposure to the combinations of (a) 0 µmol/L Cu+0 µmol/L Cd (control), (b) 0.3 µmol/L Cu+0.09 µmol/L Cd, (c) 1.5 µmol/L Cu+0.9 µmol/L Cd, and (d) 15 µmol/L Cu+9 µmol/L Cd. Data are expressed as mean ± standard deviation (n=3).

The same pattern also occurred with protein content for all combinations of Cu and Cd (Figure 6(b), (c), and (d)). A significant difference was found at the highest combined concentration of Cu and Cd, i.e. between 0 and 120 min ($p<0.05$). The decrease of protein content correlated with increasing concentration of Cu and Cd (Zulfikar, 2018). At the end of the exposure, the protein content was between 8% and 13% lower than at 0 min.

In *P. kessleri*, Cu concentrations greater than 6 µmol/L decreased protein and carbohydrate content (Nugroho and Frank, 2011). For Cd, the metal concentration of 0.07 µmol/L reduced the protein content but increased the carbohydrate content of *P. kessleri* (Ngo et al., 2009). In the present study, at the lowest combined concentration, the tested concentrations of Cu and Cd were similar to those used in studies of *P. kessleri* (Ngo et al., 2009; Nugroho and Frank, 2011). However, the results showed no significant differences, indicating that exposure to the combined metals at the lowest concentration did not result in synergistic effects. Decreased carbohydrate and protein may be associated with increased ROS, since this species can oxidize macromolecules including carbohydrates, proteins, and DNA (Leonard et al., 2004; Nikookar et al., 2005; Valko et al., 2005; Bajguz, 2011; Cadet and Wagner, 2013). As reported by Qian et al. (2009), almost the same concentration combination as the middle one used in this study (1.5 µmol/L Cu and 1.0 µmol/L Cd) increased ROS formation. This species damaged the photosynthetic apparatus, reducing the chlorophyll content and the rate of photosynthesis (Bajguz, 2011), which ultimately decreased the carbohydrate content. ROS also caused peptide chain breakage and increased susceptibility to proteolysis by proteases, leading to protein degradation (Rezayian et al., 2019).

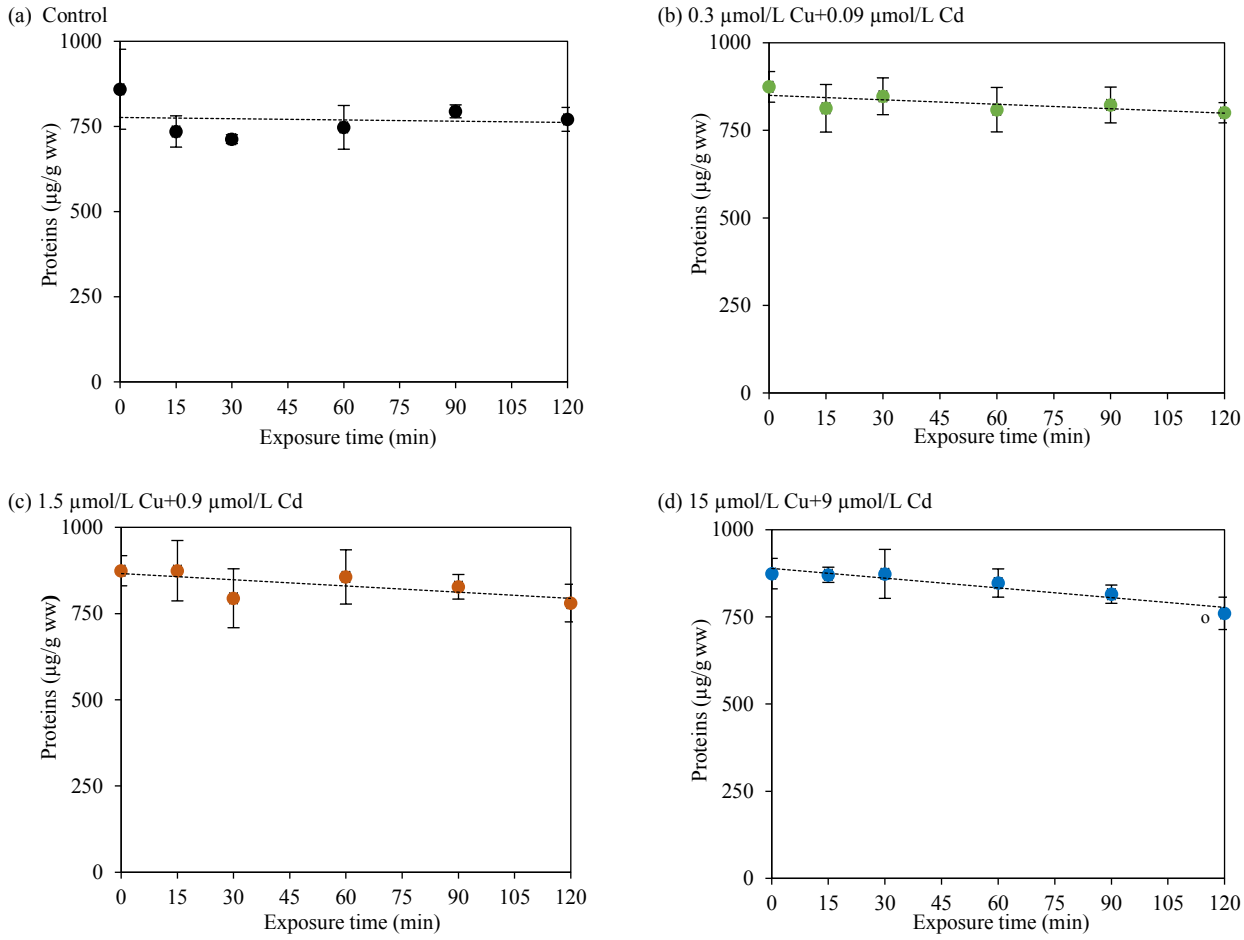

Figure 6. Protein content (µg/g ww) in *C. pyrenoidosa* after exposure to the combinations of (a) 0 µmol/L Cu+0 µmol/L Cd (control), (b) 0.3 µmol/L Cu+0.09 µmol/L Cd, (c) 1.5 µmol/L Cu+0.9 µmol/L Cd, and (d) 15 µmol/L Cu+9 µmol/L Cd. Data are expressed as mean ± standard deviation (n=3). A significant difference in comparison to control is indicated by °.

3.4 Alkaline phosphatase activity

The activity of the enzyme decreased drastically in the first 30 min after exposure ($p<0.05$), by 50% (0.3 µmol/L Cu+0.09 µmol/L Cd) (Figure 7(b)), 62% (1.5 µmol/L Cu+0.9 µmol/L Cd) (Figure 7(c)), and 62% (15 µmol/L Cu+9 µmol/L Cd) (Figure 7(d)). At the end of the exposure (120 min), the decrease of enzyme activity ranged from 54% to 68%. The results also showed that increased inhibition of the enzyme activity accompanied increased combined Cu and Cd concentrations (Zulfikar, 2018).

The studies of Awasthi (2012) and Jiang et al. (2016) showed inhibition of this enzyme activity by several metals including Cu and Cd: the higher the concentrations of the metals, the lower the activity of alkaline phosphatase. These metals may displace essential metal cofactors from the central and functional part of alkaline phosphatase, reducing the activity of the enzyme (Awasthi, 2012). Jiang et al. (2016) reported that in *Chlamydomonas reinhardtii*, the enzyme activity decreased by 50% after exposure to Cu and Cd individually (8.18 and 0.27 µmol/L) for 5 h. Another study on the effect of combined Cu and Cd at lower concentration on the activity of alkaline phosphatase of *C. vulgaris* has also been conducted by Ferro and Durrieu (2012), who demonstrated that the activity of the enzyme decreased by about 50% after exposure to Cu (0.05–0.15 µmol/L) and Cd (0.01 µmol/L) for 48 h. In this study, at a similar combined concentration, the decrease of enzyme activity by about 50% occurred in a shorter time period (30 min).

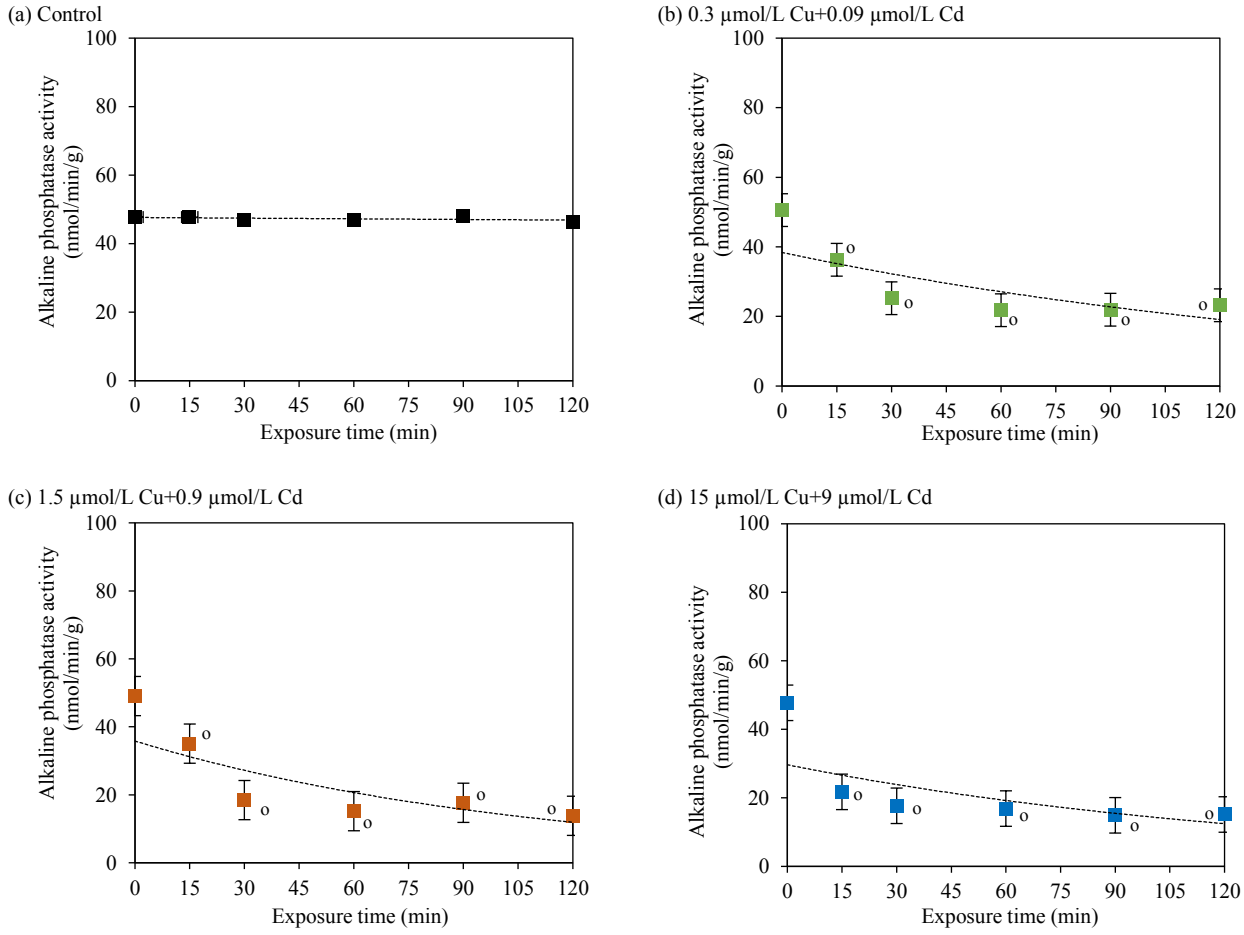

Figure 7. Alkaline phosphatase activity (nmol/min/g) in *C. pyrenoidosa* after exposure to the combinations of (a) 0 µmol/L Cu+0 µmol/L Cd (control), (b) 0.3 µmol/L Cu+0.09 µmol/L Cd, (c) 1.5 µmol/L Cu+0.9 µmol/L Cd, and (d) 15 µmol/L Cu+9 µmol/L Cd. Data are expressed as mean ± standard deviation (n = 3). A significant difference in comparison to control is indicated by °.

3.5 DNA damage

Under the combined metal administration, at the beginning of exposure (2 h), the percentages of tail comets DNA were about 6- to 11-fold higher (Figure 8(b), (c), and (d)) than in the control (Figure 8(a)) and tended to increase until the end of the exposure. Higher Cu and Cd concentrations in combination led to a stronger effect on the integrity of DNA, and the duration of the exposure also affected the level of damage (Rizki, 2019).

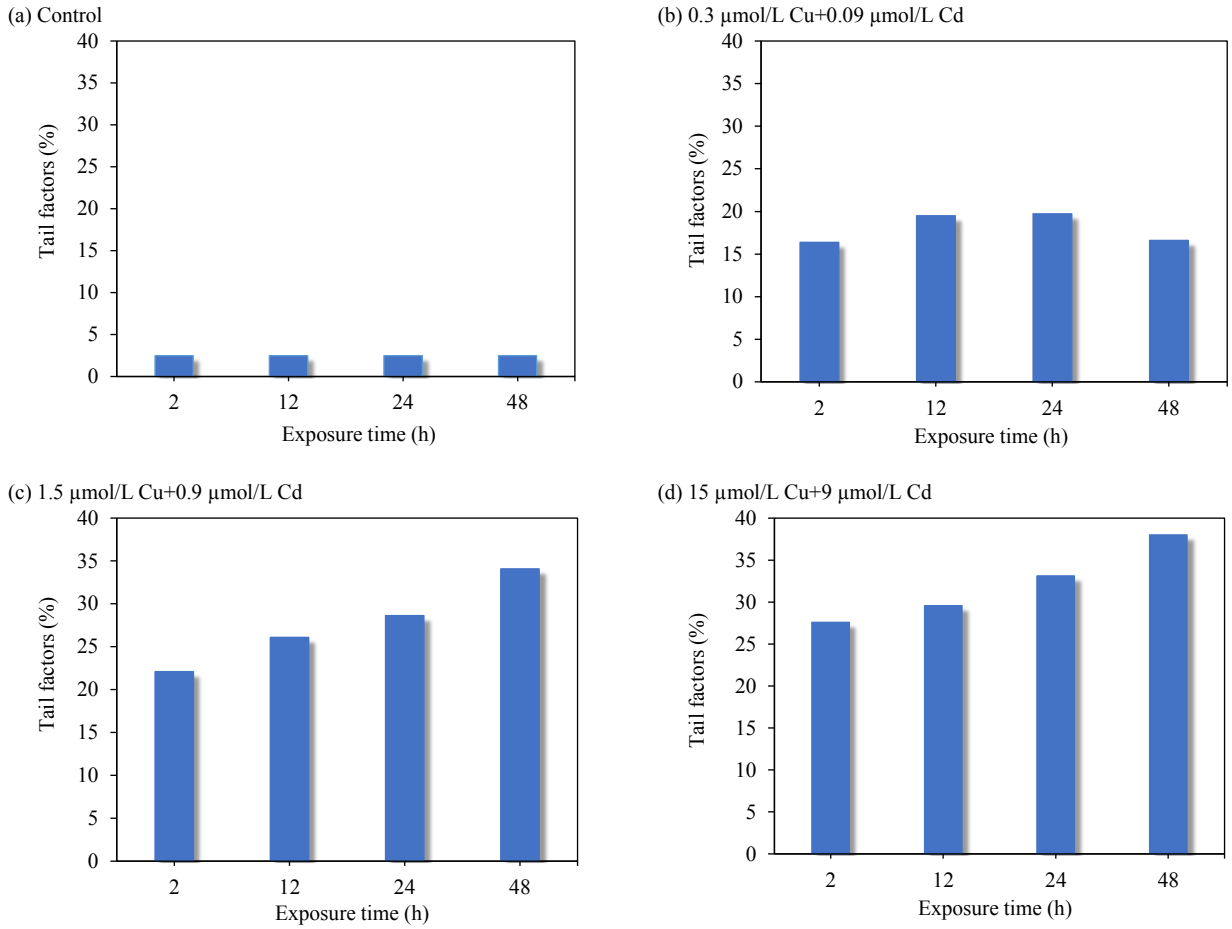

Figure 8. Tail factors of comet-nuclei of *C. pyrenoidosa* after exposure to the combinations of (a) 0 µmol/L Cu+0 µmol/L Cd (control), (b) 0.3 µmol/L Cu+0.09 µmol/L Cd, (c) 1.5 µmol/L Cu+0.9 µmol/L Cd, and (d) 15 µmol/L Cu+9 µmol/L Cd.

Qian et al. (2009) reported that exposure to combined 1.5 µmol/L Cu+1.0 µmol/L Cd for 48 h resulted in overproduction of ROS in *C. vulgaris*, which is one possible mechanism of oxidative stress in microalgae, inducing DNA damage (Pinto et al., 2003). Oxidative attack on DNA causes strand breakage, deoxyribose oxidation, and removal of nucleotides (Cadet and Wagner, 2013). Desai et al. (2006) reported that exposure of Cd to *Chaetoceros tenuissimus* at 0.09 mmol/L for 2 days caused DNA damage in up to 15% of observed cells. Prado et al. (2015) found similar results: the percentage of comet-nuclei of *C. moewusii* and *C. vulgaris* under copper administration at concentrations of 0, 0.02, 0.08, 0.16, 0.31, and 0.47 mmol/L for 3 h ranged from 15.2-95.1% and 15.1-49.2%, respectively. In comparison with the studies of Desai et al. (2006) and Prado et al. (2015), our results indicate that a short-term exposure (2 h) at lower combined metal concentrations (in µmol/L) may lead to synergistic effects.

3.6 Integrated biomarker response

In the IBR star plots, among the selected parameters, alkaline phosphatase activity had the highest S value at all combined metal concentrations: the values ranged from 1.5 to 1.6 (Figure 9(a), (b), and (c)), indicating that this biomarker was the most responsive parameter to the presence of metals in the environment. The high S value of proteins also showed that this parameter was affected strongly by exposure to the combined metals. This demonstrates that the two biomarkers are the most important weights on the IBR index.

Based on the IBR index, the combined concentration of 1.5 µmol/L Cu+0.9 µmol/L Cd resulted in greater stress on the microalgae (Figure 9(b), IBR index=5.8) than the highest combined concentration (Figure 9(c), IBR index = 4.8). These results suggest the occurrence of metal detoxification mechanisms in the microalgae, such as sequestration and compartmentalization, due to excess metals at the

highest combined concentration. Metal detoxification would decrease the concentration of soluble metals, reducing their effects on biochemical parameters (Arunakumara and Xuecheng, 2008; Levy et al., 2008). This would have implications for the S values of the observed parameters.

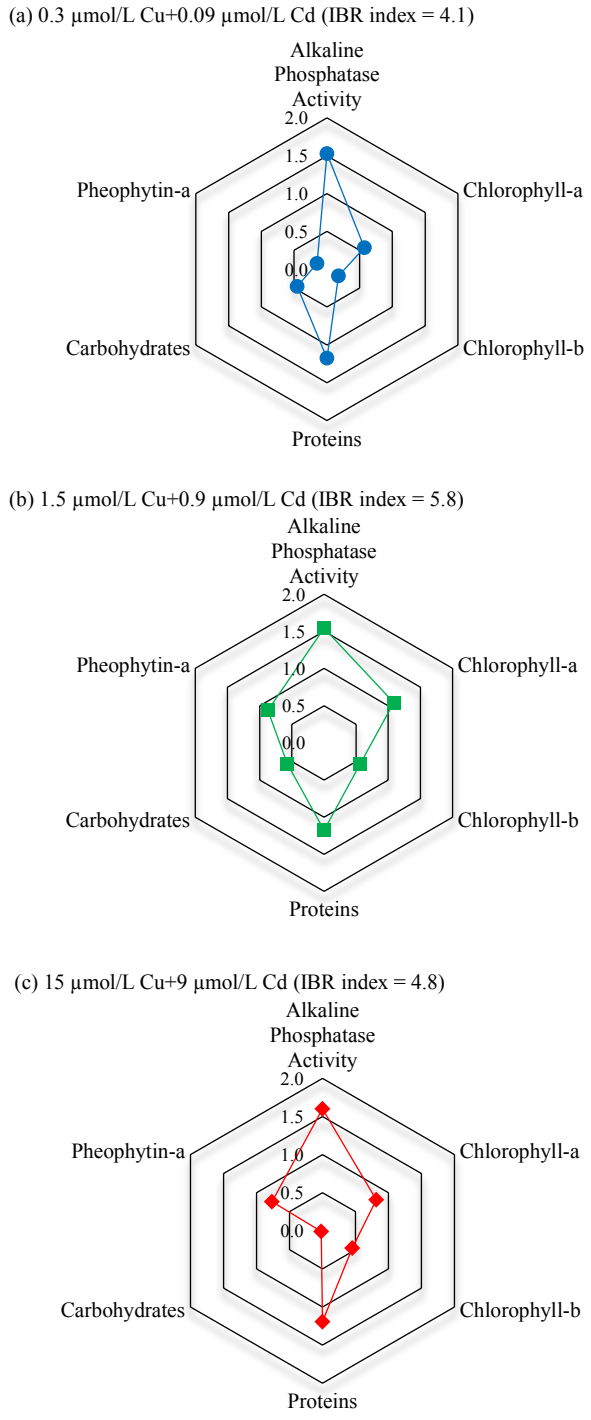

Figure 9. Star plots (IBR) for biomarker responses in *C. pyrenoidosa* exposed to the combinations of (a) 0.3 µmol/L Cu+0.09 µmol/L Cd, (b) 1.5 µmol/L Cu+0.9 µmol/L Cd, and (c) 15 µmol/L Cu+9 µmol/L Cd.

4. CONCLUSION

Short-term exposure to combined Cu and Cd at environmentally realistic levels or lower concentrations can lead to inhibition of growth and activity of alkaline phosphatase, a decrease of chlorophyll-a, chlorophyll-b, and protein content,, an increase of pheophytin-a content, and an increase of tail factors DNA. IBR analysis affirmed that inhibition of alkaline phosphatase activity and a decrease of protein level are responsive biomarkers to exposure to combinations of metals; therefore, these are promising biomarkers for biomonitoring of aquatic metal pollution.

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