

Integrated assessment of biomarker responses in algae *Chlorella sorokiniana* exposed to copper and cadmium

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Abstract. Rahayu TH, Nugroho AP. 2020. *Integrated assessment of biomarker responses in algae Chlorella sorokiniana exposed to copper and cadmium. Biodiversitas 21: 3569-3575.* Microalgae *Chlorella* as a major producer in aquatic ecosystems has high probability of being exposed to metal pollutants. Metal exposure to algae can induce the formation of reactive oxygen species (ROS), leading to oxidative stress states. ROS trigger the responses of biomarkers of antioxidative enzymes, including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). This study aims to develop an integrated biomarker response index and star plot for interpretation of the antioxidative enzyme responses in *Chlorella sorokiniana*. The microalgae were exposed to three concentrations of copper (Cu) and cadmium (Cd) for 48 h, respectively. The results showed that exposure to Cu and Cd caused an increase of protein content and activities of SOD and APX. Analysis of integrated biomarker response (IBR) showed a decrease in the value of IBR along with an increase in cadmium concentration but no similar pattern found in copper. The multiple factor analysis (MFA) approach showed that exposure to Cu 0.3 $\mu\text{mol L}^{-1}$ for 48 h had high score of protein content and SOD activity in the first dimension. For the second dimension, exposure to Cu 1.6 $\mu\text{mol L}^{-1}$ for 6 h had high score of APX and SOD activity.

Keywords: Antioxidative responses, *Chlorella sorokiniana*, integrated biomarker responses, metal, multiple factor analysis, star plot

INTRODUCTION

Freshwater microalgae such as *Chlorella* are used in environmental toxicology studies because of their abundant quantity, short life cycle, easy to be cultured, and sensitive to metal contaminants at environmentally realistic levels (Wang et al. 2018). Cu and Cd represent metals that generally pollute aquatic ecosystems and can cause toxic effects on aquatic organisms. Cu is an important micronutrient for plants and algae. It is a component of proteins and enzymes involved in various metabolic pathways (Elisabetta and Gioacchino 2004). However, it can be toxic at high concentrations (Chang and Sibley 1993). Cd is a non-essential element and toxic to humans, animals, and plants even in low concentrations.

In algae, metals stimulate defense mechanisms through the production of non-enzymatic and enzymatic antioxidants (Dat et al. 2000). Non-enzymatic antioxidants include glutathione, phenol, and ascorbate, while enzymatic defenses include superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). SOD catalyzes the conversion of superoxide ($\text{O}_2^{\bullet-}$) to peroxide (H_2O_2), while APX and CAT enzymes catalyze the reduction of H_2O_2 to H_2O and O_2 (Bhattacharjee 2005). Those antioxidants are called biomarker responses because antioxidants are produced as one of the responses of algae when they are exposed to pollutants such as metal.

A common challenge in multi-biomarker studies is to go beyond an independent interpretation of each one, and to assess the global response of individual biomarkers. This global assessment also simplifies their interpretation of

biomonitoring programs. Among the indices proposed to integrate individual biomarker responses, the most popular is the Integrated Biomarker Response (IBR) initially proposed by Beliaeff and Burgeot (2002). The IBR approach is a method that provides both a graphical synthesis of the different biomarker responses and a numeric value that integrates all these responses at once (Devin et al. 2014). This study aims to develop an integrated biomarker response index and star plot for interpretation of the antioxidative enzyme responses in *Chlorella sorokiniana*.

MATERIALS AND METHODS

Culture condition and experimental design

The freshwater algae *Chlorella sorokiniana* Shirira et Krauss was provided by Ugo Plankton Laboratory which is located in Purworejo, Central Java. It was grown in axenic conditions at 23°C, under continuous illumination (1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity), and in Guillard F/2 medium (Sandoval et al. 2015). Guillard F/2 medium consists of macronutrients ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), micronutrients ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) and vitamins (thiamine HCl, biotin, cyanocobalamin). To test the effect of Cu and Cd, stock solution was prepared by dissolving $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$, respectively.

For testing the effects of Cu and Cd, into each 1-L culture bottle containing 750 mL algal culture with the density of $6.9 \times 10^6 \text{ cell mL}^{-1}$.) was added Cu and Cd from

a stock solution, respectively, to establish the concentrations of Cu, i.e. 0.3, 1.6, and 15.7 $\mu\text{mol L}^{-1}$, and Cd, i.e. 0.2, 0.9, and 8.9 $\mu\text{mol L}^{-1}$ (n=3). Three culture bottles were used as controls. At 0, 2, 6, 24, and 48 h after the addition of Cu and Cd, 100 mL aliquots were transferred into two 50-mL polypropylene tubes for analysis of enzymatic antioxidants.

Enzyme extraction

Enzyme extraction was conducted by modified method of Moussa et al (2017). Algae was centrifuged at 4500 g for 5 min, then the pellet was added with phosphate-buffered saline and centrifuged at 4500 rpm for 5 min. Pellet was put into 2 mL microtube of known weight then weighed to measure fresh weight. Fresh algae were crushed five times using liquid nitrogen and added protease inhibitor solution each one mL of volume. Protease inhibitor solution was made from mixture of 50 g of protease inhibitor for each 50 mL aquabidest. This mixture was centrifuged at 11000 g for 5 min (4°C). The supernatant was used for analyzing protein, superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) activity assays.

Protein analysis

Protein analysis was conducted following the method of Lowry et al (1951) by reacting 300 μL supernatant to 300 μL of Lowry D reagent then vortexed for 15 sec. Lowry D reagent was made in commercial laboratory (CV Chem-Mix Pratama Jogjakarta). Sample of mixture was incubated for 10 min, reacted to 900 μL of Lowry E reagent then vortex for 30 sec. Then, the mixture was incubated for 45 min, and the absorbance was read using a spectrophotometer at λ 750 nm.

Superoxide dismutase analysis

Analysis of SOD activity was conducted following the method of Marklund and Marklund (1974) in Kim et al (2017). This method reacted 1 mL of Tris-HCl buffer (pH 8.2) with 1 mL of aquabidest and 15 μL of algae extract supernatant and added with 10 μL of pyrogallol 2 mM. Absorbance measurements were performed with a spectrophotometer using a wavelength of 470 nm with time intervals of 3 min. Blank solutions were combination of pyrogallol and aquabidest (ratio 1: 1). The measured data were expressed as units per milligram of protein (1 unit was the amount of enzyme utilized to inhibit 50% of pyrogallol auto-oxidation per minute).

Catalase analysis

Catalase activity was measured according to modified Aebi and Lester (1984) method in Kasmiyati (2016). A total of 1.99 mL of phosphate buffer solution 50 mM pH 7.0 and 10 μL of supernatant sample was mixed. One milliliter of H_2O_2 solution (3% concentration) was added to mixture then incubated for a minute. Catalase enzyme activity was measured by a UV-VIS spectrophotometer at wavelength of 240 nm for 3 min (one-minute interval). Value of H_2O_2 extension coefficient is 40 mM cm^{-1} . Enzyme activity is expressed in $\mu\text{mol H}_2\text{O}_2 \text{ gram}^{-1}$ fresh weight. Fresh weight of algae was determined by

harvesting algae using centrifuge at 11000 g for 5 min (4°C) then they were weighed.

Ascorbate peroxidase analysis

APX activity was conducted following the method of Nakano and Asada (1981) with modification. A mixture of 1.3 mL potassium phosphate buffer 0.05 mM pH 7.8 contained 0.1 mM EDTA was reacted with 10 μL supernatant, 800 μL 0.5 mM ascorbate and 800 μL H_2O_2 (3% concentration). Those were incubated for a minute then the decrease in absorbance was measured for 3 min (one-minute interval) at λ 290 nm using a spectrophotometer. The collected data were used to define the reaction rate for H_2O_2 independent of ascorbate oxidation.

Statistical analysis

Analysis of variance (ANOVA) test was carried out to identify significant difference between metal concentration and exposure time to enzyme activity. Then data were analyzed using Duncan (DMRT) test to compare differences between two independent groups when the dependent variable is either ordinal or continuous.

Integrated Biomarker Response (IBR) was calculated using four selected biomarkers of *C. sorokiniana*: protein content, SOD, CAT, and APX activities. Briefly, the mean value (X_i), the general mean (m_i), and standard deviation (SD_i) for each biomarker at each exposure condition were calculated (Iturburu et al. 2018). The value X_i was then standardized to obtain Y_i , where $Y_i = (X_i - m_i) / SD_i$. Subsequently, $Z_i = -Y_i$ or $Z_i = Y_i$ were computed in the case of a biological effect corresponding. The minimum value (mini) of Z_i for each biomarker was obtained for each exposure condition. Finally, the score S was calculated as $S_i = Z_i + |\text{mini}|$, where $|\text{mini}|$ is the absolute value. These S values thus represented the gradient of values for each biomarker in different exposure conditions, with highest values corresponding to the highest biological effects. The integrated biomarker response for each condition was calculated via the following formula:

$$\text{IBR} = S_1 * \frac{S_2}{2} + S_2 * \frac{S_3}{2} + \dots + S_{n-1} * \frac{S_n}{2} + S_n * \frac{S_1}{2}$$

In which, the obtained score for each biomarker (S_i) was multiplied with the score of the next biomarker (S_{i+1}), arranged as a set, dividing each calculation by 2 and adding-up of all values (Devin et al. 2014, Iturburu et al. 2018).

Multiple Factor Analysis (MFA) is a multivariate data analysis method for summarizing and visualizing a complex data table in which individuals are described by several sets of variables (quantitative and /or qualitative) structured into groups (Pagès 2002). Its goal is to analyze several data sets of variables collected on the same set of observations, or—as in its dual version—several sets of observations measured on the same set of variables (Lê and Pages 2010). Exploratory data analyses were performed using R software with the FactoMineR package:

<https://cran.r-project.org/web/packages/FactoMineR/> (Le et al. 2008).

RESULTS AND DISCUSSION

Results

Protein content

Exposure to Cu and Cd resulted in significant increase of soluble protein content ($p < 0.05$) (Figure 1). Exposure time (0, 2, 6, 24, 48 h) of Cu and Cd also showed significant difference on protein contents of *C. sorokiniana* ($p < 0.05$). The results of post hoc DMRT test was shown in Tables 1 and 2.

Activity of antioxidative enzymes

Exposure to Cu and Cd also had significant effects on superoxide dismutase and ascorbate peroxidase activities ($p < 0.05$), but for catalase activity was not significant ($p = 0.054$). Based on Figures 2-4, there were increasing activities of each treatment if compared to controls.

Biomarker responses

This study was conducted to understand effects of copper and cadmium on enzymatic antioxidants of *C. sorokiniana*. The calculation for copper and cadmium consisted of 4 biomarkers (B): Protein content, SOD, CAT, and APX enzyme activities. The value of B was based on standard deviation of each variable without using statistical calculations (Table 3). The four B values were then integrated into one IBR value for each concentration. IBR value for Cu ranged from 2.34 to 2.74 (shown in Figure 5. A-C). IBR value for Cd ranged from 2.49 to 2.54 (shown in Figure 5. D-F).

Multiple factor analysis

This analysis was divided into one supplementary group and two active groups (Figures 6-8). Red color referred to active groups of variables and green color was supplementary groups of variables (Figure 11). There were two dimensions, the first dimension/Dim 1 (44.4%) and the

second dimension/Dim 2 (29.35%). Dimensions counted for variations in component analysis based on eigenvalues (Matus et al. 1999). Coordinates between two active groups in the first dimension differed slightly whereas in the second dimension (Dim 2) there was only one group of variables namely enzyme activity (enzyme output). It had significant contribution to the second dimension. Briefly, Figure 7 showed the relationship between variables, quality of the representation of variables, as well as, the correlation between variables, and the dimensions.

Table 1. Significance of Cd and Cd concentration to protein content based on post hoc DMRT test

Treatments	Protein content (mg gram ⁻¹)
Control	0.076 ^a
Cu 0.3 μmol L ⁻¹	0.159 ^{ab}
Cu 1.6 μmol L ⁻¹	0.305 ^{ab}
Cu 15.7 μmol L ⁻¹	0.333 ^b
Cd 0.2 μmol L ⁻¹	0.368 ^b
Cd 0.9 μmol L ⁻¹	0.374 ^b
Cd 8.9 μmol L ⁻¹	0.385 ^b

Note: ^{abc}Different letters show significant differences between treatments

Table 2. Significance of Cd and Cd exposure time to protein content based on post hoc DMRT test

Hour	Protein content (mg gram ⁻¹)
0	0.096 ^a
2	0.133 ^a
6	0.219 ^{ab}
24	0.349 ^b
48	0.631 ^c

Note: ^{abc}Different letters show significant differences between treatments

Table 3. B value of *Chlorella sorokiniana* exposed to Cu and Cd

Treatments (μmol L ⁻¹)	B value				IBR
	Protein	SOD	CAT	APX	
Cu 0.3	0.650	0.592	0.524	0.574	2.34
Cu 1.6	0.715	0.696	0.654	0.672	2.74
Cu 15.7	0.698	1.345	0.654	0.679	2.38
Cd 0.2	0.619	0.576	0.650	0.698	2.54
Cd 0.9	0.605	0.558	0.648	0.702	2.51
Cd 8.9	0.498	1.025	0.490	0.476	2.49

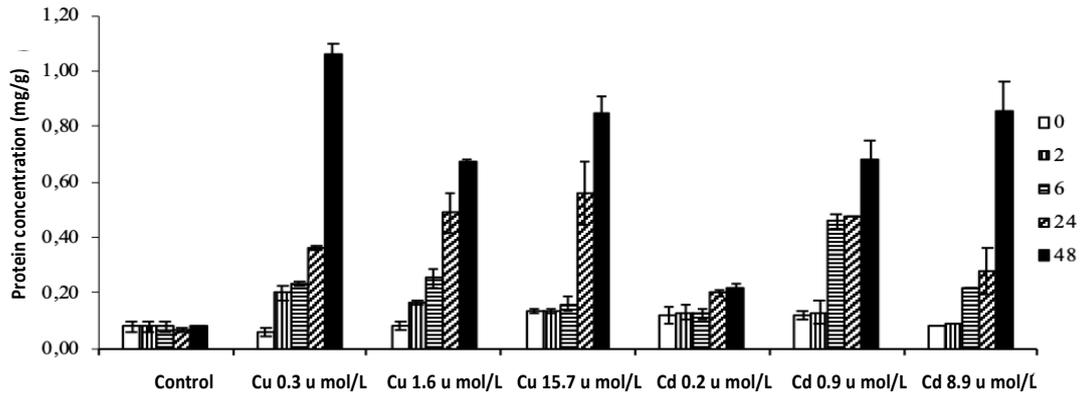


Figure 1. Protein content of *Chlorella sorokiniana* after exposure to Cu and Cd.

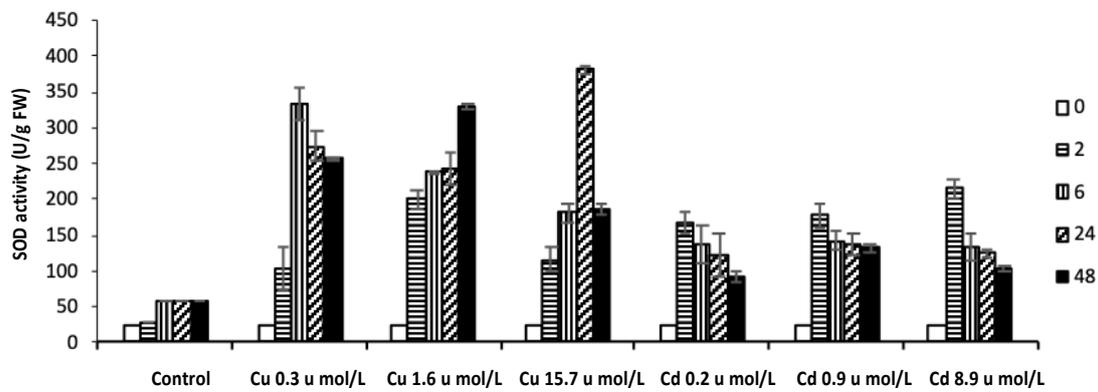


Figure 2. SOD enzyme activity of *Chlorella sorokiniana* after exposure to Cu and Cd.

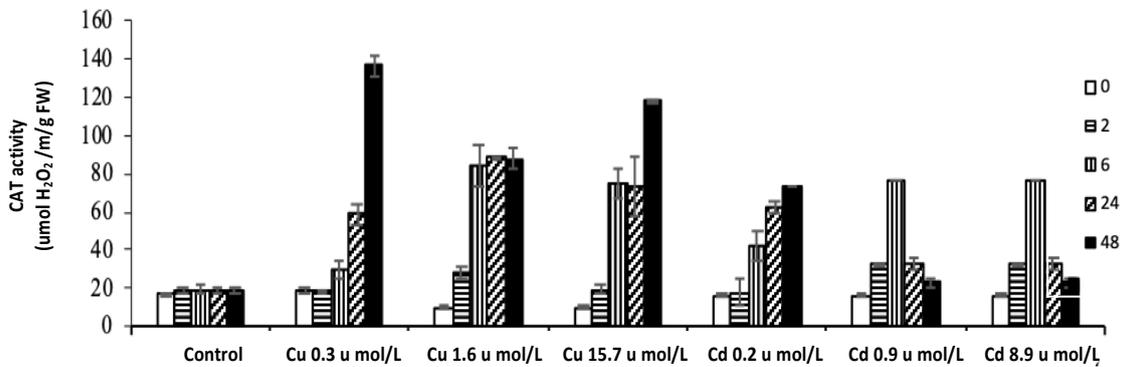


Figure 3. CAT enzyme activity of *Chlorella sorokiniana* after exposure to Cu and Cd.

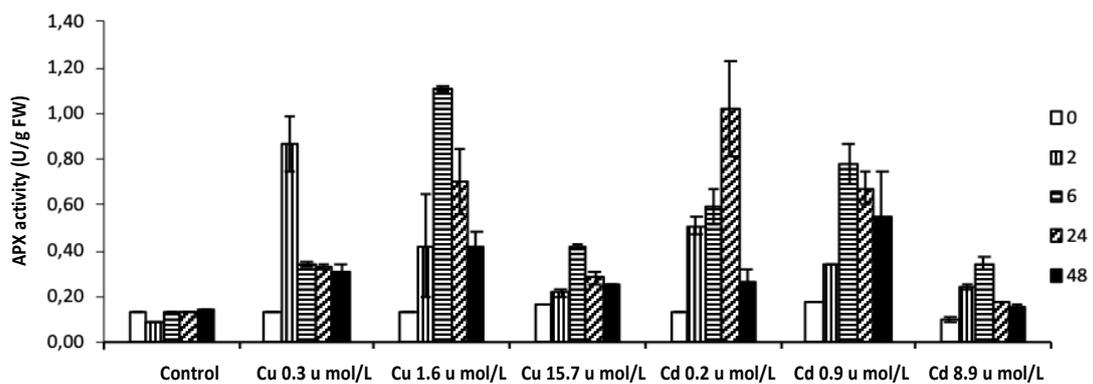


Figure 4. APX enzyme activity of *Chlorella sorokiniana* after exposure to Cu and Cd.

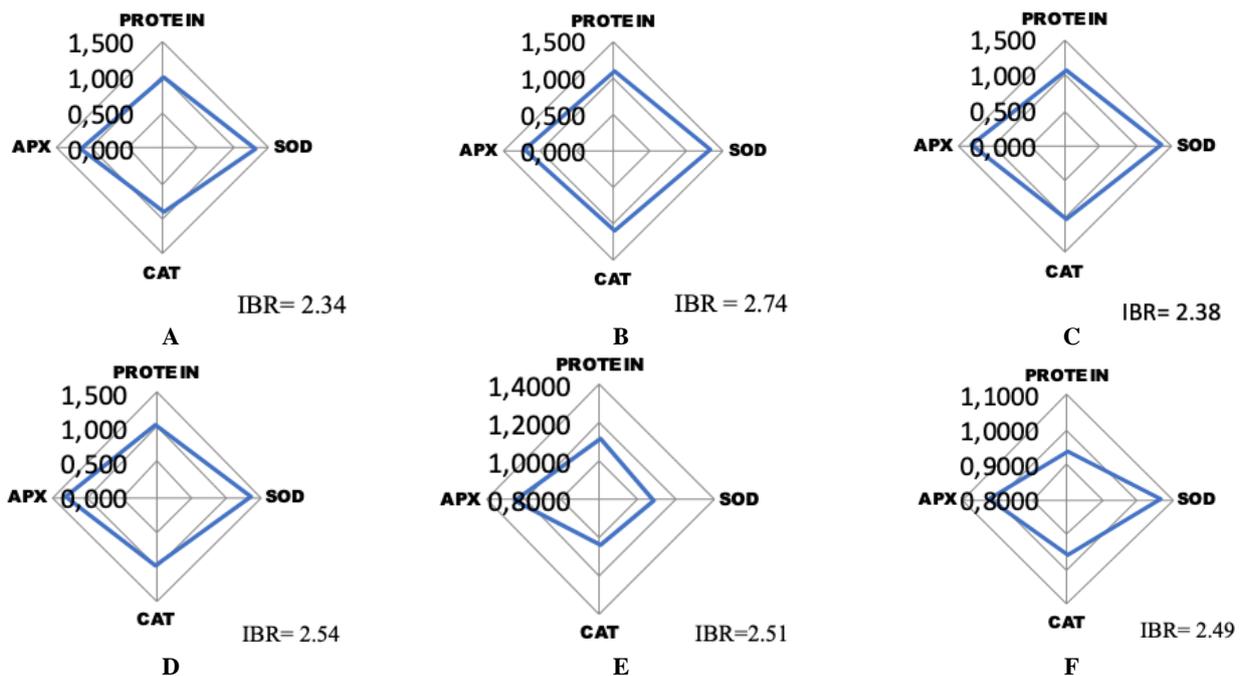


Figure 5. A. IBR value of Cu 0.3 $\mu\text{mol L}^{-1}$, B. IBR value of Cu 1.6 $\mu\text{mol L}^{-1}$, C. IBR value of Cu 15.7 $\mu\text{mol L}^{-1}$, D. IBR value of Cd 0.2 $\mu\text{mol L}^{-1}$, E. IBR value of Cu 0.9 $\mu\text{mol L}^{-1}$, F. IBR value of Cu 8.9 $\mu\text{mol L}^{-1}$

The first dimension had positive sentiment for protein content, SOD, and APX activity but it had negative sentiment for CAT activity. However, the second dimension only had positive sentiment for enzyme activity (protein content had negative sentiment in the second dimension). Not only analyzed groups variable and correlation between groups, MFA also could analyze individuals data. Figure 8 visualized 90 datasets based on values from the MFA program calculation. There were 90 individual data, a combination of 3 concentration of Cu and Cd metal exposed at 5 time periods in triplicate. Blue line referred to cadmium and yellow line referred to copper. Individual data number 61 had the highest value in the first dimension and individual data number 15 had the highest value in the second dimension.

Discussion

Antioxidative defense mechanism

Chlorella sorokiniana is used in this study because it shows higher absorption of metal than other algae. *C. sorokiniana* also shows less growth inhibition and oxidative stress response, which could possibly be caused by a more efficient antioxidant defense system, alleviating the stress induced by metal exposure (Hamed et al. 2017). Under no stress conditions, the formation and removal of reactive oxygen reactive (ROS) are balanced. However, under stress conditions, the formation of ROS exceeds the ability to eliminate them, so it is necessary to form enzymatic and non-enzymatic antioxidant compounds (Alscher et al. 2002). The first enzymatic compound formed to reduce ROS level is superoxide dismutase (SOD) enzyme. SOD will reduce ROS content by converting $\text{O}_2^{\bullet-}$ to hydrogen peroxide which easily passes through the cell plasma membrane. This hydrogen peroxide will later be

converted into a less reactive compound by the CAT and APX enzymes.

As mentioned in results, there are significant differences in protein content, SOD, and APX activities after exposure to copper and cadmium. The only exception is CAT enzyme activity. The similar result also was found in Mallick (2004). CAT and APX have same substrate, hydrogen peroxide (H_2O_2). Anjum et al. (2016) state that APX has higher affinity of H_2O_2 than CAT by using ascorbate acid as specific electron donor. Then ascorbate acid is used for reduction of H_2O_2 to H_2O in chloroplast, cytosol, mitochondria, and peroxisome organelles. It means that when APX activity is getting higher, CAT enzyme activity is not showing same pattern because they work antagonized. Those three enzymes are related to each other to reduce levels of reactive oxygen species (ROS) in organisms. The relationship between three enzymes is shown in Figure 9.

Integrated biomarker responses (IBR)

Biomarkers responses are not always clear and easy to interpret because of their different pattern and visuals. This study used four biomarker responses (Protein content, SOD, CAT, and APX activities) and IBR value interpreted in each concentration of copper and cadmium (Table 3). Figure 5. A-F showed the score of protein content, SOD, CAT, and APX activity for each metal (Cu and Cd) concentration. Those scores are drawn by using star plot and computerized as IBR.

Metal such cadmium has been known for its effect to increase antioxidative enzyme activities (Zhu et al. 2019). However, which enzyme has most sensitive response is still unknown. IBR could be one of tool to find out the question before. Cu and Cd concentration does not correlate with the

increasing or decreasing of IBR value. Based on ANOVA and post hoc DMRT test, Cu and Cd do not have significant effect on CAT enzyme activity of *C. sorokiniana*. This result is updated with the IBR value that each concentration affects CAT enzyme activity differently (See Figure 5. A-F). This can occur because the IBR is a method that provides both a graphical synthesis of the different biomarker responses and a numeric value that integrates all these responses at once (Devin et al. 2014). The IBR value is calculated using selected biomarkers in a way of integrating responses and facilitate their interpretation (Iturburu et al. 2018).

Multiple factor analysis

Multiple factor analysis (MFA) is used to compare data sets structured in groups of variables in order to compare the groups and the conclusions to be drawn from the analysis of the data set by each group (Uher et al. 2017). In this study, the data are structured into three groups: one supplementary group (factor: metal) and two active groups (protein content and enzyme activity). Both active groups have strong contribution to dimension 1 whereas only

enzyme activity had same contribution to dimension 2 (Figure 6). These results mean that there is a strong connection between protein content and enzyme activity. Change in protein content would affect enzyme production (Li et al. 2006). More explanation about protein content and enzyme activity is shown in Figure 7. Protein content and SOD have high values in dimension 1, and APX and SOD in dimension 2. Specific function of MFA besides showing the relationship between group variables is able to show the relationship between individuals in two dimensions. Individuals with similar profiles are close to each other on the factor map (Abdi and Williams 2010). Individual data number 61 has the highest protein content and SOD enzymes in dimension 1 and individual data number 15 has the highest APX and SOD enzyme activity (Figure 8). Variable which has the highest value in each dimension is the variable that most contributes to that dimension. The variables that most contribute to the first dimension and the second dimension are the most important variables that can explain the level of variability of data (Pagès 2002).

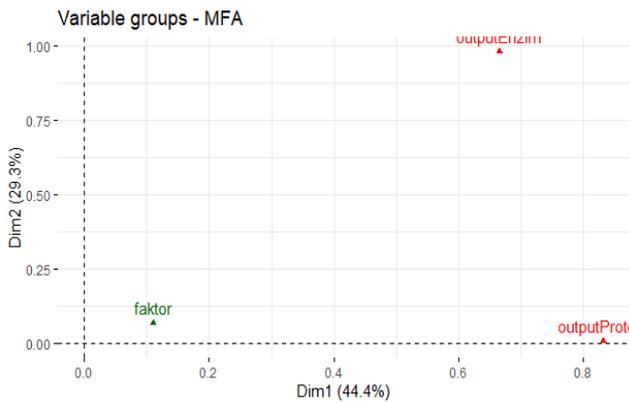


Figure 6. Representation of groups

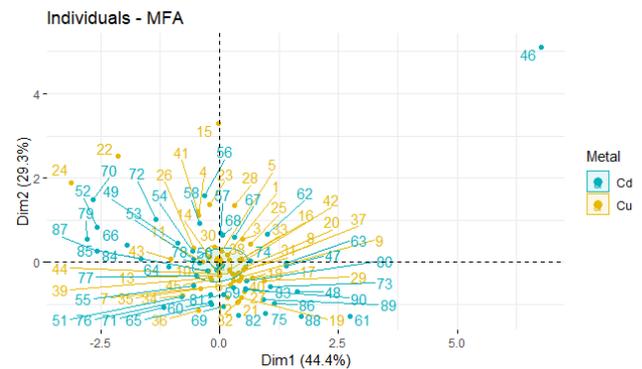


Figure 8. Individual data of each treatment and exposure time

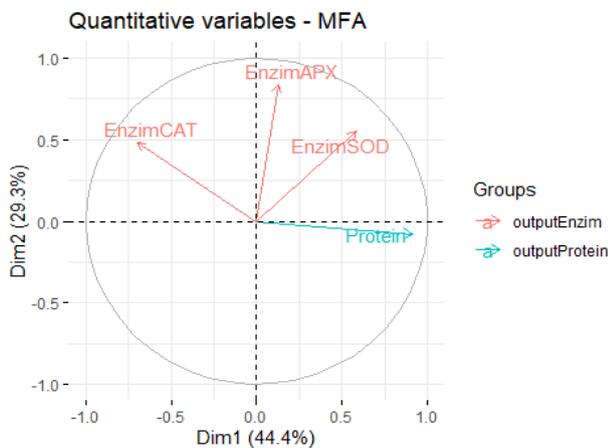


Figure 7. Relationship of quantitative variables with dimensions

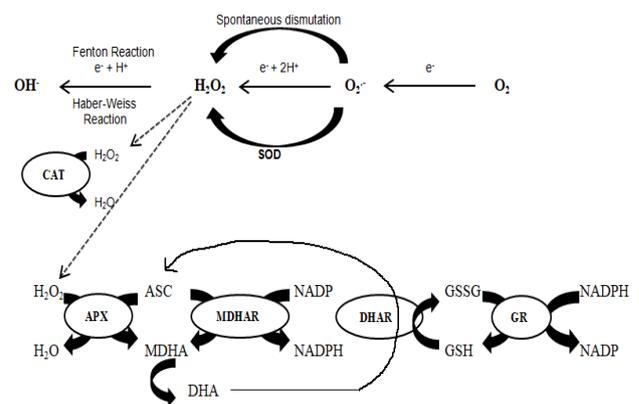


Figure 9. Defense mechanism against ROS (Anjum et al. 2015)

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