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COMBINED EFFECT OF PESTICIDES CONTAINING ACTIVE INGREDIENTS OF CHLORPYRIFOS AND MANCOZEB ON THE DNA DAMAGE OF *CHLORELLA SOROKINIANA* SHIHIRA AND KRAUSS

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ABSTRACT

The intensive use of pesticides in agricultural areas can leave residues of pesticide mixtures on the soil surface. Surface run-off can carry pesticide residues, enter water bodies, and then may affect non-target organisms. Chlorpyrifos and mancozeb are active ingredients commonly contained in pesticides applied in shallot farming. This study aims to evaluate the combined effect of pesticides containing active ingredients of chlorpyrifos and mancozeb on the growth and DNA damage of the microalgae *Chlorella sorokiniana*. The test organism was exposed to the combined concentration of chlorpyrifos:mancozeb, i.e., 0:0, 20%:20%, 20%:80%, 80%:20%, and 80%:80% of the individual EC50 of each pesticide with sampling time at hours 0, 6, 24, and 48. Microalgae growth was estimated by cell counting method, and DNA damage was analyzed by alkaline comet assay method with parameters, i.e., Tail Intensity (TI%), Head Intensity (HI%), Tail Moment (TM), Olive Tail Moment (OTM), and Tail Factor (TF). The results showed that the combined pesticides inhibited the growth of *C. sorokiniana*, with the highest growth inhibition being at a combined concentration of 80%:80%. The TM and OTM values of *C. sorokiniana* increased with the increase of combined concentrations at an exposure period of up to 24 hours. In conclusion, the combined exposure could induce growth inhibition and DNA damage of *C. sorokiniana*, mainly in the first 24 hours. The TM and OTM can be used as sensitive biomarkers for biomonitoring pesticide pollution.

Keywords: *chlorella sorokiniana*; comet assay; chlorpyrifos; DNA damage; mancozeb

INTRODUCTION

The use of pesticides is currently very extensive and sometimes not following the recommended dosage. This will increase the amount of pesticide residue in the soil (Rajmohan et al., 2020). Their high accumulative and persistent characteristics also can cause detrimental effects on soil organisms (Nie et al., 2020). Surface run-off can carry pesticide residues, enter water bodies, and affect non-target organisms such as microalgae, zooplankton, and fish. A study on the quality of freshwaters in Indonesia shows that pesticides have been detected in the rivers, such as endosulfan (Prabowo & Subantoro, 2012) and malathion (Atifah et al., 2019). The study indicated that river waters have the potential to be contaminated with various types of pesticides. In nature, two or more types of pesticides can be absorbed by organisms. Interactions between pesticides in the body or cells of organisms can produce synergistic, additive, or antagonistic effects (Zhang et al., 2012; Chamsi et al., 2019). So, it is necessary to evaluate the toxicity effect of the combination of widely used pesticides on aquatic organisms.

Pesticides containing chlorpyrifos and mancozeb as active ingredients are among the most commonly used in Indonesia, such as in shallot farming. Chlorpyrifos [O,O-diethyl O-(3,5,6-

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trichloro-2-pyridinyl) thiophosphate] belongs to the organophosphate insecticides (Huang et al., 2020), while mancozeb (Mn-Zn-ethylenebisdithiocarbamate) is one of the active ingredients of contact or non-systemic dithiocarbamate fungicides (Roede & Miller, 2014). Various studies show that these two types of active ingredients can be toxic and cause changes in freshwater organisms from the cellular to the community level. Chlorpyrifos (CP) can enter the aquatic ecosystem, be absorbed by freshwater organisms, and become toxic at various concentrations, ranging from sub-lethal or lethal concentrations (Asselborn et al., 2015). Chlorpyrifos exposure causes oxidative stress in fish in the presence of DNA damage (Sud et al., 2020), inhibits the germination and growth of *Allium cepa* (Fatma et al., 2018b), and increases the activity of antioxidative enzymes, damages DNA and affects chlorophyll levels in microalgae and cyanobacteria (Martinez et al., 2015; Chen et al., 2016). Meanwhile, exposure to pesticide residues with the active ingredient mancozeb (MZ) in surface water has a fairly high toxicity status for several aquatic species, ranging from moderate to very toxic (Dall'agnol et al., 2021).

One of the methods in the field of ecotoxicology to analyze the toxicity of pollutants to organisms at the cellular level is the Comet assay. This method can detect the occurrence of strand breaks (SB) in DNA (Langie et al., 2015). The comet assay method is widely used for genotoxicity testing of medicinal products, biomarker testing in environmental quality biomonitoring studies, and ecogenotoxicity studies of aquatic organisms (Martins & Costa, 2015; Neri et al., 2015). Several studies have shown that this method can detect the level of DNA damage in microalgae given a single exposure to pesticides (Cid et al., 2012; Esperanza et al., 2015) and heavy metals (Hazlina et al., 2019).

Microalgae, as the main primary producers in freshwater ecosystems, have the potential to be exposed and sensitive to high levels of pesticide contamination. So, it is essential to evaluate both the toxic and genotoxic effects of pollutants at the cellular level (Singh & Mittal, 2012; Habibah et al., 2020). Microalgal growth is used as a parameter in this study because it can produce more consistent results than biomass (Jiang et al., 2016). Pesticide exposure also could increase reactive oxygen species (ROS) production, which induces DNA damage through oxidation (Nugroho et al., 2020). The alkaline comet assay method is used to determine DNA damage because of its high sensitivity to detect DNA damage that occurs in single cells (Cadet & Wagner, 2013).

Previous studies have been done on the toxicity of pesticides to evaluate the effects of chlorpyrifos individually on growth, chlorophyll contents, enzyme activities (Chen et al., 2016; Bhuvaneswari et al., 2018), and microalgal cell ultrastructure (Asselborn et al., 2015; Fernández et al., 2021). Meanwhile, mancozeb induces DNA damage and necrosis in HepG2 and A549 cell lines (Lori et al., 2021). On the other hand, a study by Martinez et al. (2015) measured the DNA damage of chlorpyrifos on *Pseudokirchneriella subcapitata* and *Nannocloris ouclata.* However, studies on the toxicity and genotoxicity of the mixture of chlorpyrifos and mancozeb on freshwater organisms, mainly microalgae, have not been carried out. Focusing on the research gap, this research studies the effect of exposure to a mixture of chlorpyrifos and mancozeb on growth by using the cell counting method and DNA damage of *C. sorokiniana* using the alkaline comet assay method. The results of this study will provide new information about the toxicity of combined chlorpyrifos and mancozeb as a consideration for the environmental risk assessment of pesticides in the aquatic environment.

METHODS

This research began with preparing the culture of the microalgae *C. sorokiniana* and stock solution for exposure of the microalgae to combined pesticides. Then, it was followed by the treatment of the microalgae with various combinations of pesticides, the calculation of the density of the microalgae for growth analysis, the analysis of DNA damage by comet assay, and the data analysis (Figure 1).

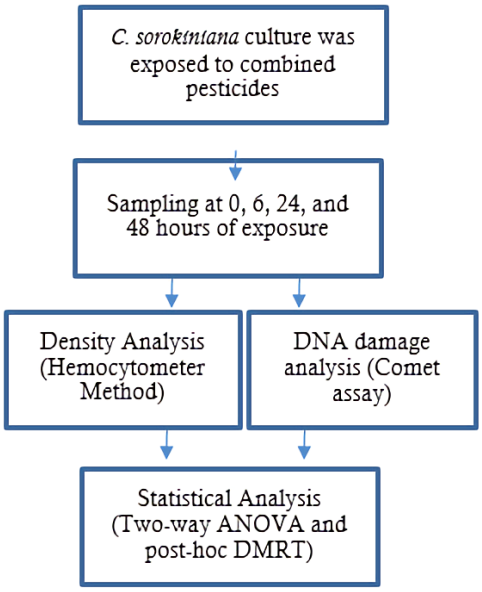

Figure 1. Research Flow Chart

For culture preparation, the glass utensil used in this study was always cleaned with distilled water and sterilized by autoclaving along with the medium before use. *C. sorokiniana* Shihira *et* Krauss culture obtained from the Center for Brackish Water Aquaculture Fisheries (BBPBAP) in Jepara, Central Java, Indonesia. The culture was grown at 23℃ with 1800 lux lighting and aeration. The pH of the medium was observed and maintained in the range of 7.0. For the combined exposure experiment, stock solutions of chlorpyrifos and mancozeb were prepared in a 500 mL volumetric flask (Pyrex) using bidistilled water by dissolving 50 mg chlorpyrifos PESTANAL, analytical grade (CAS 2921-88-2, Merck) in ethanol (2.5 mL), and 50 mg mancozeb PESTANAL, analytical grade (CAS 8018-01-7, Merck) in dimethyl sulfoxide (2.5 mL), respectively, yielding a concentration of each 100 mg/L.

The concentrations used in this experiment referred to the individual EC50 value of each pesticide on *C. sorokiniana*, the 96 hours EC50 value of CP = 3.736 mg/L (Zalizniak, 2006) and 96 hours EC50 of MZ = 11.85 mg/L (Sasmita, 2021) from previous studies. To each 500 mL culture of *C. sorokiniana* in a 500 mL-Erlenmeyer (Pyrex) flask, the pesticides were added from the stock solution with varying concentrations: respectively 0 and 0 mg/L (control); 0.75 and 2.37 mg/L (20%:20% of the EC50 CP and MZ); 0.75 and 9.48 mg/L (20%:80% of the EC50 CP and MZ); 2.99 and 2.37 mg/L (80%:20% of EC50 CP and MZ); and 2.99 and 9.48 mg/L (80%:80% of the EC50 CP and MZ) with three replication for each treatment.

The density of *C. sorokiniana* was estimated using the cell counting or hemocytometer method (BTI, 2015). At each combined concentration, 900 µL of aliquot was taken using a 1000-µL micropipette (Eppendorf) and transferred into a 2-mL microtube (Eppendorf), then added 100 µL of 70% alcohol (Merck) and homogenized. Then, the sample was placed in a counting chamber (hemocytometer) and examined under a microscope (Leica DM 100) with 40x10 magnification to estimate the density of *C. sorokiniana*.

For the preparation of the Comet assay, an aliquot of 50 mL from each combined concentration was taken using a 50-mL syringe connected to a 10-cm silicone tube and transferred into a 50-mL polypropylene (PP) conical tube. Then, the sample was centrifuged (Sorvall Biofuge Primo R) at a speed of 4500 rpm for 10 minutes at a temperature of 4℃. The sample was washed twice with phosphate-buffered saline (PBS) by centrifugation. The supernatant was discarded, and the pellet was suspended in PBS before analysis. DNA damage was analyzed by the alkaline comet assay method based on Hazlina et al. (2019) with some modifications. The slides were coated with 1% normal melting point agarose (NMA) in 1x PBS as the first layer. After that, 10 µL of cell suspension was mixed with 90 µL of 0.75% low melting point agarose (LMA) in PBS, then spread evenly on slides that had been treated with NMA and covered with cover glass. Slides were stored at 4℃ for at least 4 hours until the agarose sets.

In the lysis process, the slides were immersed in cold lysis solution (4℃), which consisted of 2.5M NaCl, 100mM EDTA, 10mM Tris; pH 10-10.5; 1% Triton X-100 and 10% DMSO for at least 1 hour. Afterward, the slides were transferred to an electrophoresis tank containing a cold electrophoresis buffer solution (4℃) consisting of 0.3 M NaOH and 1 mM Na_2EDTA, pH 12.6. The slide was placed in a horizontal position. The electrophoresis was run for 30 min at 300 mA and 25 V at 4℃. After electrophoresis, slides were neutralized/washed thrice with 0.4 M Tris Buffer, pH 7.5 for 5 min to remove the alkaline solution. Slides were stained with SYBR Green in DMSO 10000x dilution for visualization. The stained slides were observed under a confocal microscope.

About 50 cells of each slide were examined to measure the level of DNA damage. Measurement and suspension of the level of DNA damage were carried out with the help of Comet Score Pro Automatic Comet Assay Software. The DNA damage parameters included Tail Intensity (TI%), Head Intensity (HI%), Tail Moment (TM), and Olive Tail Moment (OTM). TailFactor parameters were obtained by categorizing comets based on TI% according to Focke et al. (2010). Data were analyzed using IBM SPSS Statistics 23, with a two-way ANOVA test and post hoc Duncan Multiple Range Test (DMRT) to determine the significance of differences in concentration of toxicant mixture and exposure time to the DNA damage of *C. sorokiniana*.

RESULTS AND DISCUSSION

The exposure of *C. sorokiniana* to combinations of pesticides caused a decrease in the density within 48 hours (Figure 2). At hour 48, the highest combined concentration (CP 2.99 mg/L + MZ 9.48 mg/L) decreased the cell density, reaching the lowest level compared to the other combined concentrations and being significantly different from hour 0 ($p<0.05$). The growth inhibition reached about 56%. The inhibition level

indicated that the combined exposure might not lead to a synergistic effect.

The other combinations of the two pesticides also caused the decrease in density, the lowest level occurred in the first 6 hours, being significantly different from hour 0 ($p<0.05$) (Figure 2). Exposure of *C. sorokiniana* to various combined concentrations did not result in a difference in cell density among the concentrations ($p>0.05$), but the density upon exposure differed from the control ($p<0.05$). The concentrations of CP 0.75 mg/L + MZ 9.48 mg/L had a lower density and higher inhibition than the concentrations of CP 2.99 mg/L + MZ 2.37 mg/L. It indicated that in the combination of toxicants, an increase in the concentration of MZ could be more toxic than an increase in the concentration of CP.

Many similar studies showed the effects of CP and MZ individually or in combination with other pesticides on the growth of microalgae. Chen et al. (2016), in their study on the toxicity of CP to *Chlorella pyrenodoisa,* also showed a significant decrease in growth rate along with the addition of CP concentrations in the range of 2.4 mg/L to 35.4 mg/L. Exposure to CP at a concentration of 75 mg/L was able to increase the biovolume of *A. gracilis*, which was caused by inhibition of cell division, so cell density tends to decrease, but cell size would increase (Asselborn et al., 2015). The increase in CP concentration is also related to the inhibition of the synthesis of pigments which have a major role in energy harvesting, chlorophyll a and carotenoids in *S. platensis* (Bhuvaneswari et al., 2018) and *Merismopedia* sp (Chen et al., 2016).

Combined exposure of MZ with metalaxyl was able to produce an EC50 value of 96 hours 0.273 mg/L, which is higher than the toxicity of single exposure to mancozeb in *Pseudokirchneriella subcapitata* (Abd-Allah et al., 2012). Exposure to MZ in plant cells can inhibit growth as a result of molecular damage due to excess ROS production, which is characterized by increased activity of antioxidant enzymes as a self-defense mechanism (Fatma et al., 2018a).

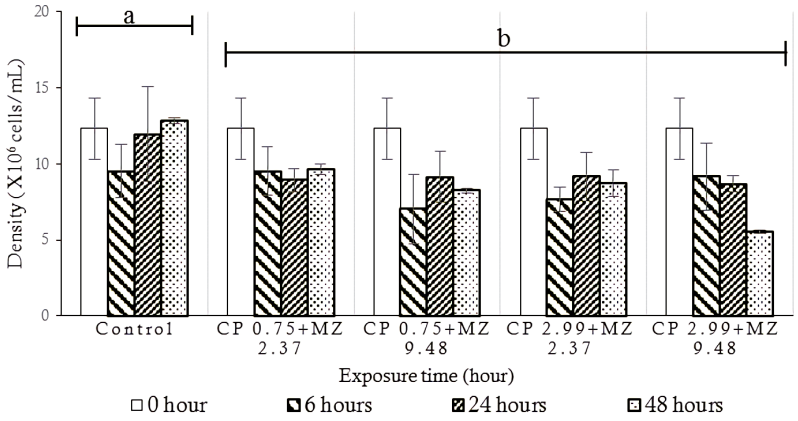

Figure 2. The Density of *C. sorokiniana* after Exposure to a Combination of Chlorpyrifos (CP) and Mancozeb (MZ). Different Letters Indicated Significant Differences between Concentration Exposures ($p<0.05$)

The comet assay visualization showed that at hour 0 of exposure, all combined exposures had not caused DNA damage, which is indicated by the absence of comet formation (Figure 3). Similar to the control treatment for all hours of treatment, the majority of cells observed were round and had not yet formed a comet tail. Meanwhile, at 6 hours of exposure, comets began to form in each toxicant treatment, except for CP 0.75 mg/L + MZ 2.37 mg/L, although the size of the comet's head was still larger than the comet's tail or still in the category of minor damage (Collins & Azqueta, 2012). At the 24th hour of exposure, comets formed in all toxicant treatments with CP:MZ treatments of 0.75:9.48 mg/L and 2.99:9.48 mg/L gave longer tails than the others. Meanwhile, at the 48th hour of exposure, comets appeared with the size of the comet head starting to shrink in the CP:MZ 0.75:2.37 mg/L and 2.99:2.37 mg/L treatments and the head color becoming darker and fading in the CP:MZ treatments of 0.75:9.48 mg/L and 2.99:9.48 mg/L. The characteristics of comets with reduced head size and faded head color are characteristics of cells with moderate-to-severe DNA damage (Hazlina et al., 2019).

A study found that phosphoryl groups that have electrophilic properties (low electron density areas) in chlorpyrifos readily form bonds with nucleophilic centers (high electron density are-

as) in DNA. This bond triggers the formation of DNA adducts. The implication is that increasing the concentration of chlorpyrifos can cause an increase in the level of DNA damage (Martinez et al., 2015). Meanwhile, the transitional metal content in mancozeb, such as manganese (Mn) and zinc (Zn), can catalyze the formation of ROS in the Fenton reaction, so that mancozeb has the potential to become a pro-oxidant substance (Mohammadi-Sardoo et al., 2018). The study of Esperanza et al. (2015) showed that exposure of microalgae *Chlamydomonas reinhardtii* to paraquat herbicide increased ROS levels, provoking the formation of oxidative 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-OHdG)-DNA adducts. The 8-OHdG is an important indicator of free radical-induced DNA damage.

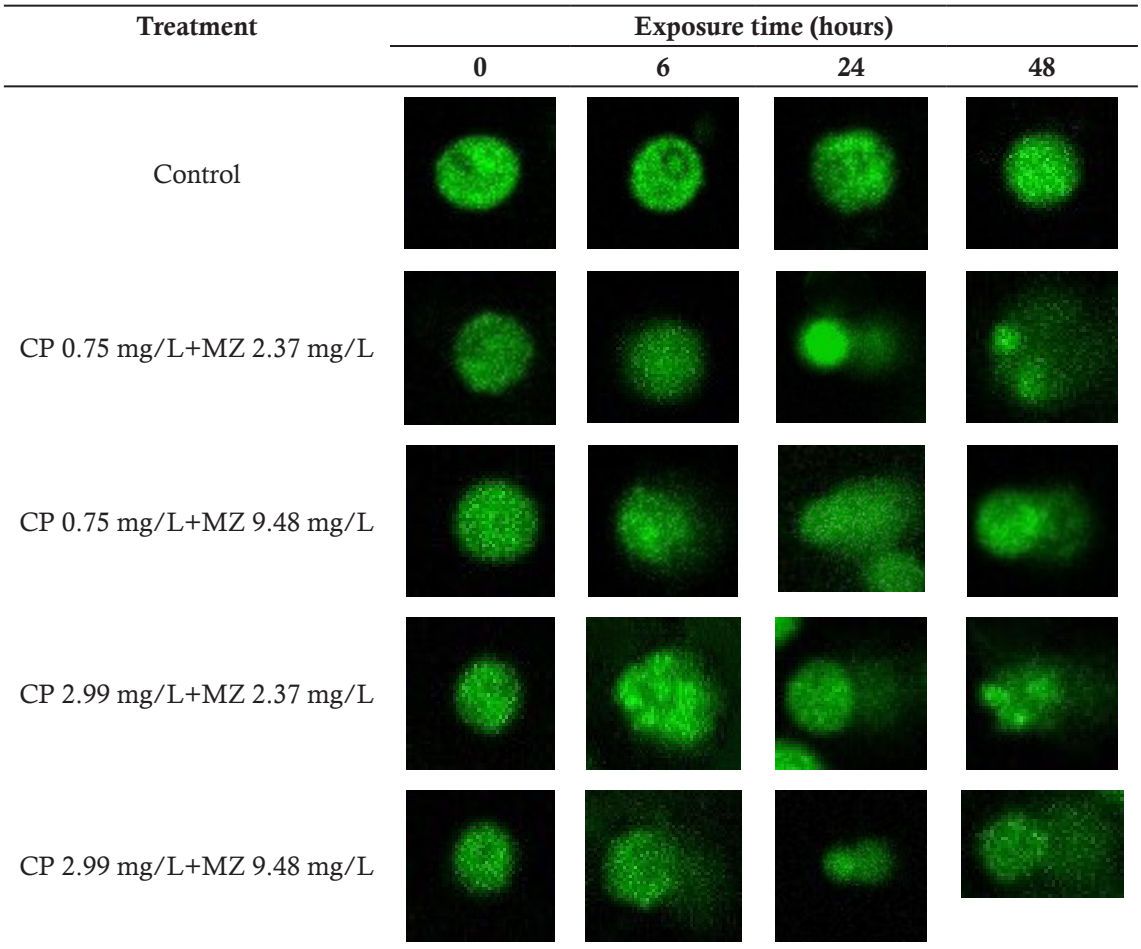

Figure 3. Comet Assay Result Visualization

Exposure to the combined pesticides resulted in increased TI% (Figure 4a), the difference in TI% values among exposure concentrations being significant ($p<0.05$). The highest value of TI% was observed at the combined concentrations of CP 0.75 mg/L + MZ 9.48 mg/L (20%:80%) and CP 2.99 mg/L + MZ 9.48 mg/L (80%:80%) at hour 24, being about 1-1.5 times higher than the other combined concentrations or 2-2.5 times higher than the control ($p<0.05$). The increase in TI% values may be caused by increased concentrations of MZ, leading to a stronger effect. Although the concentration of 80%:80% was 4 times higher than the other combined concentrations, the increased TI value was not linear. Martinez et al. (2015) reported that 0.8 mg/L CP resulted in 4 times higher DNA damage than controls in *Pseudokirchneriella subcapitata*. It indicated that the combination of CP and MZ did not lead to a synergism effect.

Concerning head intensity (HI), it occurred a decrease in the values at all concentration exposures, the difference in HI values among exposure concentrations being significant ($p<0.05$). Exposure to the CP 2.99 mg/L + MZ 9.48 mg/L (80%:80%) at all times of exposure was able to produce the lowest Head Intensity (HI) value, about 9-16% lower than the control (Figure 4b).

A decrease in the HI% values also responded to an increase in the concentration of the pesticides in combination. Meanwhile, exposure to a combination of the two pesticides for 24 hours was able to produce the lowest HI% significantly ($p<0.05$). The results were similar to the research of Erbes et al. (1997) that exposure to the genotoxicant 4-nitroquinoline-1-oxide (4-NQO) at a concentration of 5-50 nM was able to reduce HI% by 15-40%. This is an indication of increased DNA damage in cells. These results indicated that the increase in HI% was inversely proportional to the increase in the concentration of the pesticides in combination, and the increase in HI% was inversely proportional to the duration of exposure up to 24 hours.

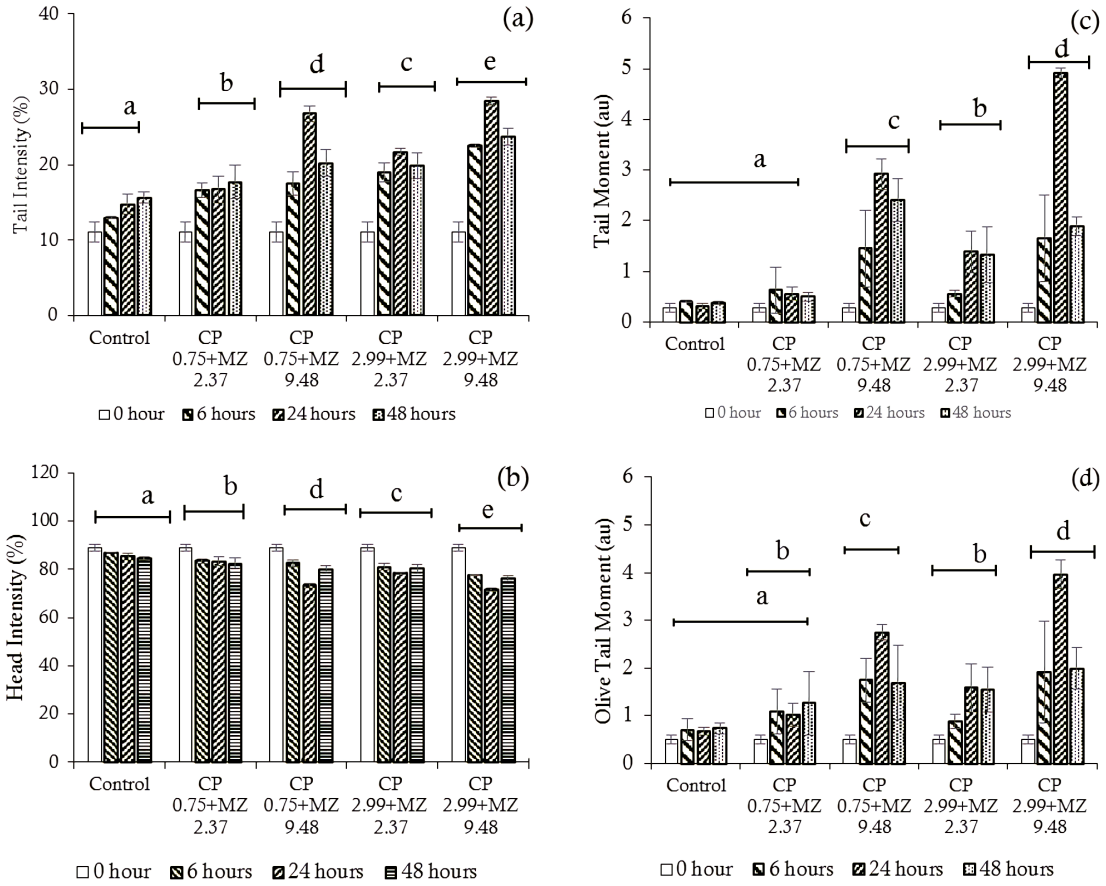

Figure 4. Quantitative Analysis of *C. sorokiniana* DNA Damage after Toxicant Exposure, i.e., (a) TI%, (b) HI%, (c) TM, and (d) OTM. Different Letters Indicate Significant Differences between Concentration Treatments ($p<0.05$)

About TM, the difference in TM values among exposure concentrations was significant ($p<0.05$). The values at hour 24 tended to be higher than at hour 48 ($p<0.05$) (Figure 4c). Similar to the TI% (Figure 2a), increased TM values may also be caused by increased MZ concentration in combination. The combined exposure to CP 2.99 mg/L + MZ 9.48 mg/L (80:80) resulted in the highest TM value of *C. sorokiniana* at an exposure time of 24 hours, 5 times higher than the control (Figure 4c). At the combined concentration of CP 0.75 mg/L + MZ 9.48 mg/L, the highest TM value was reached at an exposure time of 48 hours, about 6 times higher than hour 0. These results indicated that the overall increase in TM was directly proportional to the increase in the concentration of the combined pesticides, while the increase in TM was directly proportional to the duration of exposure to the two pesticides up to 24 hours. Based on these results, the increase in TM values can indicate a sensitive biomarker to the exposure of MZ pesticide, since the increase in TM value was linear with increasing MZ concentration.

The combination of CP 2.99 mg/L + MZ 9.48 mg/L (80%:80%) resulted in the highest OTM value of C. *sorokiniana* for the entire duration of exposure, about 2-4 times higher than

hour 0. Exposure to CP 0.75 mg/L and MZ 9.48 mg/L (20%:80%) resulted in a significant increase in OTM compared to the control (Figure 4d). Meanwhile, exposure to a combination of toxicants for 24 hours was able to produce the highest OTM, and there was a significant difference ($p<0.05$) with the duration of exposure to other toxicants. The previous study also showed that the OTM value of *Eisenia fetida* was linear with the increase in exposure duration (Ma et al., 2016). In addition, the difference in OTM values among exposure concentrations was significant ($p<0.05$).

The trend showed that the increase in OTM was directly proportional to the increase in the combined concentrations, while the increase in OTM was directly in line with the duration of exposure to the two pesticides up to 24 hours. The concentration of CP 0.75 mg/L + MZ 9.48 mg/L showed a higher OTM than CP 2.99 mg/L + MZ 2.37 mg/L. Again, this indicated that in the combination of toxicants, an increase in the concentration of MZ could be more toxic than an increase in the concentration of CP. Like TM, the OTM parameter can be considered a sensitive biomarker to MZ exposure.

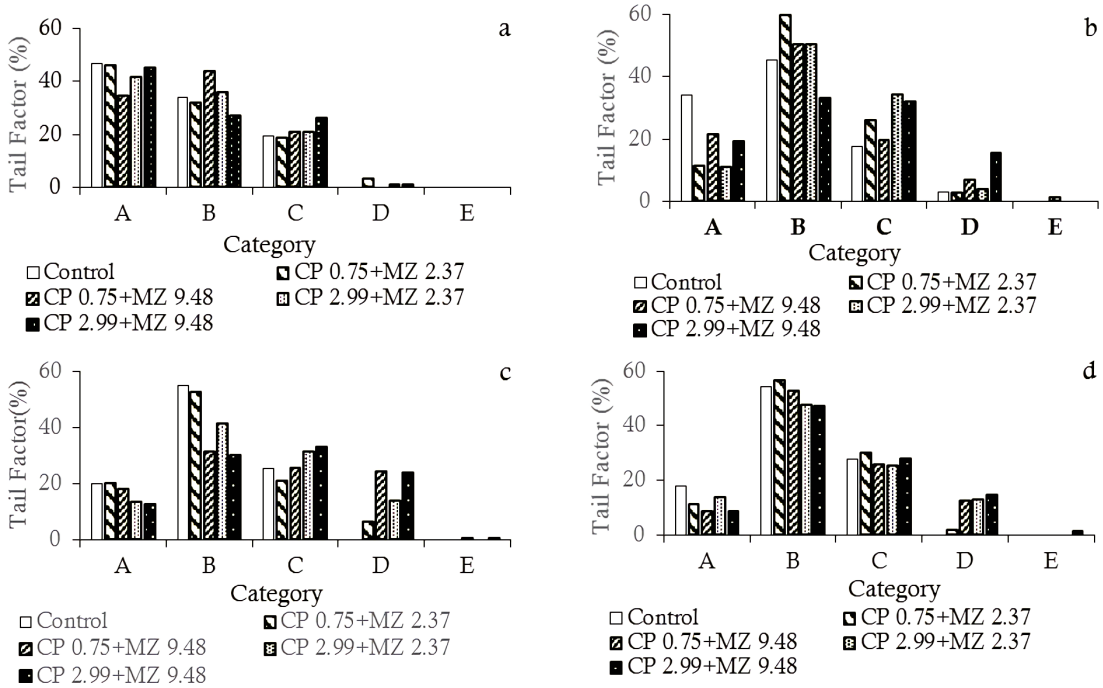

Figure 5. Tailfactor (TF%) of *C. sorokiniana* at 0 hours (a), 6 hours (b), 24 hours (c), and 48 hours (d) of Exposures

Tailfactor (TF) values for all combined concentrations at 6 hours (Figure 5) showed an increase of 7-28% in category B, increasing 6-14% in category C and 2-14% in category D, respectively, while category A decreased 14-30% from the 0 hours. At the 24 hours, there was a 3-18% increase in category D in all combined concentrations, while category B decreased by 3-18% from the 6th hour. At the 48th hour, there was an increase in category B by 4-21% and a decrease in category D by 2-12% from the 24th hour. Meanwhile, category E or cells with very severe/extreme damage were rarely observed, only at CP 0.75+MZ 9.48 mg/L at 6 hours (1.4%) and 24 hours (0.67%), and CP 2.99+MZ 9.48 mg/L at 24 hours (0.57%) and 48 hours (1.55%).

These results indicated that all combined concentrations for the entire duration of exposure caused B-D stages of DNA damage or mild to severe damage, and the duration of exposure for 24 hours had the highest DNA damage rate. These results are similar to previous studies that increasing the concentration of the oxidative agent H_2O_2 at 100-150 M was able to cause damage to human fibroblast DNA from low to severe levels (Focke et al., 2010).

The results indicated by the DNA damage parameters, namely TI, HI, TM, OTM, and TF showed a similar trend in the increase in DNA damage along with the increase in the concentrations of the two pesticides. Meanwhile, at 48 hours of the pesticide exposure based on TF, it was suspected that a DNA repair mechanism had occurred. It was caused by a shift in the trend of damage from the dominance of C-D levels at 24 hours, to B-C. Previous studies have suggested

that DNA damage can potentially activate the DNA repair pathways of the cell (Esperanza et al., 2015). Meanwhile, exposure to genotoxic substances such as ultraviolet light can induce the expression of genes related to DNA repair, such as DNA Damage-Binding Protein I (DDB I) (Wang et al., 2022). Another possibility is that there is a defense mechanism or tolerance of *C. sorokiniana* cells against the increase of ROS through the antioxidant defense (Rezayian et al., 2019).

Although the result of this study may not lead to a synergistic effect on DNA damage, the increased DNA damage in the organism upon exposure to various concentrations of combined two pesticides showed that the increased concentration of pesticides in combination mainly for MZ and duration of exposure affected the level of toxic effects, i.e., on the level of DNA damage. In application, the increased DNA damage in microalgae can indicate the presence of pesticides and other pollutants in the aquatic ecosystem (Li et al., 2014).

CONCLUSION

Exposure of *C. sorokiniana* to the combined chlorpyrifos and mancozeb can lead to growth inhibition and an increase in DNA damage. The genotoxicity tended to increase with the addition of combination concentration, especially for an increase of MZ concentration and duration of exposure, mainly at the first 24 hours. From TM and OTM values, the two parameters indicated sensitive biomarkers for biomonitoring of pesticide pollution in the aquatic ecosystem using microalgae as the object. This research was only conducted on a laboratory scale. For validation and further research development, such as rapid assessment of stream pollution, it is necessary to analyze the DNA damage of microalgae in waters contaminated with these pesticides.

REFERENCES

Abd-Allah, S. M., Goud, N. A., & Talha, M. M. (2012). Ecological Hazards of Some Pesticides on Unicellular Freshwater Green Alga; *Pseudokirchneriella subcapitata*. *Alexandria Science Exchange Journal*, *33*(1).

Asselborn, V., Fernández, C., Zalocar, Y., & Parodi, E. R. (2015). Effects of chlorpyrifos on the growth and ultrastructure of green algae, Ankistrodesmus gracilis. *Ecotoxicology and Environmental Safety*, *120*, 334–341.

Atifah, Y., Lubis, M., Lubis, L. T., & Maulana, A. (2019). Pencemaran Pestisida pada Sungai Batang Gadis, Mandailing Natal, Sumatera Utara. *BIOEDUSCIENCE: Jurnal Pendidikan Biologi Dan Sains*, *3*(2), 100–105.

Bhuvaneswari, G. R., Purushothaman, C. S., Pandey, P. K., Gupta, S., Kumar, H. S., & Shukla, S. P. (2018). Toxicological Effects of Chlorpyrifos on Growth, Chlorophyll a Synthesis and Enzyme Activity of a Cyanobacterium *Spirulina (Arthrospira) platensis*. *International Journal of Current Microbiology and Applied Sciences*, *7*(6), 2980–2990.

BTI. (2015). *Algae to Energy-Using and Re-using a Hemocytometer to Count Algae Cells* (BTI Curriculum Development Projects in Plant Biology).

Cadet, J., & Wagner, J. R. (2013). DNA Base Damage by Reactive Oxygen Species, Oxidizing Agents, and UV Radiation. *Cold Spring Harbor Perspectives in Biology*, *5*(2), a012559–a012559.

Chamsi, O., Pinelli, E., Faucon, B., Perrault, A., Lacroix, L., Sánchez-Pérez, J. M., & Charcosset, J. Y. (2019). Effects of herbicide mixtures on freshwater microalgae with the potential effect of a safener. *Annales de Limnologie*, *55*(3).

Chen, S., Chen, M., Wang, Z., Qiu, W., Wang, J., Shen, Y., Wang, Y., & Ge, S. (2016). Toxicological effects of chlorpyrifos on growth, enzyme activity and chlorophyll a synthesis of freshwater microalgae. *Environmental Toxicology and Pharmacology*, *45*, 179–186.

Cid, Á., Prado, R., Rioboo, C., Suarez-Bregua, P., & Herrero, C. (2012). Use of Microalgae as Biological Indicators of Pollution: Looking for New Relevant Cytotoxicity Endpoints. In M. N. Johnsen (Ed.), *Microalgae: Biotechnology, Microbiology, and Energy* (pp. 311–323). Nova Science Publisher.

Collins, A. R., & Azqueta, A. (2012). Single-Cell Gel Electrophoresis Combined with Lesion-Specific Enzymes to Measure Oxidative Damage to DNA. In *Methods in Cell Biology* (Vol. 112, Issue C, pp. 69–92). Academic Press.

Dall'agnol, J. C., Pezzini, M. F., Uribe, N. S., & Joveleviths, D. (2021). Systemic effects of the pesticide mancozeb – A literature review. *European Review for Medical and Pharmacological Sciences*, *25*, 4113–4120.

Erbes, M., Weßler, A., Obst, U., & Wild, A. (1997). Detection of Primary DNA Damage in Chlamydomonas reinhardtii by Means of Modified Microgel Electrophoresis. *Environmental and Molecular Mutagenesis*, *30*, 448–458.

Esperanza, M., Cid, Á., Herrero, C., & Rioboo, C. (2015). Acute effects of a pro-oxidant herbicide on the microalga *Chlamydomonas reinhardtii*: Screening cytotoxicity and genotoxicity endpoints. *Aquatic Toxicology*, *165*, 210–221.

Fatma, F., Verma, S., Kamal, A., & Srivastava, A. (2018a). Monitoring of morphotoxic, cytotoxic, and genotoxic potential of mancozeb using Allium assay. *Chemosphere*, *195*, 864–870.

Fatma, F., Verma, S., Kamal, A., & Srivastava, A. (2018b). Phytotoxicity of pesticides mancozeb and chlorpyrifos: correlation with the antioxi-

dative defence system in *Allium* cepa. *Physiology and Molecular Biology of Plants*, *24*(1), 115–123.

Fernández, C., Asselborn, V., & Parodi, E. R. (2021). Toxic effects of chlorpyrifos, cypermethrin and glyphosate on the non-target organism Selenastrum capricornutum (Chlorophyta). *Anais Da Academia Brasileira de Ciencias*, *93*(4).

Focke, F., Schuermann, D., Kuster, N., & Schär, P. (2010). DNA fragmentation in human fibroblasts under extremely low-frequency electromagnetic field exposure. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *683*(1–2), 74–83.

Habibah, R., Iswanto, B., & Rinanti, A. (2020). The Significance Of Tropical Microalgae Chlorella Sorokiniana As A Remediate Of Polluted Water Caused By Chlorpyrifos. *International Journal Of Scientific & Technology Research*, *9*(1), 4460–4463.

Hazlina, A. Z., Devanthiran, L., & Fatimah, H. (2019). Morphological Changes and DNA Damage in *Chlorella vulgaris* (UMT-M1) Induced by Hg2+. *Malaysian Applied Biology*, *48*(1), 27–33.

Huang, X., Cui, H., & Duan, W. (2020). Ecotoxicity of chlorpyrifos to aquatic organisms: A review. In *Ecotoxicology and Environmental Safety* (Vol. 200). Academic Press.

Jiang, H., Islam, M. S., Sazawa, K., Hata, N., Taguchi, S., Nakamura, S., Sugawara, K., & Kuramitz, H. (2016). Development of an Electrochemical Bioassay Based on the Alkaline Phosphatase Activity of *Chlamydomonas reinhardtii* to Assess the Toxicity of Heavy Metals. *International Journal of Electrochemical Science*, 5090–5102.

Langie, S. A. S., Azqueta, A., & Collins, A. R. (2015). The comet assay: Past, present, and future. *Frontiers in Genetics*, *6*(266).

Li, M., Gao, X., Wu, B., Qian, X., Giesy, J. P., & Cui, Y. (2014). Microalga Euglena as a bioindicator for testing genotoxic potentials of organic pollutants in Taihu Lake, China. *Ecotoxicology*, *23*, 633–640.

Lori, G., Tassinari, R., Narciso, L., Udroiu, I., Sgura, A., Maranghi, F., & Tait, S. (2021). Toxicological Comparison of Mancozeb and Zoxamide Fungicides at Environmentally Relevant Concentrations by an In Vitro Approach. *International Journal of Environmental Research and Public Health*, *18*(16), 8591.

Ma, T., Chen, L., Wu, L., Zhang, H., & Luo, Y. (2016). Oxidative Stress, Cytotoxicity and Genotoxicity in Earthworm *Eisenia fetida* at Different Di-n-Butyl Phthalate Exposure Levels. *PLOS ONE*, *11*(3), e0151128.

Martinez, R. S., di Marzio, W. D., & Sáenz, M. E. (2015). Genotoxic effects of commercial formulations of Chlorpyrifos and Tebuconazole on green algae. *Ecotoxicology*, *24*(1), 45–54.

Martins, M., & Costa, P. M. (2015). The comet assay in Environmental Risk Assessment of marine pollutants: Applications, assets, and handicaps of surveying genotoxicity in non-model organisms. *Mutagenesis*, *30*(1), 89–106.

Mohammadi-Sardoo, M., Mandegary, A., Nabiuni, M., Nematollahi-Mahani, S.-N., & Amirheidari, B. (2018). Mancozeb induces testicular dysfunction through oxidative stress and apoptosis: Protective role of *N* -acetylcysteine antioxidant. *Toxicology and Industrial Health*, *34*(11), 798–811.

Neri, M., Milazzo, D., Ugolini, D., Milic, M., Campolongo, A., Pasqualetti, P., & Bonassi, S. (2015). Worldwide interest in the comet assay: A bibliometric study. *Mutagenesis*, *30*(1), 155–163.

Nie, J., Sun, Y., Zhou, Y., Kumar, M., Usman, M., Li, J., Shao, J., Wang, L., & Tsang, D. C. W. (2020). Bioremediation of water containing pesticides by microalgae: Mechanisms, methods, and prospects for future research. *Science of The Total Environment*, *707*, 136080.

Nugroho, A. P., Handayani, N. S. N., Darmasiwi, S., Zulfikar, W. G., & Rizki, A. A. (2020). Biochemical Responses and DNA Damage of *Chlorella pyrenoidosa* H. Chick upon Exposure to Combined Cu and Cd at Environmentally Realistic Levels. *Environment and Natural Resources Journal*, *18*(3), 290–303.

Prabowo, R., & Subantoro, R. (2012). Kualitas Air dan Beban Pencemaran Pestisida di Sungai Babon Kota Semarang. *MEDIAGRO*, *8*(1), 9–17.

Rajmohan, K. S., Chandrasekaran, R., & Varjani, S. (2020). A Review on Occurrence of Pesticides in Environment and Current Technologies for Their Remediation and Management. *Indian Journal of Microbiology*, *60*(2), 125–138.

Rezayian, M., Niknam, V., & Ebrahimzadeh, H. (2019). Oxidative damage and antioxidative system in algae. *Toxicology Reports*, *6*, 1309–1313.

Roede, J. R., & Miller, G. W. (2014). Mancozeb. In P. Wexler (Ed.), *Encyclopedia of Toxicology* (3rd ed., pp. 144–146). Academic Press.

Singh, J., & Mittal, S. K. (2012). Chlorella sp. based biosensor for selective determination of mercury in presence of silver ions. *Sensors and Actuators, B: Chemical*, *165*(1), 48–52.

Sud, D., Kumar, J., Kaur, P., & Bansal, P. (2020). Toxicity, natural, and induced degradation of chlorpyrifos. *Journal of Chilean Chemical Society*, *65*(2).

Wang, C., Cai, M., Liu, Y., Yang, F., Zhang, H., Liu, J., & Li, S. (2022). Facile construction of novel organic–inorganic tetra (4-carboxyphenyl) porphyrin/Bi2MoO6 heterojunction for tetracycline degradation: Performance, degradation pathways, intermediate toxicity analysis and mechanism insight. *Journal of Colloid and Interface Science*, 605, 727-740.

Zhang, J., Liu, S. S., Zhang, J., Qin, L. T., & Deng, H. P. (2012). Two novel indices for quantitatively characterizing the toxicity interaction between ionic liquid and carbamate pesticides. *Journal of Hazardous Materials*, *239–240*, 102–109.