Individual and Combined Effects of Pesticides with Active Ingredients of Mancozeb and Methomyl on the DNA Damage of *Daphnia magna* (Straus, 1820; Cladocera, Daphniidae)

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ABSTRACT

Mancozeb and methomyl are active ingredients commonly contained in pesticides applied in shallot farming. Surface runoff can carry pesticide residues that enter water bodies and affect non-target organisms, such as *Daphnia magna*. This study evaluated the genotoxicity effects of individual and combined mancozeb and methomyl on the DNA damage of *D. magna*. Organisms at 24 h old and 48 h old were exposed to individual and combined concentrations of mancozeb and methomyl for 24 h to obtain the LC50-24 h values. These values were used to evaluate DNA damage by calculating the tail intensity (TI) (%), tail moment (TM), and tail factor (TF). Results showed that based on the LC50-24 h values, methomyl has the highest toxicity level, followed by the mancozeb:methomyl combination, and then mancozeb. The combination index of mancozeb:methomyl for both *D. magna* ages (24 h and 48 h) indicated that the two pesticides antagonistically interact (CI>1). However, based on TI%, TM, and TF values, the level of damage was almost the same between the individual and combined pesticide concentrations, and the DNA damage was more massive with increased pesticide concentration. The DNA damage of 24 h old and 48 h old *D. magna* did not significantly differ. Increased DNA damage in *D. magna* indicated that this parameter was sensitive to the presence of pesticides. In application, DNA damage can be used as a biomarker for biomonitoring pesticide pollution in the aquatic ecosystem.

1. INTRODUCTION

Methomyl and mancozeb are carbamate pesticides that control foliage and soil-borne insect pests on various food and feed crops. Mancozeb is a fungicide that can inhibit the growth of fungi and spores prior to the development of mycelium in plant tissues. This fungicide is effective against external contamination caused by fungi (Asita and Makhalemele, 2009). Meanwhile, methomyl is a broad-spectrum and systemic anticholinesterase carbamate insecticide used worldwide to protect crops from invading organisms. Unfortunately, this carbamate insecticide can also affect non-target organisms (Seleem, 2019).

These pesticides can leave residues on the soil surface that are carried away by surface water that flows into aquatic ecosystems, where they are absorbed by non-target organisms. Therefore, an unpredictable ecological risk is posed based on the individual concentrations of pesticides. The interaction of various pesticides can have toxic effects, which are additive, synergistic, or antagonistic in organisms (Aktar et al., 2009; Kaur and Kaur, 2018; Vasiljević et al., 2012). Furthermore, *Daphnia magna*, an essential planktonic crustacean in the aquatic ecosystem, is exposed to these pesticides individually or in combination (Kretschmann et al., 2011). Pesticides also affect the survival, growth, and fecundity of *D. magna* (Rajini et al., 2016). The exposure of these organisms to 0.58 ppm of mancozeb resulted in a mortality rate of 50%. This pesticide also causes damage to the nucleus, and chromosomal and micronuclear aberrations (Christin et al., 2015). According to Mayer and Ellersieck (1986), exposure

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to 7.3-20.0 g/L of methomyl caused the death of *D. magna*. Both pesticides (mancozeb and methomyl) induce damage and break chromosomal abrasion, sister-chromatid exchanges, micronuclei, and DNA bonds in terms of single- and double-strand (Hart et al., 1978; Pellegri et al., 2014). Through the food chain, the accumulation of such pesticides in *Daphnia* can lead to biomagnification, which can have detrimental effects on other aquatic organisms and humans as one of the top predators. A study of mancozeb effects on the test organism, Sprague Dawley rats, indicated that it induced genotoxic effects, metabolic alterations, and histological changes in the colon and liver (Yahia et al., 2019). In humans, carbamate pesticides, such as methomyl, can inhibit various types of esterase. The toxic effect of this pesticide is the inhibition of acetylcholinesterase, leading to excessive cholinergic overstimulation. The symptoms of pesticide poisoning include muscle twitching and weakness, decreased levels of consciousness, excessive salivation and tearing, seizures, respiratory failure, and constricted pupils (Liang et al., 2023).

D. magna is a selective filter feeder organism that feeds on unicellular algae and various organic detritus. The organism becomes a food source for the next trophic level in the aquatic ecosystems because it is naturally essential for fish larvae (Antunes et al., 2016). However, this food chain also leads to the biomagnification of pesticides. Therefore, humans become exposed to high toxic concentrations at the top of the food chain (Castro et al., 2019; Mahmood et al., 2015).

Biological responses at the molecular level provide early sensitive and specific warnings regarding environmental pollutants. In polluted environments, DNA damage of aquatic organisms is considered a sensitive biomarker to evaluate genotoxicity and ecogenotoxicological risks (Liyan et al., 2005; Pellegri et al., 2014). Furthermore, single-cell gel electrophoresis (SCGE) or comet assay is often used in DNA damage analysis because it is a simple procedure. This assay can detect damages caused by genotoxic agents, especially in eukaryotic cells, including *D. magna* (Pellegri et al., 2020; Jha, 2008). *Daphnia* sp. is widely used for water quality testing due to its characteristics, which satisfy the requirement of a model organism for ecotoxicological studies. This organism has a wide distribution range, is easily cultured in the laboratory with relatively high sensitivity to pollutants, and has known biological data (Surtikanti et al., 2017).

Previous studies using comet assays with *D. magna* (Jha, 2008; Pellegri et al., 2020) have shown and emphasized the advantage of using the comet assay method in assessing the genotoxicity of toxicants on aquatic organisms. However, studies with agricultural pesticides, such as methomyl and mancozeb (and their combination), still need to be explored. Evaluation of DNA damage of the organism upon exposure to the pesticides will reveal the pesticide risks on aquatic ecosystems. Furthermore, the age selection of *D. magna* is closely related to its high sensitivity to pollutants (Pellegri et al., 2014). Therefore, this study aims to evaluate the genotoxicity of individual and combined effects of mancozeb and methomyl on the DNA damage of 24 h old and 48 h old *D. magna*.

2. METHODOLOGY

2.1 Chemical materials

The chemicals used in the study were of analytical grade (Merck). An artificial medium (Klüttgen et al., 1994) was used as the *Daphnia* growth medium. For the individual and combined exposure experiments, stock solutions of methomyl and mancozeb were prepared in 500 mL of bidistilled water by first dissolving 50 mg methomyl PESTANAL, analytical grade (CAS 16752-77-5, Merck) in acetonitrile (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 100 mg/L each.

2.2 Breeding *D. magna*

D. magna was obtained from the Center for Aquaculture Technology Development (BPTPB), Cangkringan, Sleman, Special Region of Yogyakarta, Indonesia. The culture process was carried out by maintaining female organisms in an aerated artificial medium for 24 h, with a population density of three individuals/100 mL medium. This process provided nutrition in maltose, with aeration for water circulation. The offspring were maintained for 24 h and 48 h to obtain individuals within the age range. The offspring were further used in individual or combined tests to evaluate the toxicity of mancozeb and methomyl and analyze their effects on DNA damage. For toxicity tests and analysis of DNA damage, 10 individuals of *D. magna* (24 h old and 48 h

old) were obtained from the culture and separately transferred to a glass beaker filled with artificial medium without being fed.

2.3 Acute toxicity test

The acute toxicity test consisted of preliminary and definitive tests to obtain the LC50-24 h of mancozeb, methomyl, and a combination of both pesticides on *D. magna*. For the initial examination, mancozeb and methomyl were added to the *D. magna* culture (10 individuals, n=3). Administration of both pesticides produced concentrations of 0.0, 0.1, 1.0, 10.0, and 100.0 mg/L. The mortality of the organisms was recorded after 24 h. The concentration range that produced the mortality rate of 50% was further used as the definitive test range.

Ten individuals were used for each definitive test (Table 1), and the mortality of *D. magna* was recorded after 24 h. Probit analysis was performed based on definitive test results to obtain the LC50-24 h for individual and combined pesticides.

To evaluate the effects of the pesticides in combination, e.g., additive, synergism, or antagonism, the combination index (CI) was calculated using the following classic isobologram combination index:

$$\frac{Am}{Ai} + \frac{Bm}{Bi}$$

Where; Am is the LC50-24 h value of mancozeb in combination, Bm is LC50-24 h value of methomyl in combination, Ai is LC50-24 h value of individual mancozeb, and Bi is LC50-24 h value of individual methomyl.

The values of CI are defined as synergism (CI<1), additive (CI=1), or antagonism (CI>1). If the values were plotted graphically, then the CI point position on the above additive line represents an antagonistic effect, the under-additive line being synergistic (Markovsky et al., 2014).

2.4 DNA damage analysis

The *D. magna* cultures of age 24 h and 48 h were prepared in a 250 mL glass beaker, with a density of 10 individuals/100 mL, to evaluate the effects of mancozeb and methomyl pesticides on DNA damage. The test organisms were treated with different toxicant concentrations, with three repetitions for each concentration (Table 2). The differences in the pesticide concentrations between the two age groups of *D. magna* were due to the LC50-24 h values.

Table 1. Concentrations of mancozeb, methomyl, and combination of mancozeb:methomyl in the 24-h definitive test for two age groups of *D. magna*

Pesticides	Age of *D. magna*	Concentration (mg/L)
Mancozeb	24 h	0.0
		0.1
		0.4
		0.7
		1.0
	48 h	0.0
		0.1
		0.4
		0.7
		1.0
Methomyl	24 h	0.00
		0.01
		0.04
		0.07
		0.10
	48 h	0.00
		0.01
		0.04
		0.07
		0.10
Mancozeb: Methomyl	24 h	0:0
		0.10:0.01
		0.40:0.04
		0.70:0.07
		1.00:0.10
	48 h	0:0
		0.10:0.01
		0.40:0.04
		0.70:0.07
		1.00:0.10

2.4.1 D. magna hemolymph extraction

After exposure of *D. magna* to the pesticides, the DNA damage was analyzed following the method of Pellegri et al. (2014). The organisms were obtained from the medium, crushed by a mortar, combined with 2 mL of Buffer P solution (0.1 M phosphate buffer, 0.2% citric acid, 0.1 M NaCl, 1 mM EDTA, and pH 7.8 (Pellacani et al., 2006)) and placed into a 15 mL conical tube to maintain the viability of the extraction result. The extract was then centrifuged at a speed of 45xg for 5 min. The supernatant was discarded, and the hemolymph extract pellet was washed twice using

2 mL Buffer P and centrifuged at a speed of 45xg for 5 min. The resulting pellet (hemolymph extract) was then placed in a 2 mL microtube, added with 1 mL of PP Buffer solution (0.1 M phosphate buffer, 0.2% citric acid, 0.1 M NaCl, 1 mM EDTA, and pH 7.8) and stored in the freezer (-80°C).

Table 2. Concentrations of mancozeb, methomyl, and combination of mancozeb:methomyl in the DNA damage analysis of the two age groups of *D. magna*

Pesticides	Age of *D. magna*	Concentration (mg/L)
Mancozeb	24 h	0.0
		0.3
		0.5
		0.7
	48 h	0.000
		0.080
		0.100
		0.012
Methomyl	24 h	0.00
		0.04
		0.06
		0.08
	48 h	0.000
		0.014
		0.024
		0.034
Mancozeb: Methomyl	24 h	0:0
		0.066:0.066
		0.086:0.086
		0.106:0.106
	48 h	0:0
		0.018:0.018,
		0.028:0.028
		0.038:0.038

2.4.2 Preparations

Microscope slides were placed into a 100 mL glass beaker containing 100 mL of 1% normal melting agarose (NMA) solution until two-thirds of the slide was coated. The slides were then covered with aluminum foil and stored in the refrigerator at 4°C for 24 h.

An aliquot of 10 µL hemolymph cell extract was combined with 90 µL 0.7% low melting agarose (LMA) on parafilm and mixed two to three times using a micropipette with an angle of 45°. The mixture was carefully placed on a microscope slide coated with NMA, covered with a cover glass, and stored in a refrigerator at 4°C for 24 h. The cover glass was removed by slowly sliding until detachment was achieved.

2.4.3 Lysis

The microscope slides were vertically placed into the staining jar as the lysis solution was added (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100, and 10% DMSO at pH 10) at a temperature of 4°C. The staining jar was then closed and placed in the refrigerator at 4°C for 24 h.

2.4.4 Electrophoresis

The microscope slides were removed from the lysis solution for unwinding by immersing in an alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, and pH > 13) for 10 min. The slide was then placed horizontally in an electroporator comet assay tank and filled with alkaline buffer until entirely submerged. This tank was placed in a refrigerator at 4°C, and the power supply was regulated at 300 mA and 25 V for 10 min.

2.4.5 Neutralization

Each slide was neutralized by immersion in a neutralization buffer (0.4 M Tris-HCl and pH 7.5). This process was carried out thrice, removing the remaining alkaline buffer, with the slide being soaked for 5 min in each washing. The slides were then dried and stored in a refrigerator at 4°C until the staining process.

2.4.6 Image analysis

The electrophoresed comet assay slides were stained with SYBR Safe dissolved in DMSO at a 10,000-fold dilution. Each slide was stained with 70 µL SYBR Safe and observed under a fluorescent microscope (526 nm wavelength and 5×10 magnification). These observations were calculated with at least 50 nuclei per sample with five fields of view. In this study, the DNA damage was evaluated with the parameters of the percentage tail intensity (TI%, % of DNA in the tail), tail moment (TM, tail length x% of DNA in the tail), and tail factor (TF, a measure for the degree of DNA fragmentation in a cell population) by using the Comet Score: Automatic Comet assay Software series 2.0.0.38, which displayed the TI% and TM results.

For the TF, *D. magna* nuclei were grouped into five categories (A-E) according to the amount of DNA in the tail. Category A showed 2.5% of DNA in the

tail. It was accompanied by categories B, C, D, and E, which showed cells with DNA in the tail of 12.5%, 30%, 67.5%, and 97.5%, respectively (Focke et al., 2010). Furthermore, the grade A damage indicated that the cells were primarily undamaged, with B-E representing higher levels of DNA fragmentation (Focke et al., 2010).

2.5 Data analysis

The DNA damage data (TI% and TM) were analyzed by one-way analysis of variance, with pesticide concentrations as the independent variable. When the results showed a significant difference, the Dunnett multiple comparison tests were performed between the control and pesticide concentrations in each age group, where the comet category of TF was tested by Duncan Multiple Range Test. In addition, an independent sample T-test was performed to evaluate the effect of *D. magna*'s age on DNA damage. Linear regression analysis was performed to assess the relationships between pesticide concentrations and DNA damage (TI% and TM), followed by Pearson correlation analysis to test the strength of linear relationships.

3. RESULTS AND DISCUSSION

3.1 Acute toxicity

The acute toxicity test showed that the LC50-24 h of methomyl was lower than the methomyl: mancozeb combination and mancozeb alone (Table 3). This finding indicated that methomyl has the highest toxicity level (Alwaini, 2021; Ariyanti, 2021; Izdihar, 2021). The acute toxicity tests resulted in LC50-24 h values below 1 mg/L. Based on the European Commission (EC, 2003), an LC50 value of less than 1 mg/L is extremely toxic to aquatic organisms.

Table 3. LC50-24 h of methomyl, mancozeb, and mancozeb:methomyl combination against *D. magna*

Pesticides	Age of *D. magna*	LC50-24 h (mg/L)
Methomyl	24 h	0.060
	48 h	0.024
Mancozeb	24 h	0.529
	48 h	0.141
Mancozeb:Methomyl	24 h	0.086
	48 h	0.028

According to USEPA (2005), the LC50-24 h of mancozeb toward *D. magna* was 0.058 mg/L, which was lower than the results of this study. For methomyl, Ren et al. (2017) suggested that the LC50-24 h on *D. magna* ranged from 0.0073-0.0200 mg/L, whereas Menconi and Beckman (1996) obtained 0.0317 mg/L, results that are also lower than those obtained in this study. The LC50 values may be influenced by parent nutrition, individual genotype and size, neonate qualities, and food availability (Pellegri et al., 2014). The combination index of mancozeb:methomyl for both *D. magna* ages (24 h and 48 h) showed a value of >1, indicating that the two pesticides antagonistically interacted. In the isobologram, the values of CI are above the additive line (Figure 1).

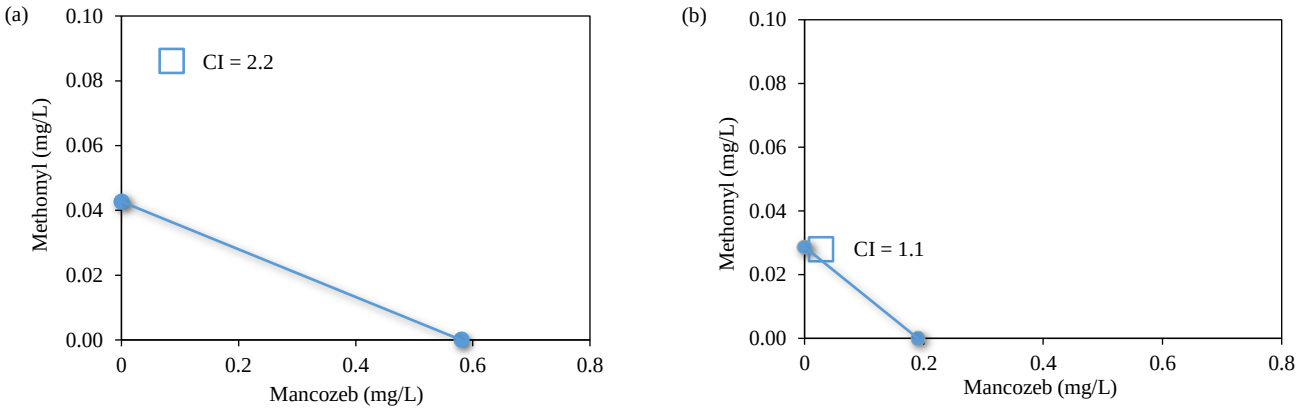

Figure 1. Isobolograms that represent the antagonistic activity of mancozeb and methomyl on (a) 24 h old and (b) 48 h old *D. magna*

Based on the toxicity test, methomyl, mancozeb, and mancozeb:methomyl combination affected the movement of *Daphnia*, which became weaker and slower. These pesticides also caused the death of some *D. magna*, which were found to have paler body color. According to Lushchak et al. (2018), pesticide exposure causes several damages to the physiological features of the organism, such as molting and the destruction of the antennae and internal body structure. This exposure also affected

reproduction, feeding, and breathing abilities, which decreased due to reduced oxygen consumption levels (Araujo et al., 2019). Furthermore, mancozeb interfered with lipid metabolism and respiration, with methomyl affecting the physiology of *D. magna*, by binding to acetylcholinesterase (AChE) and inhibiting cholinesterase at the synapse (Pereira and Goncalves, 2007). These enzymes play a role in acetylcholine (ACh) hydrolysis, the primary neurotransmitter in the peripheral and central nervous systems. The inhibition also interfered with the enzyme's ability to bind substrates by typically accumulating ACh at the nerve endings. It leads to overstimulation and the desensitization of muscarinic and nicotinic ACh receptors. Inhibition of the AChE also occurred after phosphorylation of the hydroxyl group on serine, which was observed at the enzyme's active site. In addition, cholinergic stimulation causes hyperactivity of excited tissues, fasciculations, seizures, muscle paralysis, coma, and death (Hertika and Baghaz, 2019).

D. magna at 24 h old and 48 h old were used in this research because both ages have high sensitivity to pollutants. Therefore, the organism was suitable for toxicity tests and comet assay analysis (Pellegri et al., 2014). Generally, the toxicity results on 24 h old *D. magna* produced a higher mortality percentage than the other age group. According to Pellegri et al. (2014), the 24 h old organism had more carapace fragility because it is newly hatched and possessing higher sensitivity. Therefore, the toxicants penetrated the body and carapace more easily. However, the finding is contrary to that of the present study, which found that the 48 h old *D. magna* had a higher mortality rate than the other age group. The result is consistent with that of Traudt et al. (2017), indicating that newly hatched neonates had more robust physiological defense mechanisms than older organisms, with the abilities decreasing along with their ages. Furthermore, the 24 h old *D. magna* still had leftover food reserves for body nutrition, which protected against some metals. Therefore, it indicated lower sensitivity given that 48 h old organisms no longer had yolks in their body structure (Traudt et al., 2017).

The acute toxicity test showed that the LC50-24 h for *D. magna* aged 24 h was higher than that for 48 h (Table 1). For genotoxicity analysis, the determination of individual pesticide concentrations referred to the LC50 values at, above, and below the LC50 concentrations. Thus, the individual pesticide concentration for *D. magna* aged 24 h was higher than that of 48 h. If the two age groups had the same concentration, then the organism's death is more likely to be immediate in the 48 h age group.

3.2 Genotoxicity

3.2.1 Qualitative analysis

The exposure of mancozeb, methomyl, and mancozeb:methomyl combination to the age groups at varying concentrations induced DNA damage by forming comet structures (Figures 2 and 3) (Alwaini, 2021; Ariyanti, 2021; Izdihar, 2021). The damage generally began to occur at the lowest pesticide concentration. The comet's tail increased in size with increasing concentration. Comets with a small nucleoid head and a large and long tail indicated high amounts of damaged cells and also apoptosis (Lorenzo et al., 2013). Therefore, the smaller size of the comet head indicates higher level of DNA damage.

Darlina et al. (2018) stated that the mancozeb, methomyl, and combined exposures directly caused changes in DNA structure through the indirect breakdown of strands, thereby enabling the cleavage of water molecules to produce reactive oxygen species (ROS). The presence of ROS species oxidatively damaged the DNA molecules. Therefore, the damage to the strand created a comet-like structure.

3.2.2 TI%

The TI%, which indicates the percentage of DNA in the tail, increased significantly in all individual and combined concentrations of pesticides and age groups compared with the control ($p<0.05$) (Figure 4) (Alwaini, 2021; Ariyanti, 2021; Izdihar, 2021). The increase in TI% started from the lowest level of pesticides, indicating that the lowest concentration could induce DNA damage. The greater TI value (%) indicates higher DNA fragmentation occurrence. This condition indicates that the damage was more massive in the cell. The highest values of TI%, ranging from 12.5%-13.6%, was observed at concentrations of 0.08 mg/L methomyl (*D. magna* 24 h old), 0.034 mg/L methomyl (*D. magna* 48 h old), and 0.106:0.106 mg/L mancozeb:methomyl (*D. magna* 24 h old). The regression analysis on the age group of *D. magna* 24 h and 48 h indicated the concentration-dependent effects of mancozeb (r^2=0.96 and 0.94), methomyl (r^2=0.97 and 0.99), and mancozeb: methomyl (r^2=0.93 and 0.99) on TI%. In the control, the TI value was below 10%. This level of damage is generally considered minimum (Mitchelmore et al., 1998).

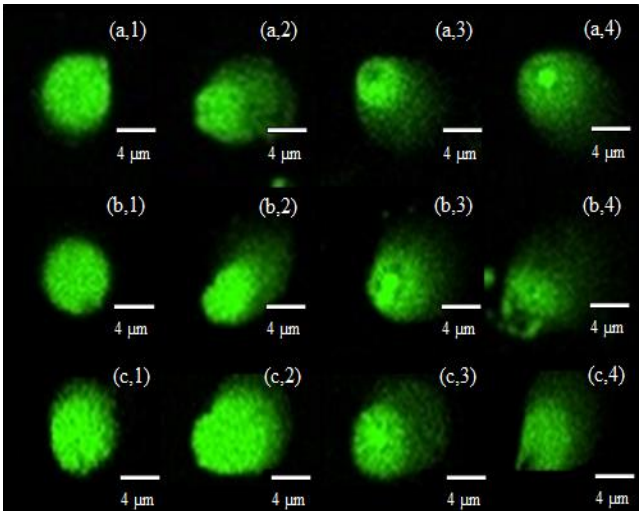

Figure 2. Comet of cell *D. magna* 24 h exposed to (a) methomyl (a,1) 0 mg/L, (a,2) 0.04 mg/L, (a,3) 0.06 mg/L, and (a,4) 0.08 mg/L; (b) mancozeb (b,1) 0 mg/L, (b,2) 0.3 mg/L, (b.,3) 0.5 mg/L, and (b,4) 0.7 mg/L; (c) mancozeb:methomyl combination (c,1) 0 mg/L, (c,2) 0.066:0.066 mg/L, (c,3) 0.086:0.086 mg/L, and (c,4) 0.106:0.106 mg/L for 24 h (5×10 magnification).

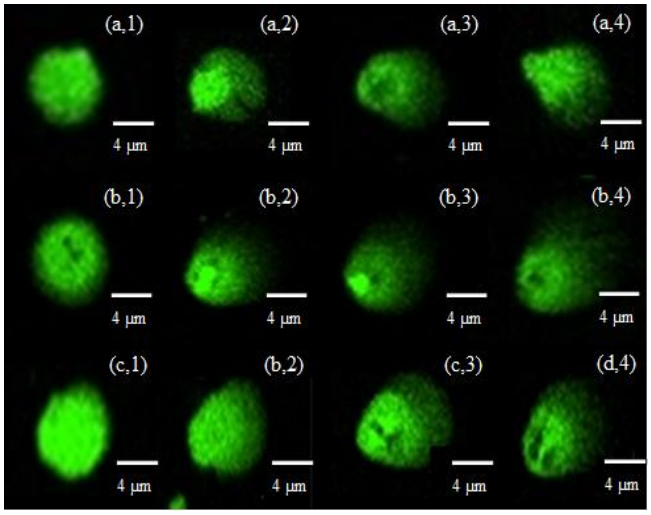

Figure 3. Comet of cell *D. magna* aged 48 h with exposure of (a) methomyl (a,1) 0 mg/L, (a,2) 0.014 mg/L, (a,3) 0.024 mg/L, and a,4) 0.034 mg/L; (b) mancozeb (b,1) 0 mg/L, (b,2) 0.08 mg/L, (b,3) 0.1 mg/L, and (b,4) 0.12 mg/L; (c) combination of mancozeb: methomyl (c,1) 0 mg/L, (c,2) 0.018:0.018 mg/L, (c,3) 0.028:0.028 mg/L, and (c,4) 0.038:0.038 mg/L for 24 h (5×10 magnification).

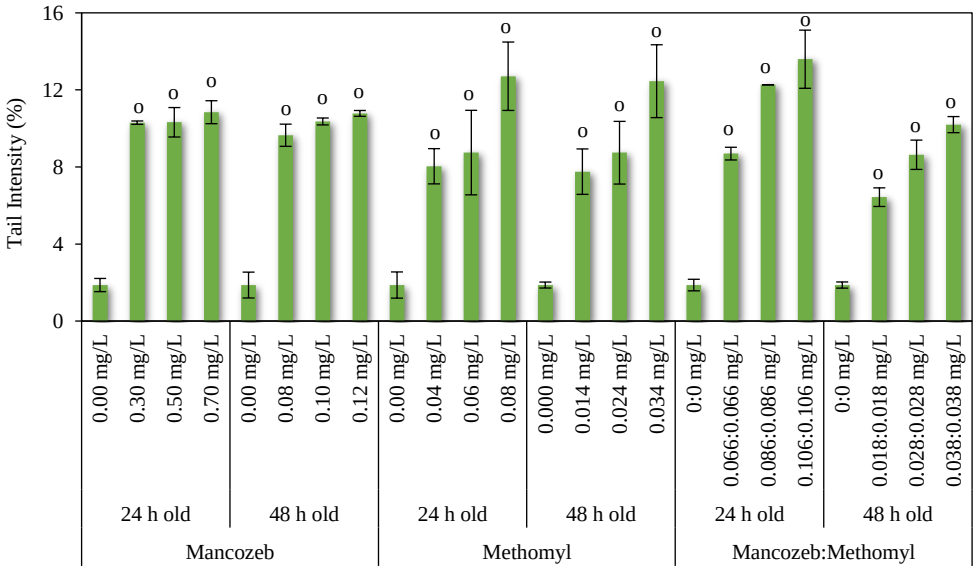

Figure 4. TI% of 24 h old and 48 h old *D. magna* upon exposure to mancozeb, methomyl, and mancozeb:methomyl combination. Significant differences in comparison to control within each age group of pesticide exposure were indicated by letter "o".

Exposure of *D. magna* to the individual pesticide showed that methomyl was more toxic to DNA than mancozeb because the concentration of methomyl was much lower than that of mancozeb but produced a similar level of TI%. A similar result was also observed in the combined pesticide exposure, confirming that the combined exposure had an antagonistic effect. Surprisingly, in 48 h old *D. magna*, the combined exposure produced the lowest impact, mainly compared with the individual pesticide (Alwaini, 2021; Ariyanti, 2021; Izdihar, 2021). Silva et al. (2019) suggested that the exposure of *D. magna* to the combination of triclosan and carbendazim also resulted in an antagonistic effect. In the *Daphnia* age group, the individual and combined pesticide concentrations administered on the 48 h old *D. magna* were two to three times lower than those of the 24 h old group. Nevertheless, the TI% of the two age groups had similar values. This finding may indicate that the 24 h old group exposed to higher

concentrations used leftover food reserves as protection from the toxicity of the pesticides (Traudt et al., 2017). However, no significant difference ($p>0.05$) was observed between the TI% of both age groups. Pellegri et al. (2014) also stated that using *D. magna* with different ages resulted in a TI% value similar to the comet assay. Knapik and Ramsdorf (2020) reported that exposure of *D. magna* to malathion at 0.23 and 0.47 µg/L for 48 h increased the intensity of fragmented DNA materials. According to Li et al. (2022) and Srivastava and Singh (2020), this genotoxic effect is caused by an increase in ROS upon exposure to pesticides, causing cell membrane damage.

3.2.3 TM

The TM values for *D. magna* 24 h old tended to be higher than the other age group (Figure 5) (Alwaini, 2021; Ariyanti, 2021; Izdihar, 2021). However, there was no significant difference ($p>0.05$) between the values of both age groups. Nebeker et al. (1986) also observed the effect of Zn on the damage of *D. magna*, as the results showed no significant difference in the genetic destruction of young and old organisms. Furthermore, the *D. magna* between the ages of 1 to 7 d had a level of DNA damage that was not significantly different; however, older organisms had lower sensitivity. Therefore, the level of DNA damage was down.

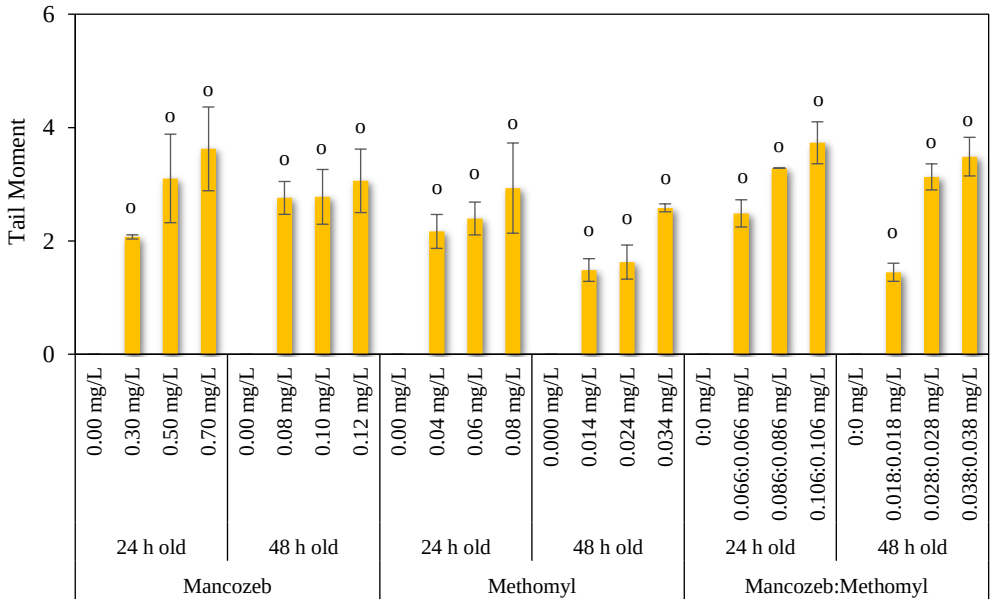

Figure 5. TM of 24 h old and 48 h old *D. magna* upon exposure to mancozeb, methomyl, and mancozeb:methomyl combination. Significant differences in comparison to control within each age group of pesticide exposure were indicated by letter "o".

The TM values also increased with higher exposure concentration. Significant differences between 0 ppm and the lowest pesticide concentration were observed in all pesticide concentrations ($p<0.05$) (Figure 5). The regression analysis represented the strong relationship between TM and pesticide concentrations. In the age groups of 24 h and 48 h, the r^2 values for mancozeb were 0.97 and 0.95, respectively; the r^2 values were 0.97 and 0.98 for methomyl, and 0.99 and 0.95 for mancozeb:methomyl combination. A concentration of 0 ppm produced a TM value below 1. This value indicated low DNA damage with a short migration distance of genetic material. For individual pesticides, exposure to methomyl generated slightly lower TM values than mancozeb and combined pesticides; it also confirmed the highest toxicity of methomyl. Combined pesticides also produced similar results to individual exposure, ensuring that combined exposure had an antagonistic effect. Prasath et al. (2016) suggested that the TM values of *Daphnia carinata* increase significantly upon exposure to 2, 4-dinitroanisole, its metabolites (2-amino 4- nitroanisole and 2,4-dinitroanisole), and 2, 4, 6-trinitro toluene for 48 h. The production of ROS due to oxidative stress is attributed to genotoxicity.

According to Shaposhnikov et al. (2008), the formation of TM was caused by areas on the double strands which experienced relaxation. This area contained double- and single-strand breaks, which migrated toward the positive pole to form a tail during electrophoresis. The migration distance of this DNA genetic material developed a TM, indicating

considerable DNA damage. At the same time, the finding suggests that the further migration distance of genetic material indicates a higher TM value.

3.2.4 TF

The TF is a parameter in the comet assay that is based on the DNA tail percentage value. Furthermore, the tail DNA indicated the percentage of DNA in the comet of a cell. This relative percentage showed the frequency of DNA breaks after the comet assay (Azqueta and Collins, 2013).

For all exposures, a concentration of 0 mg/L (24 h old and 48 h old *D. magna*) showed only a few cells that fall into category A, whereas some cells were lightly destroyed in B (Figure 6) ($p<0.05$) (Alwaini, 2021; Ariyanti, 2021; Izdihar, 2021). Based on the research of Phromchaloem et al. (2018), the value of tail DNA (%) for the control exposure of Cd toxicant against *Moina macrocopa* for 48 h belonged to categories A and B. Nunes et al. (2018) reported that upon exposure of *D. magna* to ciprofloxacin at a concentration of 0.078 mg/L, the number of damaged cells increases from category A to D. In this study, most cells belonged to category D at all concentrations, indicating the presence of DNA in the tail by 40%-95%. The percentage of cells in category D exposed to the highest concentration of pesticides was higher than those in lower concentrations ($p<0.05$). It also revealed that pesticides at lower concentrations could induce DNA damage. Category E was not observed in the cell comets at all concentrations, indicating that no cells were severely fragmented. According to Focke et al. (2010), categories A to D indicated the stress of DNA replication, whereas category E showed the apoptotic process. Thus, in this study, *D. magna* cells experienced genetic replication stress, which caused the accumulation of DNA in the tail with no apoptotic processes.

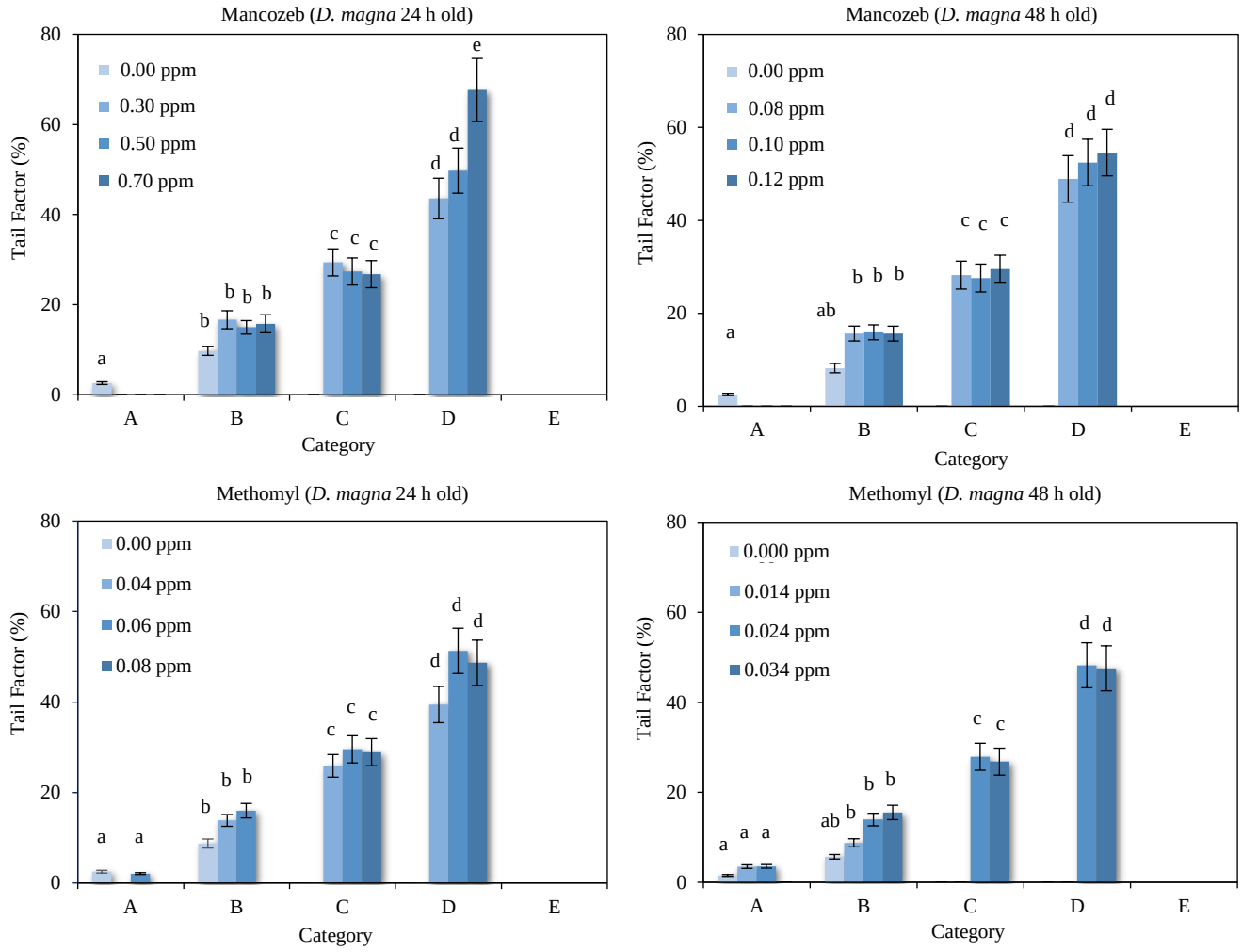

Figure 6. Percentage of *D. magna*'s cells (24 h and 48 h) in comet categories A-E upon exposure to mancozeb, methomyl, and mancozeb:methomyl. Similar letters indicate that the differences of TF were not significant.

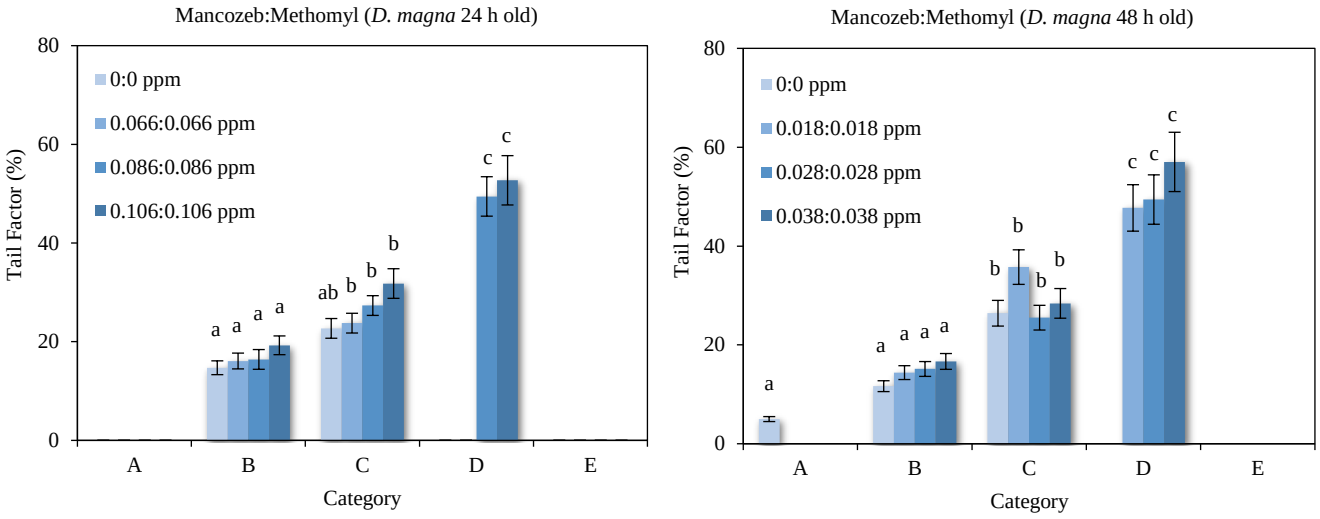

Figure 6. Percentage of *D. magna*'s cells (24 h and 48 h) in comet categories A-E upon exposure to mancozeb, methomyl, and mancozeb:methomyl. Similar letters indicate that the differences of TF were not significant (cont.).

The TF values increased with higher exposure concentration (Figure 7). In all treatments, the TF values were generally significantly different from the control. The highest TF value with the highest concentration was observed in *D. magna* upon pesticide exposure. The TI% and TM values were relatively the same between individual and combined exposures. However, the TF value indicating the level of DNA fragmentation showed that the combined exposure resulted in a slightly higher level of DNA fragmentation, followed by methomyl and mancozeb treatments. Furthermore, the TF of the 24 h old *D. magna* was more severe than that of the 48 h old *D. magna*, but the differences were insignificant ($p>0.05$).

The result of this present study indicates that, in response to the exposure to individual and combined pesticides, the values of each parameter of DNA damage, i.e., TI%, TM, and TF, showed a function of pesticide concentrations. Although the interaction of pesticides in combined exposure led to an antagonistic effect, DNA damage was massive linearly given the increasing combined concentration of pesticides. Furthermore, the three parameters can be used as sensitive biomarkers for pesticide pollution.

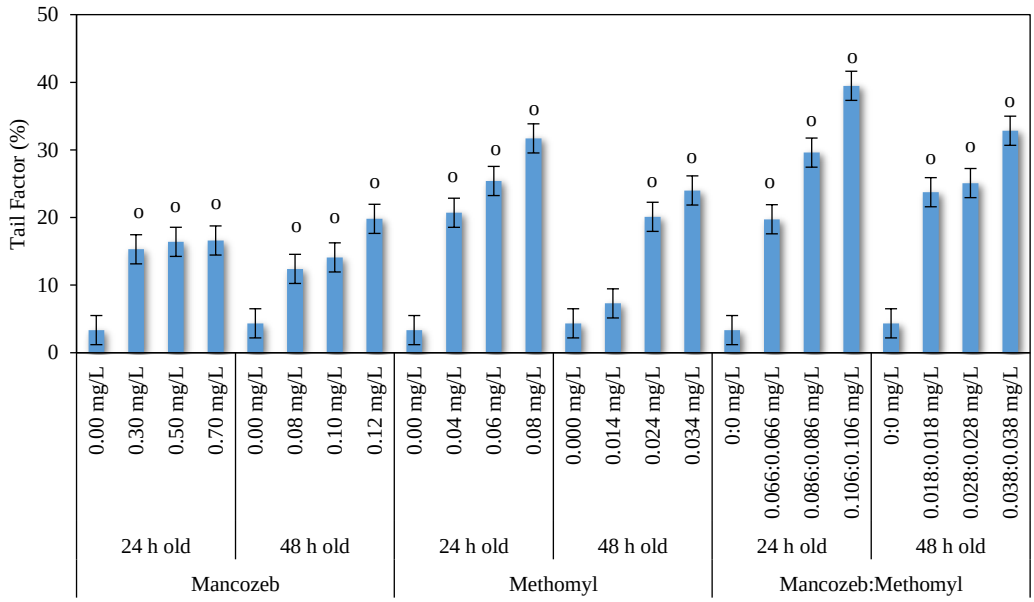

Figure 7. TF of *D. magna*'s cells (24 and 48 h old) in comet assay upon exposure to mancozeb, methomyl, and mancozeb:methomyl. Significant differences in comparison to control within each age group of pesticide exposure were indicated by letter "o".

4. CONCLUSIONS

The toxicity levels of mancozeb, methomyl, and mancozeb:methomyl combination were categorized as extremely toxic. The combination index of mancozeb:methomyl for both *D. magna* ages (24 h and 48 h) indicated that the two pesticides were antagonistically interacted (CI>1). Exposure of *D. magna* to the pesticides individually and in combination induced DNA damage. Based on TI%, TM, and TF values, the level of damage was almost the same between the individual and combined pesticide concentrations; the level of DNA damage increased with higher pesticide concentration. However, the DNA damage of 24 h old and 48 h old *D. magna* did not significantly differ. Increased DNA damage in *D. magna* indicated that this parameter was sensitive to the presence of pesticides. This study greatly contributes to the current issue of environmental pollution and environmental risk assessment in freshwater ecosystems via pesticide danger. In application, DNA damage can be used as a biomarker for biomonitoring pesticide pollution in the aquatic ecosystem. However, further field research is required to study the genotoxicity of multiple pesticides to *D. magna* in the agricultural area using the comet assay.

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