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Cytotoxic Effects of Disinfectants in Tap Water on *Hordeum vulgare* L. and *Eisenia fetida* Savigny

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Abstract

The tap water must be disinfected to remove the disease-causing microorganisms. Most disinfection methods used in water treatment are decoction, chlorination, disinfection with ozone, disinfection with ultraviolet light, with peracetic acid. The effects of some disinfectants used in tap water treatment were investigated in the seeds of *Hordeum vulgare* and the worm *Eisenia fetida*. The concentration that halves the root lenghts for the seeds and the concentration that is lethal for fifty percent of worms were calculated using these disinfectants. Within three days of application, the EC50 values for *H. vulgare* were determined as 20 µl/ml, 240 mg/ml and 80 µl/ml for NaOCl, $Ca(ClO)_2$ and PAA respectively. The lethal dose that kills the fifty percent of red worms was determined as 30µl/ml, 8 mg/ml and 25µl/ml for NaOCl, $Ca(ClO)_2$ and PAA respectively. Cytotoxic effects were examined using the mitotic index and chromosomal abnormality test in *H. vulgare*. We observed that different concentrations of NaOCl, $Ca(ClO)_2$ and PAA decreased the value of mitotic index. Also the abnormal cell frequency in mitotic divisions on the root meristematic cells of *H.vulgare* were increased. The alkaline single-cell gel electrophoresis method was used to determine the genotoxicity of NaOCl and $Ca(ClO)_2$ applications on *E.fetida*. Compared with the control group, it was determined that the degree of DNA damage after $Ca(ClO)_2$ application was higher than NaOCl. Because these chemicals can create cytotoxic effects, they should also be used cautiously at low concentrations.

1. Introduction

The basic drinking and utility water source of society is tap water. If this water is healthy and safe, many diseases may be prevented. The disinfection process is an effective barrier to many pathogens in tap water [1]. Chemicals used for disinfection in tap water are effective against microorganisms and other living organisms. Exposure to free disinfectant residues can adversely affect the lives of humans and other living things [2]. Chlorination, the most frequently used method in the disinfection of water, is still the most reliable disinfection method among existing alternatives because it has a lasting effect in a short time and is more affordable than others [3].According to the World Health Organization, the widespread use of chlorination of drinking water is one of the most important developments in the field of public health [4].

Chlorine inhibits organic matter that causes an unpleasant taste and odor in tap water. Thus, it controls biological growth [5]. The three most preferred chlorine compounds in our country are liquid chlorine (sodium hypochlorite), gaseous chlorine, and powdered chlorine (calcium hypochlorite). When any of these three chlorinated substances are left in the water for disinfection, hypochlorous acid (HOCl) formation occurs in the water, and this substance actively disinfects microorganisms [6].

Peracetic acid is also important in tap water disinfection because of its ease of application, broad activity even in the presence of heterogeneous organic matter, and absence of mutagenic residues or by-products [7]. Peracetic acid, even at low concentrations (< 0.3%), is sporicidal, bactericidal, virucidal, and fungicidal; thus, it is thought to be a more potent biocide [8]. In addition, the use of peracetic acid-based disinfectants is becoming increasingly common because of the negative effects of chlorine. Although these chemicals have positive effects, such as protecting people

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against various diseases, they can cause biotic stress if the recommended dose is exceeded [9]. During their sewage water studies in *Allium cepa* L. and *Zea mays* L., Sarkar et al. reported that trace element accumulation in the food chain may harm various kinds of organisms within the ecosystem along with human beings [10]. Tap water from a known and controlled source is safe to drink, but water of unknown origin should never be consumed in many countries.

In this study, we aimed to determine the cytotoxic and genotoxic effects of sodium hypochlorite (NaOCI), calcium hypochlorite $Ca(ClO)_2$, and peracetic acid (PAA) used in tap water disinfection on *H. vulgare* and *E.fetida*.

2. Materials and Methods

2.1. Determination of effective concentration (EC50) values in *H. vulgare*

Eight concentrations (2.5, 5, 10, 15, 20, 25, 30, and 35 µl/ml) of NaOCI, ten concentrations (1, 4, 16, 64, 128, 240, 272, 288, 320, and 360 µl/ml) of $Ca(ClO)_2$, and nine concentrations (10, 20, 30, 40, 50, 60, 70, 80, and 90 µl/ml) of PAA were used for the determination of EC50 values in *H. vulgare*. Pre-trials were carried out with at least three repetitions for three days by placing the *H. vulgare* seeds in the Petri dishes in 10 ml of each disinfection solution. Distilled water was used in the control group. Root tips of 0.5 cm and above were considered germinated. After the EC50 values were determined, the experiments were continued using three different concentrations EC50/2, EC50, and 2 as application solutions.

2.2. Mitotic abnormality tests in *H. vulgare*

H. vulgare seeds were germinated in NaOCl (EC50=20µl/ml, EC50x2=40µl/ml and EC50/2= 10µl/ml), $Ca(ClO)_2$ (EC50=240µl/ml, EC50x2=480µl/ml and EC50/2= 120µl/ml) and PAA (EC50=80µl/ml, EC50x2=160µl/ml and EC50/2=40µl/ml) concentrations in an oven at 20-25°C for 3 days. Samples 1–2 cm long from the root tips were directly fixed in Carnoy (3 parts of 70% ethyl alcohol: 1 part of %45 glacial acetic acid) and kept at +4ºC for 24 h. The root tips were then taken into 70% ethyl alcohol to be used in slide preparation and examination and stored in the refrigerator at 4°C [11].

Slides were prepared in accordance with the aceto-orsein crushing and spreading method. The roots, which were dyed and hydrolyzed by heating in a watch glass, were placed on a slide with a drop of aceto-orsein. The root tips were then cut into small pieces with a razor blade. A coverslip was covered and crushed [12]. Experiments were repeated three times for each concentration and control groups. The slides were examined using an Olympus BX-51 research microscope and observed using a camera. At least 1000 cells were counted from each slide, and the mitotic index was calculated. The rates of chromosomal damage, such as bridge formation, chromosomal adhesion, and false polarization, in dividing cells were calculated. The data obtained were transferred to a general table for each concentration. MI was calculated using the following formula.

$$\text{Mitotic index (\%)} = \frac{\text{The number of dividing cells}}{\text{The number of total cells}} \times 100$$

2.3. Determination of the LC50 (lethal concentration) values in *E. fetida*

E. fetida were purchased from commercial suppliers and incubated on moist filter paper at room temperature to defecate. 12 concentrations of NaOCl (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 µl/100ml), 9 concentrations of $Ca(ClO)_2$ (0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 µl/100ml) and 6 concentrations of PAA (5, 10, 15, 20, 25, and 30 µl/100ml) were used to determine the LC50 values in *E.fetida*. To avoid light exposure, the worms were placed in amber (dark colored) bottles containing different concentrations. Then 10 worms from each bottle were placed in a Petri dish, and it was decided whether they were alive or not by mechanical stimulation. Distilled water was used in the control group. Worms were checked after 24 h, and experiments were performed in three repetitions.

2.4. Comet Assay with Coelomocytes from *E. fetida*

2 worms were placed in 4 ml of solution in the Eppendorf tube, and the coelomocytes were allowed to be released into the environment spontaneously after 24 hours. After removing the worms in the Eppendorf tube, the cells were centrifuged at 2000 rpm for 3 min and kept on ice before electrophoresis. The slides were covered with 1% NMP agarose and left to dry overnight. 50 µl of cell solution was mixed with 75 µl of LMP, pipetted onto the NMP layer, covered with a coverslip, and kept on ice for 5 min and then in lysis solution at 4°C for 1 h. The slides were gently removed from the refrigerator and transferred to the horizontal electrophoresis tank, which was previously set to +4ºC with a cold water bath attached to it. Starting from the sides of the tank, a cold electrophoresis buffer was poured slowly on it, and the lid of the tank was closed. The slides were kept in the tank for 20 min to dissolve the DNA chain. Electrophoresis was performed at 20V, 300 mA for 20 min to observe the comets that will occur because of the different

molecular weight DNA fragments walking at different speeds depending on the damage. The slides were first kept in a cold PBS buffer for 5 min, then in 70% ethanol for 5 min and neutralized. After the neutralization process, the slides were placed vertically on the blotting paper and the excess buffer was allowed to move away from the slides. Before the slides dried, each of them was stained with 100 µl ethidium bromide solution and left for 5 min. The coverslip was closed immediately, and slides prepared for comet assay analysis were examined using a fluorescence microscope. For each dose and hour, 25 nuclei were counted in 2 repetitions. The tail DNA percentage (% tail DNA) and olive tail moment (OTM) values were measured using the Kameram comet assay analysis program [13].

3. Results and Discussion

3.1. EC50 values of NaOCl, $Ca(ClO)_2$, and PAA in *H.vulgare*

It was determined that the application of NaOCl, $Ca(ClO)_2$, and PAA had a significant effect on the germination percentage and root length of *H. vulgare*. All NaOCl concentrations had negative effects on the germination percentage of *H. vulgare* seeds (Fig. 1).

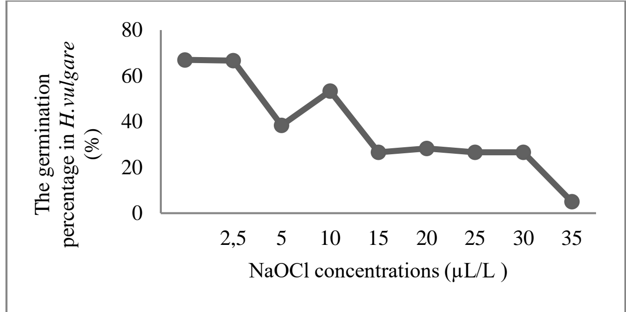

Figure 1. Effect of different NaOCl concentrations on the germination percentage of *H. vulgare* seeds.

Compared with the control group, the EC50 value for NaOCl was determined as 20 µL/mL (Fig. 2). It was observed that the germination percentage in *H. vulgare* decreased in parallel with increasing $Ca(ClO)_2$ concentrations (Fig. 3).

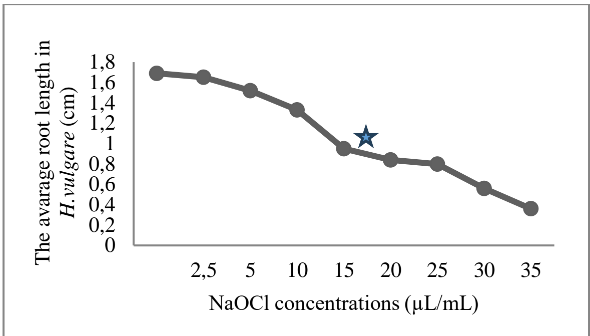

Figure 2. Effect of different NaOCl concentrations on the root growth of *H. vulgare* seeds. ★ :P<0.05

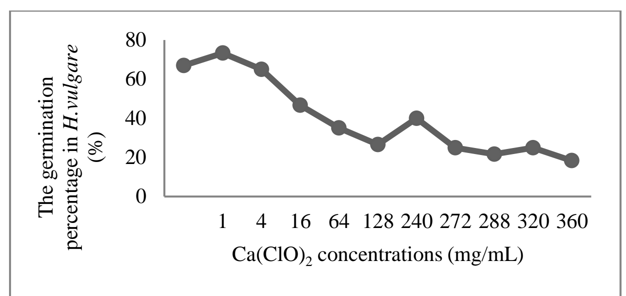

Figure 3. Effect of different $Ca(ClO)_2$ concentrations on the germination percentage of *H. vulgare* seeds.

$Ca(ClO)_2$ treatment has a significant negative effect on *H.vulgare* root growth. The EC50 for $Ca(ClO)_2$ was determined to be 240 mg/mL compared with the control group (Fig. 4).

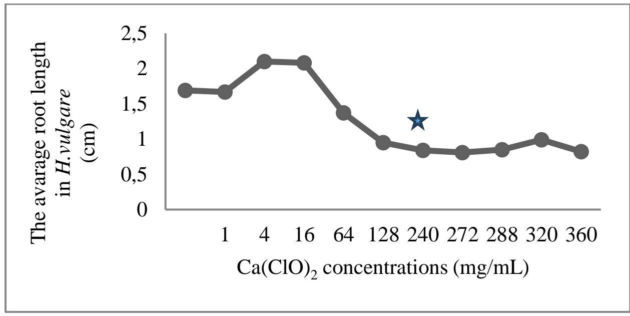

Figure 4. Effect of different $Ca(ClO)_2$ concentrations on the root growth of *H. vulgare* seeds. ★ :P<0.05

It was observed that PAA negatively affected the germination percentage of *H. vulgare* seeds (Fig. 5). PAA application was found to have a significant negative effect on the germination of *H. vulgare* seeds. Compared with the control group, the EC50 value for PAA was determined as 80 µl/ml (Fig. 6).

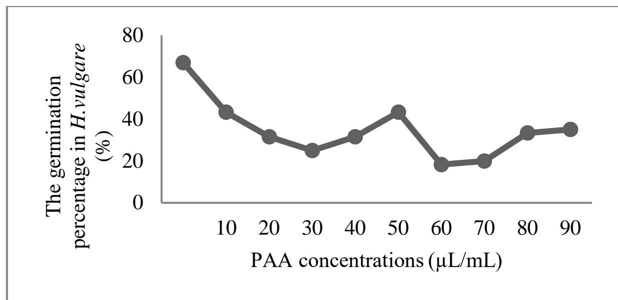

Figure 5. Effect of different PAA concentrations on the germination percentage of *H. vulgare* seeds.

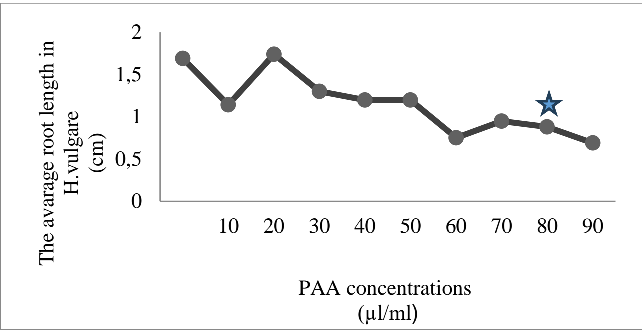

Figure 6. Effect of different PAA concentrations on the root growth of *H. vulgare* seeds. ★ :$P<0.05$

As the concentration increased, a decrease in the barley root length was detected. This decrease in cell division occurs because of some deterioration in metabolism after the treatment of the seeds with the disinfectants we used. Decreased division rates in the meristematic cells of plant roots and a negative effect on growth rates are interrelated. When EC50 values were evaluated in terms of toxicity, NaOCl showed the most toxic effect, followed by PAA and $Ca(ClO)_2$.

3.2. Effect of NaOCl, $Ca(ClO)_2$, and PAA application on mitosis and chromosomal abnormalities in *H. vulgare* root tip cells.

The mitotic index was calculated separately for each chemical and concentration. The three-day mean mitotic index rates in germinated *H. vulgare* seeds at 10 µl and 20 µl NaOCl concentrations were 8.75% and 8.16% (Table 1.).

Table 1. Effects of NaOCl concentrations on the mitotic index in *H. vulgare* root tip cells.

NaOCl concentration (µL/mL)	Number of total cells	Number of dividing cells	Mitotic index (%)
Control	1159	275	23.7
10	1165	102	8.75
20	1163	95	8.16
40	-	-	-

At 120 mg/ml, 240 mg/ml and 480 mg/ml $Ca(ClO)_2$ concentrations, the mitotic index decreased to 4.5%, 4.11%, and 4.04%, respectively (Table 2.).

Table 2. Effects of $Ca(ClO)_2$ concentrations on the mitotic index in *H. vulgare* root tip cells.

$Ca(ClO)_2$ concentration (mg/L)	Number of total cells	Number of dividing cells	Mitotic index (%)
Control	1159	275	23.7
120	1161	73	4.50
240	1240	51	4.11
480	1121	38	3.39

At 40 µl and 80 µl PAA concentrations, the mitotic index decreased to 4.04%, 1.39% (Table 3.). These data show that increasing concentrations have an inhibitory effect on cell division. The most toxic effect in the mitotic division was observed in first NaOCl and then PAA.

Table 3. Effects of PAA concentrations on the mitotic index in *H. vulgare* root tip cells.

PAA concentration (µL/mL)	Number of total cells	Number of dividing cells	Mitotic index (%)
Control	1159	275	23.7
40	1140	46	4.04
80	1222	17	1.39
120	-	-	-

Different chromosomal abnormalities were observed in the root tip cells of *H. vulgare* at different stages of mitosis. The type of each chromosomal abnormality was determined individually.

It was determined that the chromosomal abnormalities decreased with increasing NaOCl, $Ca(ClO)_2$, and PAA concentrations. The slides from each concentration were examined and photographed (Fig. 7).

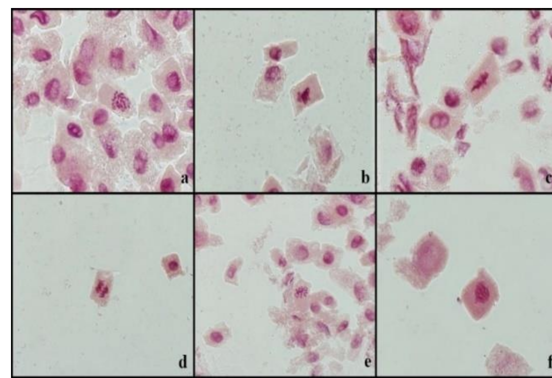

Figure 7. Chromosomal abnormalities in *H. vulgare* root tip cells germinated at 240 mg/mL $Ca(ClO)_2$ concentration (x1000); a. C-mitosis, b. Metaphase stickiness and laggard chromosome, c. Metaphase axis shift and adhesion, d. Bridge formation and axial shift in anaphase e. C-mitosis, f. Laggard chromosome in pyknotic nucleus.

3.3. LC50 values in *E. fetida* treated with NaOCl, $Ca(ClO)_2$ and PAA

The application of NaOCl, $Ca(ClO)_2$, and PAA has a significant negative effect on the survival of *E. fetida*. Compared with the control group, the LC50 value was determined as 30 µl/ml for NaOCl, 8 mg/ml for $Ca(ClO)_2$ and 25 µl/ml for PAA. NaOCl, $Ca(ClO)_2$, and PAA used in low concentrations showed toxic effects on *E. fetida*, and the highest toxicity among these disinfectants was detected in PAA application.

3.4. Determination of DNA damage by the comet assay in *E. fetida* treated with NaOCl, $Ca(ClO)_2$ and PAA

We used coelomocytes during our experiments to detect DNA damage because it was determined that coelomocytes with PAA treatment did not preserve their viability in microscopic examinations. Therefore, PAA was excluded from comet assay studies. Because it showed high toxicity and caused the death of coelomocytes' DNA damage cannot be determined. While less DNA-damaged nuclei called Type 0 and Type 1 were observed in the comet control groups, Type 2 and Type 3 DNA damage was observed in the majority of NaOCl applications, whereas more Type 3 and Type 4 DNA damage was observed in $Ca(ClO)_2$ applications. DNA damage was photographed (Fig. 8).

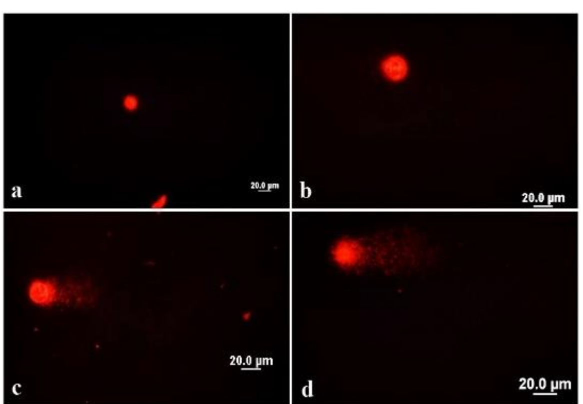

Figure 8. Comet assay analysis images; a. Type 0: Flawless (control), b. Type 1: Tail < 0.5 (control), c. Type 2: Tail > 0.5 (30 µl/ml NaOCl), d. Type 3: Tail larger than the head diameter (8 mg $Ca(ClO)_2$).

Compared with the control group, the DNA damage degree of NaOCl and $Ca(ClO)_2$ application increased. It was determined that the degree of DNA damage was higher in the $Ca(ClO)_2$ application groups than in the NaOCl application groups.

Tail DNA percentages were compared to detect DNA damage in the cells of the control group, NaOCl, and $Ca(ClO)_2$ treatments. DNA percentages were found to be significantly higher in the treatment groups than in the control. It was determined that the applied chemicals had a higher average DNA percentage in $Ca(ClO)_2$. Olive tail moment values were also calculated in addition to the tail DNA percentages. The OTM values of $Ca(ClO)_2$ were increased compared with the NaOCl and control groups.

All concentrations of NaOCl, $Ca(ClO)_2$, and PAA decreased the mitotic index of *H.vulgare* root tip meristematic cells. The decrease in the mitotic index indicates that the disinfectants have cytotoxic effects due to cell cycle disruption, such as blocking the G1 phase, suppressing DNA synthesis in the S phase [14, 15], or mitotic phase duration changes [16]. In addition, disruption of specific protein synthesis involved in the cell cycle [17] and ROS formation [18] have similar effects. Vardar and Ünal studied the effects of tralkoxydim and prochlorazin and found that they caused stress in *H. vulgare,* especially above the commercial doses [19]. In this study, NaOCl and PAA showed the most toxic effect in mitotic divisions on *H.vulgare* root tip cells. This effect may have been observed among the three chemicals because their usage rates were lower than $Ca(ClO)_2$ depending on the effective concentration EC50.

The laggard chromosome formation observed in our study is the precursor of micronucleus formation and was also observed in barley root tips treated with Al in previous studies [20]. Huri and Vardar reported that after short-term Al treatment, there was a visible accumulation of fragmented DNA in *H. vulgare* root meristematic tip cells, and cell death and DNA fragmentation increased by approximately 50% after long-term exposure [21].

Increasing NaOCl, $Ca(ClO)_2$, and PAA concentrations compared with the control showed genotoxic effect by increasing chromosomal abnormalities. Some chemicals prevent the spindle fibers from performing their job and prevent the chromosomes from being pulled to the poles, thus blocking the metaphase. Chromosomes are prevented from migrating to the poles from a conjoined image, which occurs because of the chromatin threads attaching to sister chromatins during the chromosome bridge stage and not separating until late anaphase or telophase [22]. C-mitosis may occur because of the damage to the spindle fibers because of the toxicity of the disinfectants we use. Chromatins may be broken or appear unified in anaphase, Luo et al. reported that anaphase bridges can occur due to chromosome breakage, chromatid exchange, or adhesion [23].

NaOCl, which has disinfectant properties, is used in industries such as agriculture, chemicals, food, and medicine to destroy harmful substances [24]. In addition, in the symbiotic germination of orchids, sodium hypochlorite

is used for disinfection and to stimulate the germination of seeds at concentrations of 0.5% to 3% [25, 26, 27]. NaOCl is a toxic compound that can have effects on living tissue and cells if not used correctly [28]. Similar to our study, DNA damage was determined by studying DNA tail percentage and olive tail moment parameters to determine the toxicological effect of microplastics accumulated in the intestines of *E. fetida*. The comet assay suggested that exposure to microplastics induced DNA damage in earthworms [29]. The toxicity of five pesticides used in rice fields was evaluated by experiments on the earthworm *E. fetida*. Carbendazim has been found to be highly toxic to *E. fetida*, significantly reducing earthworm weight, and exhibiting an avoidance response at soil concentrations close to those predicted in rice fields and surrounding ecosystems. This study showed that *E. fetida* may enhance its chemoreceptors [30]. In our study, avoidance behavior was observed after disinfection chemicals were applied to *E. fetida*. This may also explain our findings that the receptors in *E. fetida* combat toxicity.

Worms have a high reproduction rate and are as strong as they are vulnerable to toxic and harmful substances in the environment. Therefore, worms, especially *E. fetida*, are widely used to test the toxicity of contaminants [31]. When DNA damage exceeds the DNA repair capacity, it causes short-term changes in the amount and molecular structure of deoxyribonucleotide triphosphate, disruption of the replication process, inhibition of transcription and protein synthesis, and cessation of proteolytic activity. In the long term, it causes mutations and chromosomal abnormalities [32].

According to the data presented in this study, because genotoxic effects were observed at all the concentrations used, it was determined that both NaOCl and $Ca(ClO)_2$ chemicals caused chromosome breaks at various levels in *E. fetida*. In fact $Ca(ClO)_2$ caused more DNA damage than NaOCl.

4. Conclusion

The effects of disinfectants, especially PAA, which has just started to be used in drinking water disinfection in Turkey have not been studied Although these methods are important for water purification, they can affect non-target organisms. Because disinfectants will interfere with the food chain and affect the development and genetics of living things, it will be better to choose the less harmful ones. In this regard, future generations should be considered, and the damage to nature and living things should be minimized. Therefore, the effects of substances used for the disinfection of tap water should be investigated on living organisms. It is thought that our study will contribute to other studies on this subject and shed light on the effects of the disinfectants recently preferred in the disinfection of tap water.

Declaration of Ethical Standards:

The authors of this article declare that the materials and methods used in this study do not require ethical committee and/or legal-special permission.

Conflict of Interests:

There are no conflicts of interest.

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