Ground and surface water for drinking: a laboratory study on genotoxicity using plant tests

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Abstract

Surface waters are increasingly utilized for drinking water because groundwater sources are often polluted. Several monitoring studies have detected the presence of mutagenic activity in disinfected drinking water, especially from surface sources, due to the reaction of natural organic matter with disinfectant. The choice of the sources as well as the disinfectants for drinking water is one of the most important topics of public health because of chronic exposure to mutagenic compounds. A study model for simulating natural conditions of both deep water and surface water may be a good method for evaluating the genotoxic potential of the products of reaction between humic substances, which are naturally present in surface water, and disinfectants using short-term mutagenicity tests.

Introduction

Surface waters are increasingly utilized for drinking water because groundwater sources are often polluted by persistent organic pollutants. Several monitoring studies have detected the presence of mutagenic activity in disinfected drinking water, especially from surface sources, due to the reaction of natural organic matter with disinfectant. Many studies show that natural organic substances (humic and fulvic acids) present in surface waters may react with disinfectants utilized to potabilize waters to produce numerous disinfection by-products (DBPs) that are potentially harmful to human health.

Numerous DBPs have a mutagenic and/or carcinogenic activity, and a large number of DBPs are able to cause cancer in experimental studies. Furthermore, they may play a role in adverse reproductive outcomes such as inability to conceive, spontaneous abortion and low birth weight. Epidemiological studies in populations using chlorinated drinking water obtained from surface sources have shown some cancer hazards. For this reason, disinfectants such as ozone and chlorine dioxide are used as alternatives to chlorine for water treatment. Humic substances can also react with ozone to produce aldehydes, ketoacids, and carboxylic acids, which contribute to the biodegradable organic carbon content of ozonated water. Yet aldehydes, formed by ozonation of humic substances (such as formaldehyde, acetaldehyde, glyoxal, glyoxylic acid and methylglyoxal), show a clear mutagenic activity.

Humic and fulvic acid concentrations are related to soil and vegetation which are located near water sources, to algae living in water and to seasonal flowering.

Humic compounds are amorphous, brown or black, hydrophilic, acidic, polydispersed substances, with very different molecular weights, and constitute the majority of organic materials present in surface water. These substances derive from both living and decayed organisms, as well as from the reaction between them. Humic and fulvic acid concentrations are related to soil and vegetation which are located near water sources, to algae living in water and to seasonal flowering.

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or molluscs or plants, e.g. Zea mays, Vicia faba, Allium cepa and Tradescantia spp. The aim of this study was to investigate the genotoxic potential of the products of reaction between humic substances, which are naturally present in surface waters, and different disinfectants. In order to simulate natural conditions of both deep water (TOC = 2.5 mg/L) and surface water (TOC = 7.5 mg/L), two different concentrations of commercial humic acids were treated with three different disinfectants: two widely used drinking water disinfectants – sodium hypochlorite (NaClO) and chlorine dioxide (ClO₂) – and peracetic acid (PAA, CH₃CO-COOH), a disinfectant not yet utilized for drinking water. These humic acid solutions were studied using genotoxicity plant tests: the chromosomal aberration test in Allium cepa root cells, and the micronucleus test performed in Tradescantia pollen mother cells and in Allium cepa and Vicia faba root cells (Figure 1).

Materials and Methods

Sample preparation

Stock solutions of humic acid (Sigma-Aldrich Corporation, St. Louis, MO, USA) were prepared as follows: two solutions of humic acid, 12.5 mg/L and 37.5 mg/L in distilled water, were stirred for 1 h at room temperature. Each litre of solution was diluted to 2.5 litres with distilled water and stored overnight at 4°C. These solutions were prepared to obtain 2.5 mg/L and 7.5 mg/L TOC concentrations simulating a ground water and a surface water, respectively (according to Agarwal and Neton, with some modifications). A solution of commercial sodium hypochlorite (14.65%) used for drinking water treatment was obtained from Solvay Chimica Italia, S.p.A. (Rosignano, LI, Italy). A chlorine dioxide solution (0.23 g/L) was prepared by passing in distilled water a flow of ClO₂ produced in a generator provided by Sanipur S.r.l. (Brescia, Italy). A peracetic acid solution containing 15.20% of PAA in equilibrium with hydrogen peroxide (15.20%) was used (Promox S.r.l., Leggiano, VA, Italy). Humic acid solutions with low and high TOC values were treated with 2.5 and 7.5 mg/L, respectively, of each disinfectant to attain a C:disinfectant molar ratio of 1:1.

The disinfectant residues were monitored in the solutions using colorimetric methods. NaClO concentrations were measured as free dissolved chlorine using a DR 2000 Hach photometer (Hach Company, Loveland, CO, USA) at 530 nm (Hach 14070/99 method, adapted from Standard Methods, 1998, CI G 4500). ClO₂ was determined by the N,N-diethyl-p-phenylendiamine (DPD) method at 575 nm (Hach 22423/00 method, adapted from Standard Methods, 1998, CI G 4500). PAA concentrations were measured as free chlorine using a DR 2000 Hach photometer (Hach Company, Loveland, CO, USA) at 530 nm (Hach 14070/99 method, adapted from Standard Methods, 1998, CI G 4500). PAA calibration curves were obtained by comparing the DPD absorbance of known concentrations of PAA. The measures were performed using a Hach DR 2000 photometer after every hour, 10 times, to detect the trend of the disinfection process. Another measure was performed 24 h after the beginning of the treatment. The sample solutions were stored at 4°C until the genotoxicity plant tests were carried out.

Sample solutions were used also for AOX (Adsorbable Organic Halogens) analysis using the HPLC method. AOX value is a measurement used to estimate the total quantity of dissolved halogenated organic material in a water sample. The presence of halogenated organic molecules is indicative of disinfection by-products.

Plant genotoxicity tests

Allium cepa tests

In a preliminary assay, equal-sized (2-2.5 cm in diameter) young bulbs of Allium cepa were exposed for 72 h in the dark to undiluted, 1:1 and 1:10 solutions, and root length was measured to determine the EC₅₀ (the concentration which gives a 50% reduction in root growth). Root length and other macroscopic parameters (durability, change in colour, root tip shape) were used as an index of toxicity. A solution of commercial sodium hypochlorite (NaClO) and chlorine dioxide (ClO₂) – and peracetic acid (PAA, CH₃CO-COOH), a disinfectant not yet utilized for drinking water. These humic acid solutions were studied using genotoxicity plant tests: the chromosomal aberration test in Allium cepa root cells, and the micronucleus test performed in Tradescantia pollen mother cells and in Allium cepa and Vicia faba root cells (Figure 1).

![Figure 1. Scheme of the research.](https://example.com/f1.png)
(MCN) were counted from five slides for each experimental group. Over 1500 tetrads were scored for each sample. The data were expressed as MCN/100 tetrads (mean±standard deviation) and analyzed for significance using analysis of variance and Dunnett’s t-test. Negative control was carried out using distilled water. Two humic acid solutions with different TOC concentrations were also used as negative controls. A positive control with 5 mg/L of maleic hydrazide was performed concurrently.

**Vicia faba** micronucleus test

Micronucleus test in *Vicia faba* (*Vicia faba/MCN*) root tips was performed according to standard protocols. After germination of *Vicia faba* seeds in Hoagland’s solution, primary roots were removed for a faster secondary root production, which were then exposed in the dark to the treatment solutions (1:10 dilution in Hoagland’s solution). Two exposure times were studied: a short interval of 6 h, followed by 66 h recovery in fresh Hoagland’s solution, and 72 h exposure, until fixation. At the end of treatment, secondary roots were removed and fixed in 1:3 acetic acid-ethanol mixture. Hoagland’s salt solution and two humic acid solutions with different TOC concentrations were used as negative controls. Positive control treatment was also performed using maleic hydrazide 10^{-4} M (11.2 mg/L) in Hoagland’s solution (4 h treatment + 44 h recovery).

Feulgen staining of the roots was performed and the cut tips were squashed onto slides. The mitotic index was estimated on 1000 cells/tip and the frequency of micronuclei was determined in 5x10^4 cells per sample (mean±standard error), analysing 5000 cells per root tip, 2 secondary roots per plant, and 5 plants per experimental point (10 root tips/experimental point, 5x10^4 cells/point). Statistical analysis of the data was carried out by means of the Mann-Whitney non-parametric test. The Kruskal-Wallis non-parametric ANOVA was also used for control comparison purposes.

**Results**

**Chemical analyses**

The results of the chemical analyses for AOX detection carried out on the samples of undiluted humic acids are shown in Figure 2. In the presence of 7.5 mg/L TOC the AOX values are always higher than 2.5 mg/L TOC, particularly for NaClO treatment (220 µg/L). It is noteworthy that the highest AOX values are detected in NaClO and PAA treated humic acid solutions, at both TOC concentrations.

**Plant genotoxicity tests**

*Allium cepa* tests

The results of the preliminary toxicity test in *Allium cepa* carried out on undiluted and diluted (1:1 and 1:10) solutions of humic acid showed very high toxicity in the undiluted solution, therefore the plant genotoxicity tests were performed only at 1:1 and 1:10 diluted humic acid solutions. Exposures to 1:1 and 1:10 humic acid dilutions were carried out for 3, 6 and 24 h.

The results of the *Allium cepa* test on the 1:1 solutions of humic acid are shown in Table 1. Microscopic evaluation of *Allium cepa* roots was not possible due to the presence of toxicity that hides mutagenic activity in bulbs exposed for 24 h to the 1:1 dilution; therefore no data are reported for this experimental point. After 3 h of exposure chromosomal aberrations in *Allium* root cells were higher compared to the negative control for all samples, the untreated humic acid solutions includ-

![Figure 2. Chemical analyses (AOX) performed on undiluted samples of humic acids (HA) and disinfected humic acids.](image-url)

Table 1. Mitotic index, anaphase aberrations and micronuclei in *Allium cepa* root tips exposed for 3 and 6 h to solutions of treated and untreated humic acids diluted 1:1.

<table>
<thead>
<tr>
<th>Samples</th>
<th>1:1 dilution</th>
<th>6 h exposure time</th>
<th>MCN</th>
<th>Mitotic index</th>
<th>Anaphase aberrations (%)</th>
<th>6 h exposure time</th>
<th>MCN</th>
<th>Mitotic index</th>
<th>Anaphase aberrations (%)</th>
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<tbody>
<tr>
<td>Negative control</td>
<td>12.1</td>
<td>4.4</td>
<td>0.8±0.8</td>
<td>11.0</td>
<td>2.8</td>
<td>10.8±1.2</td>
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<tr>
<td>TOC 2.5 mg/L</td>
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<tr>
<td>Humic acids</td>
<td>9.9</td>
<td>8.3**</td>
<td>1.2±1.3</td>
<td>7.6*</td>
<td>34.3***</td>
<td>2.4±3.4</td>
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<td></td>
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<tr>
<td>Humic acids+ClO₂</td>
<td>9.1</td>
<td>7.4***</td>
<td>1.0±1.2</td>
<td>8.8</td>
<td>40.5*** °°°</td>
<td>1.6±1.5</td>
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<td>Humic acids+NaClO</td>
<td>10.1</td>
<td>14.2*** °°°</td>
<td>0.8±0.8</td>
<td>8.0*</td>
<td>57.1*** °°°</td>
<td>6.2±4.0*</td>
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<td>Humic acids+PAA</td>
<td>9.8</td>
<td>10.2***</td>
<td>0.2±0.4</td>
<td>8.9</td>
<td>68.4*** °°°</td>
<td>3.2±4.7</td>
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<td>TOC 7.5 mg/L</td>
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<tr>
<td>Humic acids</td>
<td>9.5</td>
<td>7.2*</td>
<td>0.6±0.9</td>
<td>10.3</td>
<td>42.5***</td>
<td>2.8±0.8*</td>
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<td>Humic acids+ClO₂</td>
<td>9.3</td>
<td>7.5**</td>
<td>1.2±1.6</td>
<td>9.3</td>
<td>42.2***</td>
<td>1.4±1.1</td>
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<tr>
<td>Humic acids+NaClO</td>
<td>9.5</td>
<td>15.2*** °°°</td>
<td>1.2±1.1</td>
<td>9.4</td>
<td>50.0***</td>
<td>1.6±1.1</td>
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<tr>
<td>Humic acids+PAA</td>
<td>9.8</td>
<td>13.8*** °°°</td>
<td>0.8±1.3</td>
<td>8.1</td>
<td>52.0***</td>
<td>1.6±1.5</td>
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</table>

*P<0.05 statistically significant vs negative control; **P<0.01 statistically significant vs negative control; ***P<0.001 statistically significant vs negative control; °°°P<0.001 statistically significant vs humic acid; positive control: maleic hydrazide (10 mg/L) 1.3% of anaphase aberrations and 7.5±2.1 of micronuclei.
ed. Moreover, NaClO treatment with 2.5 mg/L TOC and NaClO and PAA treatments with 7.5 mg/L TOC induced an increase in mutations compared to humic acid without disinfectant. When using a longer exposure time (6 h), genotoxic damage was much greater but the reduction in mitotic index indicated a strong toxicity, particularly for 2.5 mg/L TOC samples. For this reason the genotoxicity data may be invalidated. On the other hand, no significant MCN increase is detected from any disinfectant-treated humic acid solution, apart from that induced by NaClO treatment, but only for humic acid solution with 2.5 mg/L TOC. In 7.5 mg/L solution a mild increase in MCN was only seen in humic acid solution without disinfectant.

Actually, a consistent MCN increase is the expected consequence after the dramatic mutagenic effect as that evidenced by the anaphase aberration test. Indeed, the clear cytotoxic effect observed in Allium roots after 3 and 6 h exposure in all the tested humic acid solutions did not allow cell cycle progression, thereby preventing the production of micronucleated daughter cells. Therefore the Allium test was repeated after 1:10 dilution of all test solutions.

Table 2 shows the results of the Allium cepa test on the 1:10 dilution of humic acid after 6 and 24 h of exposure. The results showed that all the disinfected samples were positive vs. negative control (undisinfect- ed humic acid solution) after 24 h of exposure: both humic acid solutions (TOC 2.5 mg/L and TOC 7.5 mg/L) treated with ClO2 induced a significant increase in MCN frequency, whereas NaClO- and PAA-treatments induced anaphase aberrations.

Several samples (disinfected and undisinfected) induced mutations compared to the negative control.

**Tradescantia micronucleus test**

The results of the Trad/MCN test are set out in Table 3. Genotoxic effects were found for the solution of humic acid with TOC 2.5 mg/L treated with NaClO and PAA after 6-h exposure to dilutions 1:1 and 1:10, respectively. The solution with higher TOC disinfectected with NaClO induced a very high level of MCN frequency (dilution 1:1). ClO2 did not induce any genotoxicity in this organism. A slight MCN increase, yet significant at the 1:10 dilution, is registered for untreated humic acid solution with TOC 7.5 mg/L.

**Allium cepa micronucleus test**

Mitotic index values ranged from 7.23±0.85% to 9.32±0.28% (mean±standard error) for the treated samples and controls (data not shown). A statistical analysis of these data (Kruskal-Wallis non-parametric ANOVA) indicated no alterations in the proliferating activity of the roots of the controls and treated groups at the same pH (data not shown).

Significant MCN increases are detected in roots exposed to all the NaClO treatment solutions compared to both Hoagland solution (negative control) and the corresponding humic acid concentration, at both exposure times. ClO2 also induces a significant micronucleus increase, but only at 6 + 66 h of exposure to both solutions (2.5 and 7.5 TOC) (Table 4).

The MCN frequencies observed after 6 h exposure plus 66 h recovery time were almost always higher than those detected after 72 h exposure. Analysis of micronucleus induction in control roots indicated that both humic acid concentrations had no significant effect on Vicia faba micronucleus frequency.

**Conclusions**

The aim of this research was to study the formation of genotoxic, and potentially carcinogenic, agents in the products of reaction between commercial humic substances and three disinfectants for drinking water, by means of in vivo short-term plant genotoxicity tests, and to compare the effects of two widely used disinfectants, ClO2 and NaClO, with a new disinfectant, PAA, a potent antimicrobial agent, with many applications in hospitals, laboratories, factories, and wastewater disinfection, but not yet used for drinking water.

All the tested disinfectants induced a clastogenic/aneugenic effect in plant cells, even in the presence of concentrations of organic substances similar to those frequently present in drinking water. All disinfectants determined genotoxic effects in Allium cepa tests. In particular, NaClO- and PAA-treated water samples induced chromosomal aberrations, while ClO2, induced mainly MCN in Allium cepa. Furthermore, NaClO treatment induced MCN increase in Tradescantia pollen cells and also Vicia faba root cells. Besides, PAA induced MCN in

Table 2. Mitotic index, anaphase aberrations and micronuclei in Allium cepa root tips exposed for 6 and 24 h to solutions of treated and untreated humic acids diluted 1:10.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mitotic index (%)</th>
<th>1:10 dilution 6 h exposure time</th>
<th>Anaphase aberrations (%)</th>
<th>MCN mean±SD</th>
<th>Mitotic index (%)</th>
<th>1:10 dilution 24 h exposure time</th>
<th>Anaphase aberrations (%)</th>
<th>MCN mean±SD</th>
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<tbody>
<tr>
<td>Negative control</td>
<td>10.6</td>
<td>2.1</td>
<td>0.8±0.8</td>
<td>12.1</td>
<td>4.7</td>
<td>0.8±0.8</td>
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<tr>
<td>TOC 2.5 mg/L</td>
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<tr>
<td>Humic acids</td>
<td>8.8</td>
<td>5.4***</td>
<td>0.4±0.5</td>
<td>9.5</td>
<td>4.7</td>
<td>1.2±1.6</td>
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<tr>
<td>Humic acids+ClO2</td>
<td>10.3</td>
<td>2.6</td>
<td>0.4±0.5</td>
<td>10.9</td>
<td>4.1</td>
<td>6.2±1.9*** °°°</td>
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<tr>
<td>Humic acids+NaClO</td>
<td>9.4</td>
<td>3.5</td>
<td>0.4±0.5</td>
<td>9.3</td>
<td>12.9*** °°°</td>
<td>0.2±0.4</td>
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<tr>
<td>Humic acids+PAA</td>
<td>9.3</td>
<td>4.4**</td>
<td>0.2±0.4</td>
<td>7.8**</td>
<td>8.9*** °°°</td>
<td>0.6±1.3</td>
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<td>TOC 7.5 mg/L</td>
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<td>Humic acids</td>
<td>10.4</td>
<td>3.8*</td>
<td>2.8±0.8*</td>
<td>10.7</td>
<td>4.0</td>
<td>1.2±1.6</td>
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<tr>
<td>Humic acids+ClO2</td>
<td>11.4</td>
<td>4.7**</td>
<td>1.4±1.1</td>
<td>7.8***</td>
<td>5.1</td>
<td>6.2±1.9*** °°°</td>
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<td>Humic acids+NaClO</td>
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<td>9.6*** °°°</td>
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<td>Humic acids+PAA</td>
<td>8.9</td>
<td>5.3***</td>
<td>1.6±1.5</td>
<td>8.0***</td>
<td>10.7*** °°°</td>
<td>0.6±1.3</td>
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</table>

*P<0.05 statistically significant vs negative control; **P<0.01 statistically significant vs negative control; ***P<0.001 statistically significant vs negative control; °P<0.05 statistically significant vs humic acid; **°P<0.01 statistically significant vs humic acid; °°P<0.001 statistically significant vs humic acid; positive control: maleic hydrazide (10 mg/L) 5.9% of anaphase aberrations and 7.5±2.1 of micronuclei.
Humic acids + NaClO 4.9±0.96** ° 3.8±0.71* °
Humic acids + PAA 2.8±0.51 2.6±0.52
Humic acids + ClO2 3.4±0.43* 3.5±0.90
Humic acids + ClO2 3.2±0.49* 2.5±0.67

Samples 1:1 dilution 1:10 dilution
Negative control 5.7±1.3 2.9±1.3
TOC 2.5 mg/L
Humic acids 6.5±1.7 4.7±0.4
Humic acids + ClO2 6.4±2.1 5.9±2.0*
Humic acids + NaClO 8.2±1.8 8.4±1.9*** °
Humic acids + PAA 11.3±4.1*** ° 5.8±3.1
TOC 7.5 mg/L
Humic acids 6.6±2.7 7.7±4.6 *
Humic acids + ClO2 6.5±1.9 10.9±4.5** *
Humic acids + NaClO 20.2±12.5** * 4.6±1.7
Humic acids + PAA 11.9±7.8 6.0±3.5

*P<0.05 statistically significant vs negative control according to Dunnett’s test; **P<0.01 statistically significant vs negative control according to Dunnett’s test; ***P<0.001 statistically significant vs negative control according to Dunnett’s test; °P<0.05 statistically significant vs humic acid according to Dunnett’s test; positive control: maleic hydrazide (5 mg/L) 15.4±2.8 of micronuclei.

Table 4. Mean micronucleus frequencies ±SE (per 5000 cells) in Tradescantia inflorescences exposed for 6 h to solutions of treated and untreated humic acids diluted 1:1 and 1:10.

<table>
<thead>
<tr>
<th>Samples</th>
<th>I:1 dilution</th>
<th>I:10 dilution</th>
</tr>
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<tbody>
<tr>
<td>Negative control</td>
<td>5.7±1.3</td>
<td>2.9±1.3</td>
</tr>
<tr>
<td>TOC 2.5 mg/L</td>
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<tr>
<td>Humic acids</td>
<td>6.5±1.7</td>
<td>4.7±0.4</td>
</tr>
<tr>
<td>Humic acids + ClO2</td>
<td>6.4±2.1</td>
<td>5.9±2.0*</td>
</tr>
<tr>
<td>Humic acids + NaClO</td>
<td>8.2±1.8</td>
<td>8.4±1.9*** °</td>
</tr>
<tr>
<td>Humic acids + PAA</td>
<td>11.3±4.1*** °</td>
<td>5.8±3.1</td>
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<tr>
<td>TOC 7.5 mg/L</td>
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<tr>
<td>Humic acids</td>
<td>6.6±2.7</td>
<td>7.7±4.6 *</td>
</tr>
<tr>
<td>Humic acids + ClO2</td>
<td>6.5±1.9</td>
<td>10.9±4.5** *</td>
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<td>Humic acids + NaClO</td>
<td>20.2±12.5** *</td>
<td>4.6±1.7</td>
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<td>Humic acids + PAA</td>
<td>11.9±7.8</td>
<td>6.0±3.5</td>
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adopt effective strategies for reducing genotoxic compounds in disinfected drinking water.

In conclusion, in agreement with previous studies, NaClO in particular, but also ClO₂ and PAA, was genotoxic in plants and these tests were useful for evaluating the mutagenicity of different drinking water disinfectants using humic acid solutions as a study model.

References


