



Phytotoxicity of trihalomethanes and trichloroacetic acid on *Vigna radiata* and *Allium cepa* plant models

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Abstract

Disinfection by-products (DBPs) are a concern due to their presence in chlorinated wastewater, sewage treatment plant discharge, and surface water, and their potential for environmental toxicity. Despite some attention to their ecotoxicity, little is known about the phytotoxicity of DBPs. This study aimed to evaluate the individual and combined phytotoxicity of four trihalomethanes (THMs: trichloromethane (TCM), bromodichloromethane (BDCM), dibromochloromethane (DBCM), and tribromomethane (TBM) and their mixture (THM4)), and trichloroacetic acid (TCAA) using genotoxic and cytotoxic assays. The analysis included seed germination tests using *Vigna radiata* and root growth tests, mitosis studies, oxidative stress response, chromosomal aberrations (CA), and DNA laddering using *Allium cepa*. The results showed a progressive increase in root growth inhibition for both plant species as the concentration of DBPs increased. High concentrations of mixtures of four THMs resulted in significant ($p < 0.05$) antagonistic interactions. The effective concentration (EC_{50}) value for *V. radiata* was 5655, 3145, 2690, 1465, 3570, and 725 mg/L for TCM, BDCM, DBCM, TBM, THM4, and TCAA, respectively. For *A. cepa*, the EC_{50} for the same contaminants was 700, 400, 350, 250, 450, and 105 mg/L, respectively. DBP cytotoxicity was observed through CAs, including C-metaphase, unseparated anaphase, lagging chromosome, sticky metaphase, and bridging. Mitotic depression (MD) increased with dose, reaching up to 54.4% for TCAA (50–500 mg/L). The electrophoresis assay showed DNA fragmentation and shearing, suggesting genotoxicity for some DBPs. The order of phytotoxicity for the tested DBPs was TCAA > TBM > DBCM > BDCM > THM4 > TCM. These findings underscore the need for further research on the phytotoxicity of DBPs, especially given their common use in agricultural practices such as irrigation and the use of sludge as manure.

Keywords Contaminant exposure · DNA laddering · Ecotoxicity · Environmental contaminants · Genotoxin · Oxidative stress · Plant stressor · Toxins

Introduction

Reuse of treated wastewater for irrigation is a common practice. Wastewater is often disinfected with chlorine before it is discharged into surface water bodies or land for irrigation. However, chlorination generates disinfection by-products (DBPs) due to the high organic concentrations in wastewater. The two primary groups of DBPs formed after chlorination are trihalomethanes (THMs) and haloacetic acids (HAAs). DBP concentration of 4.12 to 2337.2 $\mu\text{g/L}$ was observed in treated wastewater around the world (Heng et al. 2021; Albolafio et al. 2022; Wang et al. 2022a). The toxic effects of DBPs on microbes and humans have been extensively documented in previous literature, with some of these DBPs being proven or probable carcinogens (Kali et al. 2021; Kumar et al. 2023). Despite

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this, the impact of DBPs on plants and aquatic organisms has not been adequately studied.

Recently, the ecotoxicity of DBPs to aquatic organisms has become a concern due to the widespread use of chlorine for disinfection, particularly during the COVID-19 pandemic (Parveen et al. 2022a). Elevated levels of DBPs have been found in many surface water bodies after the widespread use of chlorinated disinfectants, leading to concerns about ecotoxicity (Chu et al. 2021; Liu et al. 2022). For instance, Cui et al. (2021) evaluated the toxicity of seventeen DBPs to freshwater organisms at three trophic levels and found that phytoplankton was more sensitive to HAAs, zooplankton was sensitive to THMs and haloacetonitriles, and DBPs caused abnormalities in the embryo development of *Danio rerio* (zebrafish). Similarly, Liu and Zhang (2014) reported a median effective concentration of 9.12 μM for 2,5-dibromohydroquinone (an emerging DBP) to cause toxicity in *Platynereis dumerilii*, a marine worm, and Gordon et al. (2015) found the median effective concentration of tribromoacetic acid to microalgae to be in the range of 194–240 mg/L (Liu and Zhang 2014; Gordon et al. 2015). These studies suggest that DBPs have the potential for acute toxicity to aquatic organisms.

Although the effects of DBPs on animals and algae have been studied, their impact on plants has been largely overlooked. Since chlorinated reclaimed water and DBP-containing surface water are commonly used for irrigation, the possibility of adverse effects on plants is high. Additionally, the use of chlorinated sewage sludge as fertilizer can also contribute to the problem. Ranjan et al. (2019, 2021) studied the physiological impacts of trichloromethane (TCM), tribromomethane (TBM), and trichloroacetic acid (TCAA) on plants and observed significant phytotoxic effects. Akande et al. (2016) found a decrease in biomass gain in *Solanum lycopersicum* (tomato) seedlings with higher degrees of bromination in THMs and an increase in oxidative damage and a decrease in essential nutrient concentration with higher degrees of chlorination in THMs. However, currently, there are no studies on the relative and combined effects of all four THMs (TCM, bromodichloromethane (BDCM), dibromochloromethane (DBCM), and TBM).

Since the 1940s, *A. cepa* and *V. radiata* have been utilized as model plant species in toxicity studies (Grant 1982). These species have gained popularity due to their year-round availability, cost-effectiveness, straightforward testing procedures, and ability to easily detect chromosomal aberrations (CA) owing to their size. *A. cepa* bulbs have been extensively used in genotoxic and cytotoxic assays since 1938. CA in *A. cepa* cells has become the primary indicator of cytotoxicity. Similarly, certain nuclear abnormalities and mitotic indices are utilized to evaluate cytotoxicity, while the presence or absence of micronuclei

serves to determine the mutagenicity of compounds (Leme and Marin-Morales 2009).

Seed germination tests are widely employed for evaluating phytotoxicity, particularly concerning inhibition of germination and root growth, caused by individual contaminants or multi-compound matrices such as compost. For instance, Lin and Xing (2007) utilized rapeseed, ryegrass, corn, lettuce, and cucumber seeds to investigate the phytotoxicity of zinc and aluminum nanoparticles, dispersed separately in deionized water (Lin and Xing 2007). Such an approach proves useful for understanding the toxicity of individual compounds. However, in the case of multi-compound matrices, evaluating the toxicity of each compound present in compost on plants is a time-consuming, expensive, and impractical process. Furthermore, assessing their combined effect on plants via individual compound analysis is not feasible. As a result, seed germination bioassays using these combined matrices have become the preferred solution for researchers (Luo et al. 2018). Therefore, in the present study, we assessed the phytotoxicity of four THMs individually and in combination, as well as of one HAA, using two plant model systems. The specific objectives were to (i) understand the impact of DBPs on seed germination using *V. radiata*, (ii) study the effect of DBPs on root growth using *V. radiata* and *A. cepa*, and (iii) investigate oxidative stress and abnormalities during mitosis in *A. cepa* roots due to exposure to DBPs.

Materials and methods

Experimental setup

Four THMs, namely, TCM, BDCM, DBCM, and TBM, along with one HAA, namely, TCAA were selected for the study. TCAA was selected as it is one of the most frequent HAA in water along with dichloroacetic acid (DCAA) (Albolafio et al. 2022; Carter et al. 2019; Dong et al. 2023) and the most frequent HAA in wastewater (Li et al. 2021). To prepare the treatment doses for the experiment, concentrations ranging from 100–10,000 mg/L of TCM, BDCM, DBCM, TBM, THM4 (a mixture of the four THMs in equal proportion), and TCAA were prepared in ultra-pure water. Although the highest doses of the sample treatment are unlikely to be found in the real world, they were utilized to assess the model plant species' tolerance to disinfection by-products (DBPs). Previous studies have employed similar doses, as described by Ranjan et al. (2021). To estimate variability in test results, each treatment dose was replicated three times.

Vigna radiata

Locally available *V. radiata* (green grams or mung beans) seeds were washed with 0.1% sodium hypochlorite (NaOCl; Merck Life Sciences) solution followed by rinsing with ultra-pure water. The germination protocol was based on past studies that employed *V. radiata* for bioassays (Banerjee et al. 2014; Aziz et al. 2020; Ranjan et al. 2021; Vanapalli et al. 2021). To initiate the germination process, 18 *V. radiata* seeds were evenly spaced between two germination sheets (20 cm × 30 cm) and wetted with equal volumes of treatment doses (0 – 10,000 mg/L). These sheets were then rolled and placed in a dark incubator at 25°C for five days. Control sheets were prepared in the same manner but without DBP dosing, utilizing ultra-pure water. Both control and treatment sheets were triplicated to ensure accuracy. To maintain adequate moisture levels, the sheets were rewetted every other day. After five days, the lengths of the roots were measured using thread and scale. The seed radicles were weighed after being dried for 48 h in an oven at 80°C.

Allium cepa

A. cepa (red onion) bulbs were collected from local vendors and cleaned with water and 0.1% NaOCl like that in the case of *V. radiata*. They were sprouted in ultra-pure water for 24 h, then exposed to treatment doses of 0 – 1000 mg/L of the same DBPs used in the *V. radiata* experiments for 5 days. The outer shell of the onion bulb was removed as they are highly antioxidants and can limit the toxic effects and change the properties of the germination mixture (Sidhu et al. 2019; Kaya et al. 2023; Vujčić Bok et al. 2023). Control samples were treated with ultra-pure water. The treatment doses and protocols followed those of previous studies (Kumari et al. 2009; Banerjee et al. 2014; Ranjan et al. 2019; Aziz et al. 2020). Each dose was tested with three bulbs, and the treatment solution was replaced daily to maintain sufficient DBP concentration. After 5 days of exposure, root lengths were measured using thread and scale, and the samples were prepared for further analysis.

Analytical methods

Phytotoxicity

Various growth indices and effective concentration at 50% root length inhibition (EC₅₀) were calculated using the measured root lengths of *A. cepa* and *V. radiata*. Tolerance index (TI), relative seed germination (RSG), relative radicle growth (RRG), and germination index (GI) were calculated for *V. radiata*, and EC₅₀ was determined for both species as follows (Luo et al. 2018; Sardar et al. 2022).

$$TI(\%) = \frac{\text{dry weight of treated seeds}}{\text{dry weight of control seeds}} \times 100 \quad (1)$$

$$RSG(\%) = \frac{\text{no. of germinated seeds in treatment}}{\text{no. of germinated seeds in control}} \times 100 \quad (2)$$

$$RRG(\%) = \frac{\text{Radicle length of germinated seeds in treatment}}{\text{Radicle length of germinated seeds in control}} \times 100 \quad (3)$$

$$GI(\%) = RSG \times RRG \times 100 \quad (4)$$

Mitotic index and abnormalities

A. cepa root tips harvested on the 5th day of growth were firm in glacial acetic acid and ethanol (Merck Life Sciences) solution (1:3) for at least 3 h and subsequently were stored at 4°C (Nefic et al. 2013; Sabeen et al. 2020). Six root tips (two from triplicate bulbs) were used per treatment dose and control. Fixed root tips were hydrolyzed in 1N hydrochloric acid (Merck Life Sciences) for 20 min at 60°C prior to squashing. The squash method prescribed by (Sharma and Sharma 1980) was adopted after staining the root tips with 10% acetocarmine for 30 min. Temporary microscopic slides were prepared by sealing the squashed cells with clear nail polish and they were observed under the microscope (× 100). A total of 2500 cells were counted for mitotic endpoint evaluation and the cells with various chromosomal and cytological abnormalities were noted and photographed.

The mitotic index (MI) is characteristic of the total number of dividing cells in the cell cycle. Therefore, it has been an indicator of cell cytotoxicity by various agents. The DBP dosage for MI analysis was selected as 100–6000 mg/L (for THMs), and 10 – 1000 mg/L (for HAAs) based on the results of root growth analysis. MI values of *A. cepa* root cells were calculated using Eq. 5 and percentage aberrant cells were calculated in subjects with total no. of total cells (Sabeen et al. 2020).

$$MI(\%) = \frac{\text{No. of dividing cells}}{\text{total no. of cells}} \times 100 \quad (5)$$

DNA laddering

DNA laddering is a widely used assay for detecting apoptosis and genotoxicity caused by chemicals or pathogens (Ghosh et al. 2010, 2015; Petriccione et al. 2013). In DNA laddering assay, DNA fragments of varying sizes are separated based on their molecular weight by gel electrophoresis, with smaller fragments migrating farther than larger ones.

For analysis, *A. cepa* root tips were processed by cetyltrimethylammonium bromide (CTAB) method using a

plant DNA isolation kit (MB 502, Himedia). DNA sample preparation procedure followed previous studies (Ranjan et al. 2019, 2021). Root trips were ground using a mortar and pestle. 9 ml of prewarmed CTAB extraction buffer was added to 300–400 mg of the lyophilized ground tissue in a sterile centrifuge tube. The mixture was gently mixed by inversion. It was ensured that CTAB powder and β -mercaptoethanol were added to CTAB extraction buffer prior to use. The samples were incubated for 60–90 min at 65°C, with occasional inversion, and subsequently cooled at room temperature (15–25°C) for 5 min. Following this, 5 ml of chloroform: octanol (24:1) was added to each tube, and the samples were gently mixed by inverting the tubes for 5 min. The samples were then centrifuged at $850 \times g$ (≈ 3000 rpm) for 2 min at room temperature (15–25°C). The top aqueous layer was transferred into a fresh tube, and 25 μ l of RNase A (RNase A is added to remove RNA since the extract contains DNA and RNA; 20 mg/ml) was added. The sample was gently mixed by inversion and incubated for 30 min at room temperature. 6 ml of isopropanol was added to each tube, with gentle mixing until a white fluffy DNA precipitate appeared. Subsequently, the samples were centrifuged at $850 \times g$ (≈ 3000 rpm) for 5 min to pellet the DNA, and the supernatant was discarded. The pellet was resuspended in 8 ml of cold diluted CTAB wash buffer. The resuspended mixture was incubated at room temperature for 20 min and then centrifuged at $850 \times g$ (≈ 3000 rpm) for 5 min. To further purify the DNA, 8 ml of cold 70% ethanol was added to the tube containing DNA and centrifuged at $850 \times g$ (≈ 3000 rpm) for 5 min.

The DNA pellet was air-dried to eliminate any residual traces of ethanol. Finally, the DNA sample was transferred to a tube containing 1 ml of elution buffer, and the pellet was gently dissolved by pipetting (Southern 1975; Saghai-Marouf et al. 1984; Tai and Tanksley 1990; Williams et al. 1990; Guillemaut and Maréchal-Drouard 1992; Narzary et al. 2015; Gray et al. 2023; Sumi et al. 2023; You et al. 2023).

The extracted DNA samples were kept at -20°C till the electrophoresis analysis. Purity of the DNA samples was assessed by calculating the ratio of absorbance at 260 nm to 280 nm using a NanoDrop™ One/OneC Microvolume UV–Vis spectrophotometer. For loading, 1% agarose gel prepared in $1 \times$ tris acetate ethylenediaminetetraacetic acid (EDTA) buffer containing 2 μ l ethidium bromide (EtBr; 10mg/ml) was used. Electrophoresis was carried out at 100 V at room temperature for a duration of 45 min (Green and Sambrook 2012; Lee et al. 2012). A 1kb DNA ladder (ProxiO Ladder Plus) was loaded as reference. DNA ladders were visualised using a gel imaging system (Bio Rad ChemiGel Doc™ Touch).

Hydrogen peroxide content

Generation of hydrogen peroxide (H_2O_2) in plants is a symptom of oxidative stress due to cellular injury (Wahid et al. 2007). *A. cepa* roots (100 mg) were ground to paste in 0.1% trichloroacetic acid (Merck Life Sciences) in a water bath under cold conditions. Following Ranjan et al. (2019), this mixture was homogenized and centrifuged for 20 min at 10,000 rpm and 4°C. The supernatant (0.5 mL) was mixed with 1 mL 1M potassium iodide (Sigma Aldrich) and 0.5 ml phosphate buffer (SRL Chemicals) to maintain pH at 7. Absorption at 390 nm was determined using a spectrophotometer (Mortars India) and the H_2O_2 concentration was calculated by dividing the absorption value by the extinction coefficient of $0.28 \mu\text{m}^{-1} \cdot \text{cm}^{-1}$ (Singh et al. 2007).

Statistical analysis

All the data in the present research work were represented as mean \pm standard deviation (SD). Moreover, 2 sample t-test ($p < 0.05$) was performed to compare the mean values for determining the significance of the differences using Minitab 19.1.

Results and discussion

Germination and root growth inhibition

The presence of DBPs in the treatment water had a visible impact on the germination of *V. radiata* seeds. The length of the radicles in treated seeds was noticeably shorter, thinner, and, in some cases, appeared yellowish compared to control seeds (Fig. 1a-i). Furthermore, the roots of treated seeds were observed to be more folded than those of control seeds. Several treatments at different doses displayed the development of root hairs. At higher doses of DBPs, the epidermis (the outermost layer of the root) was appeared to be thicker than in the control group. This suggests that water uptake in the plant was hindered since the epidermis in healthy plants is thin and semipermeable to allow for the movement of water.

In the present study, *A. cepa* bulbs were initially placed in deionized water before being transferred to the desired DBP doses to assess changes in root growth compared to control bulbs. Similar to *V. radiata*, the roots of *A. cepa* also exhibited characteristics such as thickness, shortness, and discoloration at higher doses of DBPs (Fig. 1j). The root tips of *A. cepa* bulbs treated with higher DBP doses, especially of TBM, required additional heating time while preparing microscopic slides for mitosis analysis. Further, as the DBP doses increased, the root length of both plant



Fig. 1 **a** Root length of control *V. radiata* seed; **b** non-germinated *V. radiata* seed in treatment; **c** – **h** *V. radiata* seeds treated with 4000 mg/L of TCM, BDCM, DBCM, TBM, THM4, and TCAA; **i** Root length of *V. radiata* increasing dose of TCAA from 0 mg/L – 6000 mg/L; and **(j)** *A. cepa* roots at increasing dose of TCAA from 0 mg/L – 1000 mg/L

species tested was significantly reduced. At elevated doses, *A. cepa* exhibited the development of C-tumour roots and stunted root growth, similar to the response observed in *A. cepa* exposed to bilge water containing heavy metals, ammonia, and nitrates (Olorunfemi et al. 2015). Interestingly, individual THMs were found to have a greater impact on germination and root length than the THM mixture for both plant species treated. The seeds and bulbs treated with TCM and THM4 were similar in appearance.

Impact on germination of *V. radiata*

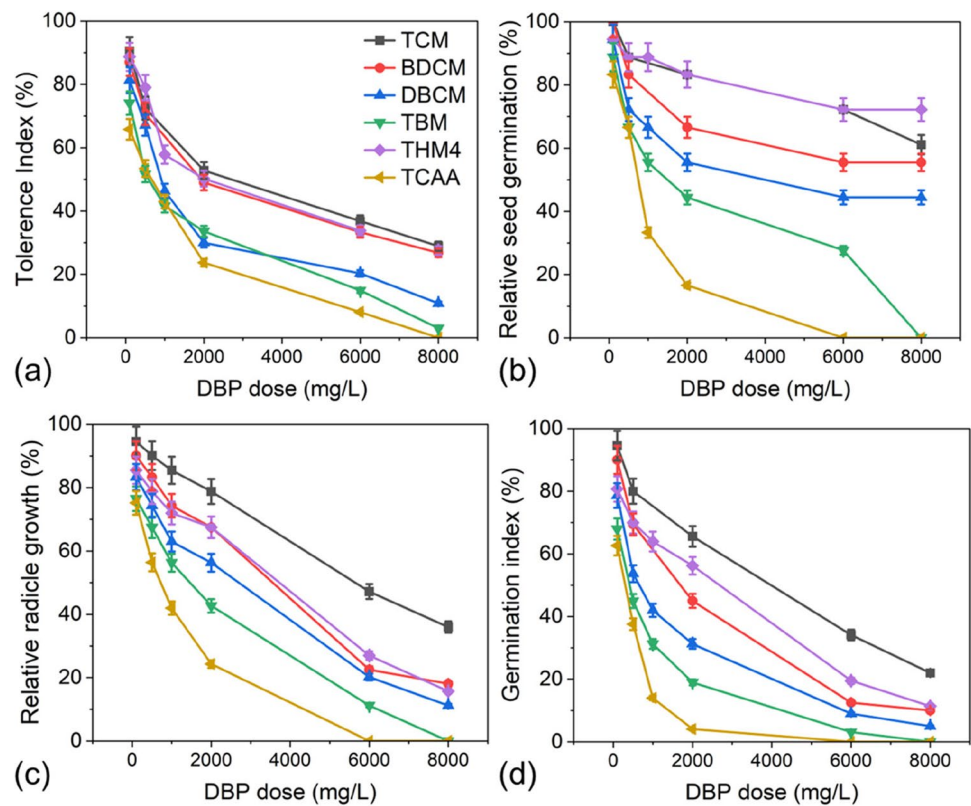
TI is the measure of tolerance of a plant towards a contaminant. It is important to note that the same plant species may exhibit different levels of tolerance to different contaminants based on their toxicity. In the case of *V. radiata* seed germination, it was evident that the tolerance varied for different DBPs. The order of TI for the various DBPs tested was TCM > THM4 > BDCM > DBCM > TBM > TCAA (Fig. 2).

The RSG, which indicates the relative number of seeds germinated in treatments compared to control, was evaluated. Control seeds showed an average of 18 ± 0.5 germinated seeds from triplicate sheets containing 20 seeds each. TCM and BDCM treatments at 100 mg/L showed similar no. of seed germination (Fig. 2). Notably, individual THMs showed lower RSGs than THM4 for the same dose. TCAA exhibited the highest toxicity to the seeds, with a significant decrease in RSG to 33.3% at a dose of 1000 mg/L, in contrast to other DBPs that maintained at least 50% relative germination at the same dose. The significant reduction in RSG observed for TCM and TBM beyond the 6000 mg/L threshold may be attributed to several factors, such as the potential toxicity exceeding the tolerance of the seeds, a depletion of oxygen in the reaction mixture due to the high toxin concentration, potential interference with enzymes in the seeds, or the formation of a barrier hindering water uptake due to toxin development (Bewley 1997). The drop in TI for TBM beyond 6000 mg/L aligns with this observation, indicating a sudden decrease in tolerance. However, it's worth noting that the TI of TCM did not exhibit a similar pattern. Consequently, the specific cause of the sharp decline in RSG for TCM warrants further investigation, and additional research is needed to confirm the validity of these potential reasons.

Since no pH adjustments were made to the treatment solutions, the TCAA solutions were acidic (pH ranging from 4.1 ± 0.5 to 6.5 ± 0.4) compared to the near-neutral or slightly alkaline pH (6.7 ± 0.4 to 8.3 ± 0.2) of the other DBPs. Acidic pH is known to negatively affect *V. radiata* seed germination (Kannan and Upreti 2008), and the adverse effects of TCAA on germination may be attributed to the low pH as well.

Among TI, RSG, RRG, and GI, GI is commonly used to evaluate the phytotoxicity of chemical compounds and composts (Wang et al. 2022b). Alternatively, some studies use RRG for the same application. However, since GI is a function of both RRG and RSG, it serves as a more comprehensive representation of phytotoxicity. Figure 2 presents the GI values for *V. radiata* seeds exposed to various DBPs. A higher GI value represents better germination and, hence, low toxicity. GI above 80% is indicative of a healthy plant environment with no phytotoxicity due to the exposed substances. In contrast, GI values below 80% indicate that the compound is phytotoxic to seed germination

Fig. 2 **a** Tolerance index, **b** relative seed germination, **c** relative radicle growth, and **(d)** germination index of *V. radiata* at varying DBP doses



and growth (Yuan et al. 2016; Luo et al. 2018). While initial doses of TCM, BDCM, and THM4 did not cause phytotoxicity, the GI decreased significantly with increasing DBP concentrations. Moreover, the GI values for DBCM, TBM, and TCAA were below 80% at the initial dose of 100 mg/L, suggesting that these compounds are phytotoxic to *V. radiata* seeds and adversely affect germination and growth. The order of GI for the tested DBPs was TCM > THM4 > BDCM > DBCM > TBM > TCAA, with TCAA, TBM, and DBCM being the three most phytotoxic DBPs. These results are consistent with those reported by Ranjan et al. (2021), who found that the order of phytotoxicity for the tested DBPs was TCAA > TBM > TCM. However, no other studies have investigated the phytotoxicity of BDCM, DBCM, and THM4 to date.

Studying the nature of interactions between contaminants and their impact on plant physiology is crucial as contaminants can induce stress in plants. The classification of these interactions is determined by the plant's response to multiple stressors, with interactions being categorized as either synergistic or antagonistic. If the combined effect of stressors is greater than the additive sum of the individual stressors acting independently, then the interaction is considered synergistic. Conversely, if the combined effect is less than the additive sum, then the interaction is considered antagonistic. The chemical interactions between

individual THMs and THM4 were analysed based on their GI values, which are reported in Table 1.

The observed GIs for THM4 values represent the combined effect, while the calculated GIs for THM4 values represent the arithmetic average of GIs for TCM, BDCM, DBCM, and TBM. Therefore, the calculated GIs values depict the phytotoxic effects of different THMs without considering the interactions between them. The arithmetic difference between the observed and calculated GIs for THMs was calculated to understand the interactive effects of individual THMs. A negative difference between the calculated and observed GIs for THMs depict synergistic interactions between the individual THMs, while the positive difference between them indicates antagonistic effects. Moreover, the statistical significance of the differences between the means of calculated and observed THM4 was evaluated through 2 sample t-test ($p < 0.05$) using Minitab 19.1. The statistical analysis was performed considering each GI value for the corresponding concentration of calculated and observed THM4 as an independent variable. At low tested concentrations (100, 500 mg/L), there was no significant difference between the mean values of observed and calculated THMs ($p > 0.05$), indicating no additional stress to the plants. However, as the THM dose increased, an antagonistic effect was observed with a significant difference between the means ($p < 0.05$), suggesting that at higher doses of mixtures,

Table 1 Synergistic or antagonistic effects of THMs on germination of *V. radiata*

Dose (mg/L)	Germination index (%)						
	TCM	BDCM	DBCM	TBM	Calculated THM4	Observed THM4	Difference
100	94.6±4.7	90.1±4.5	78.6±3.9	67.9±3.3	82.8±4.1	80.7±4	-2.1±0.1
500	80.0±4	69.4±3.4	53.6±2.6	45.0±2.2	62.0±3.1	70.0±3.4	+8.0±0.4
1000	73.3±3.6	55.0±2.7	42.0±2	31.2±1.5	50.4±2.5	63.9±3.1	+13.6±0.68*
2000	65.6±3.28	45.0±2.2	31.2±1.5	19.0±0.94	40.2±2	56.2±2.8	+16.0±0.8*
6000	34.1±1.7	12.5±0.62	9.0±0.44	3.1±0.15	14.7±0.7	19.5±0.97	+4.8±0.24*
8000	22.0±1.1	10.0±0.5	5.0±0.24	0	9.3±0.4	11.3±0.56	+2.1±0.1*

*Indicates a significant difference between the means of calculated and observed GIs of THMs (As per 2 sample t-test; $p < 0.05$)

THMs reduced each other's effects. With further increases in dosage to greater than 2000 mg/L, the rate of antagonistic effect significantly decreased, likely due to low germination at higher doses of individual brominated THMs. The statistical significance of these differences indicates the prominence of the synergistic or antagonistic phytotoxic effects of THMs at higher concentrations (≥ 1000 mg/L) on germination of *V. radiata*.

Plant stressors can either have antagonistic or synergistic effects and often interact with other abiotic and biotic factors. Understanding these interactions is gaining more attention nowadays. The use of transcriptomics, metagenomics, and metabolomics data has proven pivotal in addressing challenges in plant-stress interaction research (Nadeem et al. 2023). The results of a metadata analysis of 286 freshwater ecosystems for plant diversity and biomass revealed that the net effect of stressors was frequently more antagonistic (41%) than synergistic (28%), additive (16%), or reversed (15%) (Jackson et al. 2016). Similar to the findings of the current study, the interactions between two herbicides were observed to be antagonistic on plant health (Zhang et al. 1995). Further, the plant stressors that activate systemic acquired resistance (SAR; a potent innate immunity system in plants that provides protection against a broad range of pathogens) as well as many abiotic plant stressors were antagonistic than synergistic (Yasuda et al. 2008; Bansal et al. 2013).

Zandalinas and Mittler (2022) found that simultaneous or sequential abiotic and biotic stressors significantly impair plant growth, crop yields, and overall plant health. Interestingly, when multiple stressors affecting a plant increase in number and complexity, the plant's growth and survival decline significantly, even if each individual stressor is considered low impact. Studies on interactions between various plant stressors and nutrients have shown that the antagonistic effect of one stressor/nutrient on another can be stronger than the reverse in root absorption and transport within plants (Rietra et al. 2017; Aboyeji et al. 2020). For instance, the antagonistic effect of potassium (K) on magnesium (Mg) was stronger than that of Mg on K in higher plants (Xie et al.

2021). Therefore, further research is needed to understand the impacts of individual THMs on each other. Recently, biochemometric approaches, which combine the biochemistry of various substances with chemometric models, have been employed as effective tools for investigating synergy (Ma et al. 2018).

Root length inhibition and effective concentration

The concentration that causes a 50% reduction in root length, known as the EC_{50} , is typically expressed as mg/L of toxin. Figure 3 presents the EC_{50} of TCM, BDCM, DBCM, TBM, THM4, and TCAA for *V. radiata* and *A. cepa*. The order of EC_{50} was $TCM > THM4 > BDCM > DBCM > TBM > TCAA$ for both plant species. Plants can take up soluble toxic contaminants along with water at all stages of their growth cycle. If the plant fails to degrade the contaminant, it can adversely affect the plant directly and/or indirectly. During germination, direct effects can include disturbed water balance, inhibition of cytoplasmic enzymes, and damage to cell structure resulting from oxidative stress. Indirect effects can include the replacement of nutrients with toxins (Bansal 2018). Both direct and indirect effects can negatively impact the germination and growth process.

The lower EC_{50} values for *A. cepa* indicate relatively higher sensitivity to DBPs compared to *V. radiata*. This tendency was observed in two previous studies that assessed the EC_{50} of TCM, TBM, and TCAA on *A. cepa* and *V. radiata* roots (Ranjan et al. 2019, 2021). These studies reported that *A. cepa* was relatively more sensitive to these three DBPs by 37.5, 33.3, and 18.5 times, respectively.

As there is no available literature on the phytotoxicity of DBPs and their interactions with plant enzymes, heavy metal(loid)s were used as a reference to understand the direct and indirect effects of toxins on germination and root growth. The EC_{50} of lead, copper, arsenate (AsV), and cadmium were 924.8 mg/kg of soil (on *Hordeum vulgare*), 28.9 μ M (on *Zostera marina*), 18.8 μ M (on *Triticum aestivum*), and 2268.4 μ M (on *Zostera marina*), respectively (Liu et al. 2021; Li et al. 2022; Qin et al. 2022; Qiao et al. 2022).

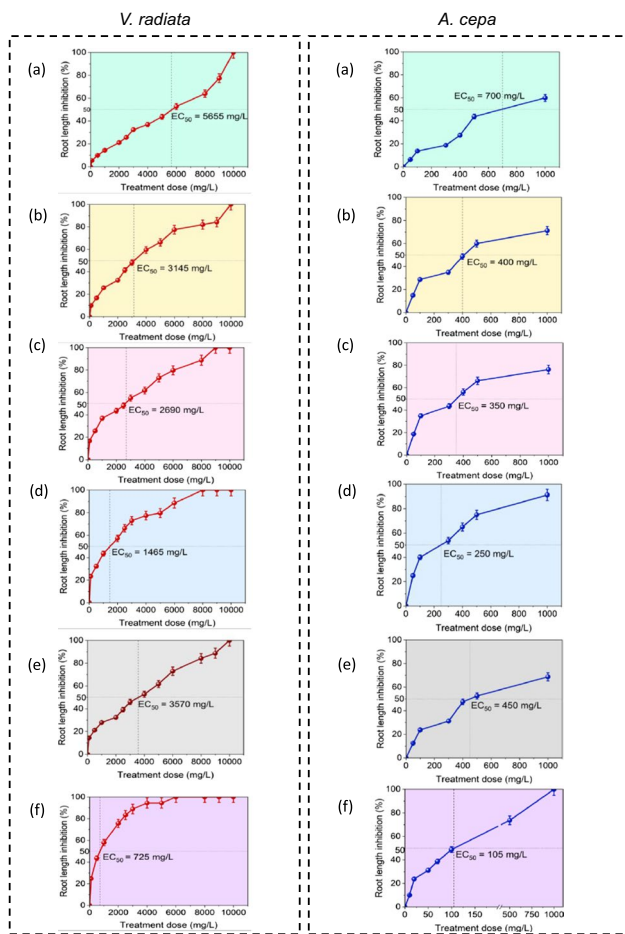


Fig. 3 EC_{50} of (a) TCM, b BDCM, c DBCM, d TBM, e THM4, and (f) TCAA on *V. radiata* and *A. cepa* root growth

Similarly, the EC_{50} of ionic silver (Ag^+) was 0.27 μM (on *Triticum aestivum*) and that of hydrogen sulphide was 500 μM (on *Arabidopsis*) (Zhang et al. 2017; Luo et al. 2021).

Previous studies reported an EC_{50} of 160 mg/L for TCM on *A. cepa* and 6000 mg/L on *V. radiata*. Similarly, TBM exhibited an EC_{50} of 120 mg/L on *A. cepa* and 4000 mg/L on *V. radiata* and TCAA impacted *A. cepa* at EC_{50} = 100 mg/L and *V. radiata* at EC_{50} = 1850 mg/L (Ranjan et al. 2021, 2019). Although heavy metals and metal ions have lower EC_{50} values compared to DBPs, indicating their higher toxicity, their toxin pathways were assumed to be similar. For example, *Elsholtzia argyi* and *Triticum aestivum* seeds and *A. cepa* roots exposed to lead (Pb) exhibited cell wall thickening, folds, protuberances, and nicks, similar to the observations in the current study (Fahr et al. 2013). Additionally, cytotoxicity and morphological effects were reported in *Oryza sativa* L. seedlings treated with arsenic (As) and cadmium (Cd), especially in lateral root development (Ronzan et al. 2018). Roots treated with As or Cd or both showed severe damage to the lateral root primordia

(the nascent developing unit of lateral roots) resulting in incorrect evolution of them to lateral roots, which was comparable to the irregular patterns of lateral roots observed in the current study, despite the difference in the root development period (10 days vs 5 days in the present study) and the plant species.

The phytotoxicity pathway of Pb involves its binding with the physical barriers of roots such as the cell wall or callose in higher plants, followed by the breaking of this barrier. At higher doses, Pb enters the cells through ion transport. Inside the cell, Pb forms complex compounds by bonding with phytochelatin, which are cysteine-rich peptides. These complexes are transported into the vacuoles, which are the main storage site for toxins in plants. Vacuoles in plant cells play an essential role in maintaining cell acidity and turgor pressure, regulating the storage and transport of substances, controlling the transport and localization of key proteins, and responding to biotic and abiotic stresses. Therefore, the presence of a toxin-bound compound in the vacuole can disrupt cell function. Since the effect of DBPs on *A. cepa* roots showed similar morphological and physical changes as Pb, the pathway of the toxin may follow the same stages.

Mitotic abnormalities to *Allium cepa*

Mitotic index and depression

The stained and sealed slides of *A. cepa* root tip cells from control and treatment groups were examined under a microscope to assess cell division. The control roots grown in ultra-pure water had a MI of $14.1 \pm 0.2\%$, which decreased significantly for the various DBP treatments (Table 2). A lower MI value suggests a slower rate of mitosis, and a significantly lower MI than that of the control indicates alterations in the growth and development of the organism due to the contaminant exposure. The MI of control *A. cepa* roots was reported to be 60.3% in tap water and 8.6%, 8.8%, and 7.3% in de-ionized water in previous studies, which is in accordance with the current study (Kumari et al. 2009; Ranjan et al. 2019; Kuloğlu et al. 2022; Macar et al. 2022).

The MI values for 6000 mg/L of TCM, BDCM, and THM4 were 64.5%, 65.9%, and 65.8% lower than the MI of control roots, respectively. Similarly, DBCM (4000 mg/L), TBM (4000 mg/L), and TCAA (500 mg/L) resulted in 68.5%, 72.5%, and 84.7% lower MI than that of the control MI, respectively. The cytotoxic effects of TCM and BDCM were similar, while DBCM and TBM exhibited much lower MI values than the control, suggesting that increased bromination increases cytotoxicity. This trend is similar to the cancer slope factors (CSF) of THMs that are derived from dose–response animal studies and epidemiology. As per the Integrated Risk Information System (IRIS) by US Environmental Protection Agency (USEPA), the oral CSF of

Table 2 Mitotic index and abnormalities due to various DBPs

Compound and dose (mg/L)	Number of dividing cells	Mitotic index (%)	Mitotic depression%	Chromosomal aberrations (%)	
Control	353 ± 5.4	14.1 ± 0.2	–	1.35 ± 0.11	
TCM	100	248.3 ± 5.3	9.9 ± 0.2	29.7	2.23 ± 0.08
	2000	199.1 ± 5.4	7.9 ± 0.2	43.6	3.88 ± 0.05
	6000	125.3 ± 1.8	5.0 ± 0.0	64.5	4.17 ± 0.07
BDCM	100	243.1 ± 1.9	9.7 ± 0.0	31.1	2.50 ± 0.07
	2000	181.1 ± 2.6	7.2 ± 0.1	48.7	3.98 ± 0.05
	6000	120.3 ± 2.8	4.8 ± 0.1	65.9	4.19 ± 0.06
DBCM	100	217.1 ± 4.3	8.6 ± 0.1	38.5	2.78 ± 0.06
	2000	153.1 ± 1.9	6.1 ± 0.1	56.6	4.10 ± 0.07
	4000	111.1 ± 2.1	4.2 ± 0.0	68.5	4.26 ± 0.07
TBM	100	178.5 ± 2.4	7.1 ± 0.0	49.4	3.46 ± 0.07
	2000	123.0 ± 1.7	4.9 ± 0.0	65.1	4.22 ± 0.07
	4000	97.0 ± 1.26	3.8 ± 0.1	72.5	3.70 ± 0.04
THM4	100	252.0 ± 1.41	10.1 ± 0.05	28.7	2.18 ± 0.08
	2000	191.6 ± 1.63	7.67 ± 0.06	45.7	3.44 ± 0.09
	6000	120.8 ± 2.2	4.83 ± 0.08	65.8	4.10 ± 0.07
TCAA	50	161.0 ± 1.78	6.44 ± 0.07	54.4	3.06 ± 0.07
	100	108.5 ± 2.4	4.34 ± 0.09	69.2	3.51 ± 0.09
	500	53.8 ± 2.92	2.15 ± 0.11	84.7	1.88 ± 0.05

TCM, BDCM, DBCM, and TBM are 1×10^{-02} , 6.21×10^{-02} , 8.4×10^{-02} , and 7.9×10^{-03} (mg/kg-day)⁻¹, respectively (Parveen et al. 2022b). The corresponding value for TCAA is 7.2×10^{-02} (mg/kg-day)⁻¹. Although TCAA has a higher CSF than TBM, the drastic change in pH due to the introduction of TCAA may contribute to its higher phytotoxicity.

Mitotic inhibition or depression (MD) is a valuable indicator of cytotoxicity, providing insights into the extent to which a toxin slows down mitosis in treated roots compared to untreated ones. The mathematical formula for MD is (MI of control—MI of treatment) / MI of control, expressed as a percentage (Alaguprathana et al. 2022). The MD of DBPs was observed to increase with dose (Table 2). Notably, the MD of BDCM was higher than that of TCM for the same increase in dose from 100 mg/L to 2000 mg/L. However, with a further increase from 2000 mg/L to 6000 mg/L, TCM-treated roots showed higher inhibition than BDCM-treated ones. In addition, the same increase in DBCM and TBM dose resulted in slightly higher inhibition of DBCM than TBM. This observation is supported by the MD of these two compounds, where DBCM exhibited a 38.5% to 56.6% increase in inhibition from 100 mg/L to 2000 mg/L (an 18.1% increase), while TBM showed a 49.8% to 65.1% increase in inhibition for the same dosage (a 15.7% increase). It is worth noting that the MD of TCAA was the highest of all DBPs, with even lower doses of TCAA than THMs resulting in the highest MD among all.

Previous research reported low MI for *A. cepa* root cells at lower dose of TCM (2.64 ± 0.006 at 800 mg/L), TBM

(4.92 ± 1.08 at 400 mg/L), and TCAA (2.48 ± 0.01 at 200 mg/L) than what observed in the current study (Ranjan et al. 2019). The difference in MI between the previous and current studies suggest further exploration on this topic. Based on the MI values observed for several other environmental pollutants such as herbicides like quizalofop-p-ethyl (MI = 9.6 ± 0.6 at 15,000 mg/L, control = 30.2 ± 1.8), cycloxydim (MI = 15.9 ± 0.3 at 15,000 mg/L, control = 34.1 ± 1.9), and atrazine at 3 mg/L (MI showed no significant difference from the control cells), di(2-ethylhexyl)phthalate (MI = 68.47 ± 13.00 at ≈ 200 mg/L, control = 100.00), triclosan (MI = 1.46 ± 20.14 at ≈ 150 mg/L, control = 100.00), propylparaben (MI = 43.50 ± 13.08 at ≈ 6 mg/L, control = 100.00), bilge water containing various heavy metals, ammonia, and nitrate (MI ≈ 2 at 50% volume of bilge water, control ≈ 14), and heavy metals such as chromium (MI = 36.66 ± 1.44), copper (MI = 46.66 ± 1.44), lead (MI = 32.50 ± 0.00), and zinc (MI = 50.00 ± 0.00) at 200 mg/L (control = 60.00), indicates that the toxicity of DBPs is similar to certain herbicides and notably lower than that of heavy metals (Herrero et al. 2012; Olorunfemi et al. 2015; Abubacker and Sathya 2017; Silveira et al. 2017; Rosculete et al. 2019).

Chromosomal aberrations

The cell cycle comprises various stages such as interphase, prophase, metaphase, anaphase, and telophase. Interphase involves growth and DNA replication, which is followed

by prophase which initiates mitosis with condensed chromosomes, metaphase aligns chromosomes for cytogenesis and toxicity assessments, anaphase sees chromatids split and move to poles, and telophase clusters chromosomes, forms nuclear membranes, and initiates cytokinesis, yielding two identical cells (Boden and Ziebold 2006; Qi and Zhang 2020). The chromosomes temporarily stop moving at metaphase and only correctly aligned chromosomes enter the next stage, anaphase (Registre and Proudlock 2016). At anaphase, the non-aligned chromosomes fail to get separated properly and end up in various aberrations (Potapova and Gorbisky 2017). The normal stages of cell division in control onion cells are displayed in Fig. 4a.

Any alterations in the normal stages of mitosis are considered CA. The extent of CA was determined for *A. cepa* roots treated with various DBPs and control roots as a percentage of abnormal cells to the total number of cells counted.

$$CA(\%) = \frac{\text{no. of aberrated cells}}{\text{total no. of cells}} \quad (6)$$

In control roots, CA was relatively low compared to treated roots (Table 2). Most number of the CAs observed were in metaphase followed by anaphase and telophase. Different CAs included C—metaphase, sticky metaphase, unaligned metaphase, unseparated anaphase, lagging chromosome and bridging in anaphase, un-oriented chromosomes in anaphase, irregular anaphase, sticky anaphase, multipolar anaphase, binucleated and extended cell, prolonged prophase and unseparated chromatids, and unequal and faulty polarized telophase (Fig. 4). The findings in the current study align with those of Ranjan et al. (2019) and affirm that the majority of the CAs were observed in the metaphase. Additionally, their study indicated that the CAs of TCAA surpassed that of TBM and TCM similar to what observed in the present study.

DBP doses for CA analysis were decided based on the EC₅₀ values with an increase in DBP concentration, CA was found to increase differently for different compounds. For example, the CA observed for 4000 mg/L of DBCM was higher than the CA for 6000 mg/L of TCM, DBCM, and THM4. This is even higher for TBM and TCAA (Table 2). CA was calculated as the percentage of cells with CA out of the total number of cells (Eq. 6). CA can happen only to the cells that are under mitosis. For higher doses of TBM and TCAA, the mitosis was very less (Table 2). Since there were very few cells that were dividing, the cells with CA were very less in number and hence the numerator in Eq. 6 was low compared to the rest of the doses. This explains the low CA for high doses of TBM and TCAA.

To understand the extent of aberrations, CA was further calculated against the number of cells dividing instead of

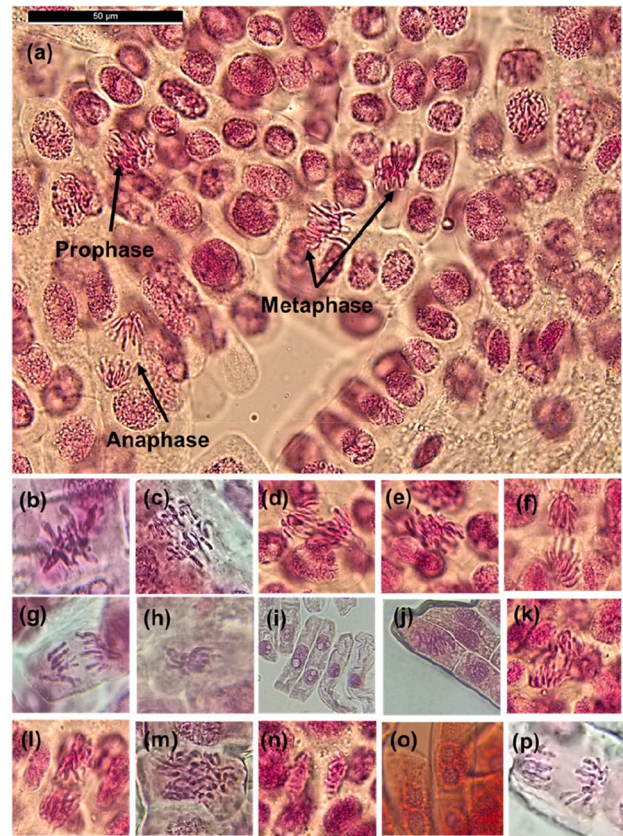


Fig. 4 a Photomicrograph of mitosis of control onion cells with normal mitotic stages; b C—metaphase; c unseparated anaphase; d lagging chromosome; e sticky metaphase; f unoriented chromosomes and bridging; g irregular anaphase; h sticky metaphase; i binucleated and extended cell; j unaligned metaphase; k unoriented chromosomes; l sticky anaphase; m prolonged prophase; n faulty polarization in telophase; o lobulated nucleus; p multipolar anaphase

the total number of cells (i.e., in Eq. 6, the denominator was ‘number of dividing cells’). In this case, the CA (%) for the tested DBP doses (doses are the same as noted in Table 2) are as follows: TCM (22.4 ± 1.0 , 48.8 ± 1.2 , 83.2 ± 1.8), BDCM (25.7 ± 0.6 , 54.9 ± 0.6 , 87.1 ± 2.7), DBCM (32.0 ± 1.0 , 66.9 ± 0.5 , 95.8 ± 1.3), TBM (48.4 ± 1.1 , 85.7 ± 1.7 , 95.3 ± 1.2), THM4 (21.6 ± 0.9 , 44.9 ± 1.5 , 84.8 ± 2.0), and TCAA (47.5 ± 0.8 , 81.0 ± 4 , 87.9 ± 6.7).

DNA laddering assay of *Allium cepa*

Figure 5 displays the DNA ladder of *A. cepa* root cells that are subjected to increasing concentrations of DBPs, harvested from specific doses based on the EC₅₀. The brightest bands in the ladder correspond to the highest concentration of a particular DNA fragment size. In the control cells, the

bright, thick fragment at the top of the agarose gel indicates intact DNA. DNA shearing was observed for root tip cells treated with several DBPs, indicating genotoxicity, DNA fragmentation, and cell death. Among the five DBPs tested, TBM-treated roots exhibited the darkest (completely smeared) ladders. A bright patch at the bottom of lane 5 in Fig. 5b indicates fragmentation into smaller pieces in roots treated with 100 mg/L TBM, which disappears in lane 6 which represent roots treated with 500 mg/L. The DNA ladders of BDCM (Fig. 5a, Lane 5 – 7) and DBCM (Fig. 5b, Lane 2,3) were visually similar indicating similarity in genotoxicity. These findings are consistent with previous studies demonstrating DNA fragmentation caused by TCM, TBM, and TCAA (Ranjan et al. 2019).

The DNA laddering assay of DBPs showed the genotoxicity and subsequent apoptosis cell death in *A. cepa* roots. Apoptosis and necrosis are two different types of cell deaths that have fundamental differences in their mechanisms and consequences. Apoptosis is an active, programmed process that avoids inflammation, while necrosis is a passive, accidental cell death that results in the uncontrolled release of inflammatory cellular contents (Bortner et al. 1995). Cells undergoing apoptosis undergo non-random DNA cleavage, resulting in the characteristic ladder pattern of fragmented DNA, whereas non-apoptotic cells exhibit a single, large DNA fragment (Fink and Cookson 2005). Similarly, cells undergoing necrosis have random and general cleavage as they are completely digested and appears smeared in agarose gel electrophoresis (Maines 2002). However, according to Fink and Cookson (2005), necrosis pertains to the observable features in cells once they have perished and stabilized in their environment, and apoptotic bodies may lose their structure and progress to a secondary stage known as apoptotic

necrosis. Even these cells are characterized by a smeared DNA ladder. Therefore, in the case of TBM, the smeared ladder might be representing either direct and sudden cell death or apoptotic necrosis. The presence of bright patch at the end of Lane 5 in Fig. 5b suggest towards the second possibility as the cells were fragmented (apoptosis) first and were then digested (apoptotic necrosis). In this context, it could be understood that the genotoxicity and cell death due to TBM occurred faster than the other DBPs as TBM-treated cells underwent apoptosis and necrosis while the others are still at the apoptosis stage.

Hydrogen peroxide content in *Allium cepa* roots

Plants may generate H_2O_2 as a result of various stressors such as UV stress, pathogen invasion, toxic contaminants, or oxygen shortage, leading to oxidative stress and cellular damage (Blokhina et al. 2003; Singh et al. 2007). At lower concentrations, H_2O_2 acts as a signaling molecule to help plants acclimatize to abiotic stressors, while at higher concentrations, it can be fatal, causing cell damage and death. Figure 6 shows the accumulation of H_2O_2 in *A. cepa* roots at different doses of DBPs. The results of this study align with previous findings by Ranjan et al. (2019) which revealed a progressive increase in H_2O_2 concentration with an increase in plant stressor (DBP) doses. Conversely, Singh et al. (2007) observed no significant change in the H_2O_2 concentration of *V. radiata* cells when exposed to arsenic. This indicates the potential for H_2O_2 to function as a signal molecule, triggering the defense response in plants to avoid cell damage and reducing further H_2O_2 production. Therefore, the increasing H_2O_2 concentration in the current study indicates cell damage due to DBP-induced H_2O_2 production.

Fig. 5 DNA laddering of *A. cepa* roots treated with different levels of DBPs. L1: DNA marker; **a** 1,8: control, 2 – 4: TCM 10 – 1000 mg/L, 5 – 7: BDCM 10 – 1000 mg/L, **b** 1,8: control; 2,3: DBCM 10, 1000 mg/L; 4: THM4 1000 mg/L; 5,6: TBM 100, 500 mg/L; 7: TCAA 100 mg/L

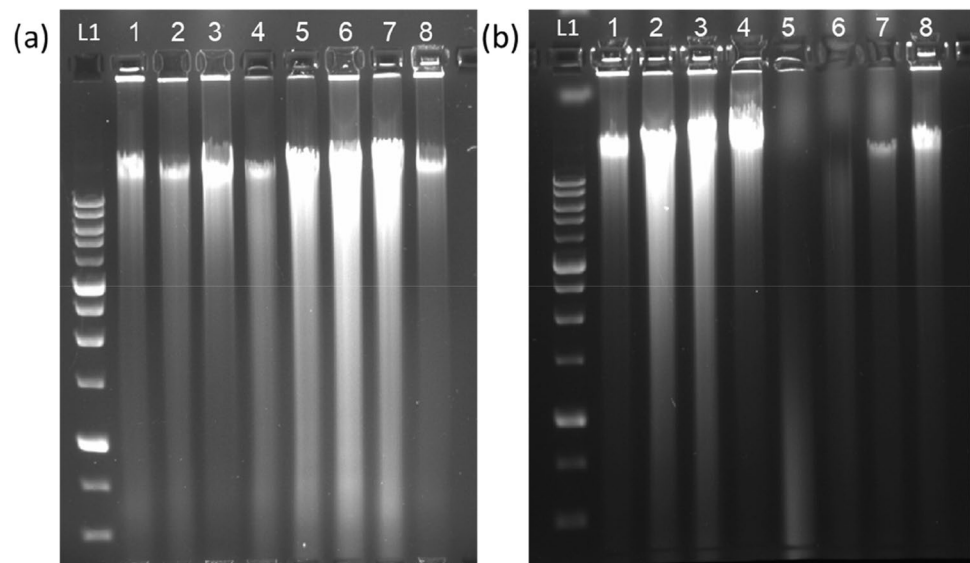
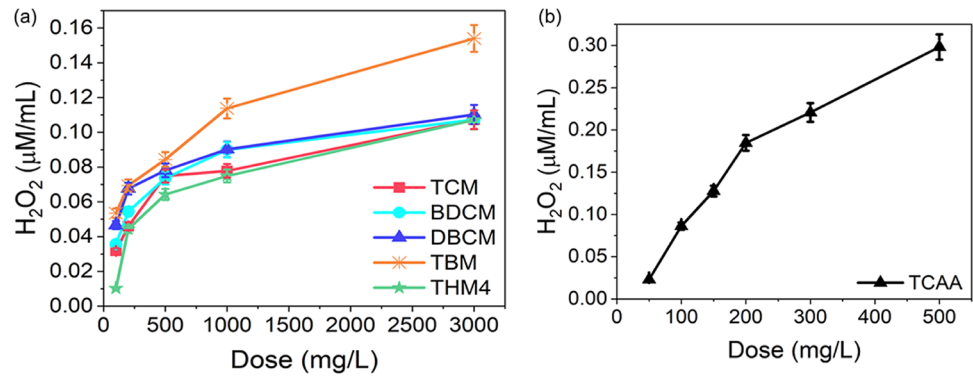


Fig. 6 Hydrogen peroxide content developed in *A. cepa* roots at various doses of (a) TCM, BDCM, DBCM, TBM, THM4, and (b) TCAA



Understanding the significance of H₂O₂ in plant growth and the cell cycle requires clarification of its production in plant cells under abiotic stress.

Plants produce reactive oxygen species, also known as reactive oxygen intermediates (ROIs), when under abiotic stress. ROIs are partially reduced forms of atmospheric oxygen that are traditionally believed to be highly reactive, toxic byproducts of aerobic metabolism, and can exist as free radical (O₂⁻, OH[·]) or non-radical molecular (H₂O₂, 1O₂) forms. Unlike molecular oxygen, ROIs are highly oxidative and can damage various cellular components, including proteins, lipids, carbohydrates, and DNA (Mittler 2002; Gill and Tuteja 2010). Recent studies confirm the role of RIOs as signaling molecules in controlling programmed cell death, pathogen defense, and abiotic stress response. Thus, RIOs serve as dual-purpose cellular indicators of stress and secondary messengers in stress-response signal transduction pathways (Mittler 2002). To serve these roles, cells must maintain a minimum ROI concentration. Plants use at least two different scavenging mechanisms to achieve this balance: maintaining low levels of ROIs for signalling applications and eliminating excess ROIs for cellular detoxification. Balancing these mechanisms determines the level of ROIs, especially with H₂O₂, which serves as the primary scavenging ROI in plant cells. Therefore, any increase in its concentration disrupts cellular components (Polle 2001; Asada 2006).

Although the tested doses of DBPs may not occur naturally in the environment, there is a potential for unusually high concentrations of these compounds to form in highly chlorinated reclaimed water and discharges from wastewater treatment plants, which are often used for irrigation (Munasinghe-Arachchige et al. 2021; Hong et al. 2022; Liang et al. 2022; Souza-Chaves et al. 2022; Xiao et al. 2023). Consequently, plants grown in such conditions should be monitored for phytotoxicity. In addition, it is important to investigate the potential accumulation of DBPs in plants, particularly in edible plant parts. Plants grown with reclaimed water need to be assessed

for potential risks associated with the ingestion of these compounds.

Conclusions

The present study established the phytotoxicity of TCM, BDCM, DBCM, TBM, and their mixture, as well as of TCAA. High doses of DBPs negatively impacted seed germination and root growth of *V. radiata* and *A. cepa*. The observed increasing toxicity with higher DBP concentrations emphasizes the need for careful monitoring of DBP concentrations in the environment to prevent potential harm to plants and ecosystems. The presence of CAs in onion root cells indicated cytotoxicity and the rising H₂O₂ concentration suggested oxidative stress and cell disruption. The individual THMs posed higher toxicity to both plant species than their mixture. Based on the root inhibition assay and mitosis assay results, TCAA was found to be the most toxic of all five DBPs tested, followed by TBM, DBCM, BDCM, and TCM. This implies that brominated THMs were more toxic to plants than TCM. The study provided suggestive evidence of the genotoxicity of various DBPs, particularly TBM and TCAA. Although the tested DBP doses are unlikely to occur in the natural environment, this study offers some insights into the phytotoxicity of DBPs and enables a comparative assessment of individual THMs and their phytotoxicity.

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Declarations

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Consent to participate Not applicable.

Consent to publish Not applicable.

Informed consent Not applicable.

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