

The halogen effects of disinfectant by-products on nutrient concentration, oxidative stress, fatty acids and α -tocopherol concentrations in membrane lipids of two *Solanum lycopersicum* cultivars

Babatunde Akande · Olalekan Fatoki ·
James Odendaal · Jeanine Marnewick ·
Patrick Ndakidemi

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Abstract A 30-day exposure time experiment was designed to investigate whether the extent of physiological impacts of trihalomethane exposure on two *Solanum lycopersicum* cultivars correlated with either the number of bromine or chlorine atoms in the trihalomethane molecules, focusing on biomass gain, nutrient concentration, oxidative stress, fatty acids and α -tocopherol contents in membrane lipids. The gain of biomass in tomato seedlings decreased with an increase in the degree of bromination. Concentrations of essential nutrients required for plant growth such as nitrogen, phosphorus, potassium, sulphur and boron decreased in plant dry matter that correlated with the increasing number of chlorine atoms in the trihalomethane molecules. Trihalomethane exposure resulted in an increase in oxidative stress response in

both tomato cultivars with parameters such as total phenolic content, ferric reducing antioxidant power, oxygen radical absorbance capacity and the activities of antioxidant peroxidase enzymes, all correlating with an increasing number of chlorine atoms in the trihalomethane molecule; hence, oxidative damage increased with a higher degree of chlorination. However, no such correlations were observed in superoxide dismutase activity, general lipid peroxidation, α -tocopherol content and total soluble proteins. In plant membrane lipids, an increase in the saturated fat hexadecanoic acid was observed in both tomato cultivars that correlated with the degree of chlorination in the trihalomethane molecule. The increase in α -linolenic acid stress signaling correlated with an increase in the degree of chlorination in only one tomato cultivar suggesting variable tolerance between cultivars to chemical action.

B. Akande (✉) · O. Fatoki · J. Odendaal
Faculty of Applied Science, Cape Peninsula University of
Technology, Cape Town Campus, Keizersgracht,
P.O. Box 652, Cape Town 8000, South Africa
e-mail: bcakande@gmail.com

J. Marnewick
Faculty of Health and Wellness Sciences, Institute of
Biomedical and Microbial Biotechnology, Oxidative
Stress Research Centre, Cape Peninsula University of
Technology, Bellville Campus,
P.O. Box 1906, Bellville 7535, South Africa

P. Ndakidemi
School of Life Sciences, Nelson Mandela African Institute
of Science and Technology, P.O. Box 447, Arusha,
Tanzania

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1 Introduction

Treated wastewater reuse in agriculture has become a viable economic option in many semi-arid water-stressed regions that have incorporated wastewater use in the planning and development of water resources to irrigate different crops (Batarseh et al. 2011; El Ayni

et al. 2011). Wastewater by itself is known to contain pharmaceuticals and refractory organics with phytotoxic potentials capable of altering crop physiology (Carvalho et al. 2014; Ma et al. 2014). Organic pollutants cause their damage after uptake and transport into plant tissues by altering enzyme function, promoting DNA adduct formation and inducing oxidative stress (Pilon-Smits 2005; Sharma et al. 2012; Verkleij et al. 2009).

The exposure of crop plants to organic pollutants has become a source of concern as plants are in direct contact with these chemicals during irrigation (Calderón-Preciado et al. 2011). The disinfection of wastewater is an important step in the control of biological contamination. However, the consequences of such treatments have led to the formation of disinfection by-products (DBPs) mainly from the reaction between organic/inorganic matter and disinfectants (Richardson et al. 2007; Sedlak and Von Gunten 2011). The trihalomethanes (THMs) and the haloacetic acids (HAAs) represent the two major classes of halogenated disinfection by-products (Krasner et al. 2009). The trihalomethanes which include bromodichloromethane (CHBrCl_2), bromoform (CHBr_3), chloroform (CHCl_3) and dibromochloromethane (CHBr_2Cl) are classified as persistent bioaccumulative toxic chemicals (PBT) (Wong et al. 2012).

Trihalomethanes have received attention based on their carcinogenic and mutagenic health risks to mammals, yet their potential physiological impacts on agricultural cash crops have not been previously studied. As concerns increase regarding the formation of organo-bromine and organo-chlorine compounds in treated waters, some studies have reported plants that have the ability to stimulate the removal of chlorinated organic pollutants through plant uptake, phyto-volatilization and/or phytodegradation (Chen et al. 2014; Doucette et al. 2013; Imfeld et al. 2009). The molecular structure of trihalomethanes shows an increasing order of both chlorination and bromination. With chlorination an increasing order of $\text{CHBr}_3 < \text{CHBr}_2\text{Cl} < \text{CHBrCl}_2 < \text{CHCl}_3$ is observed, while the order of bromination increases in the sequence $\text{CHCl}_3 < \text{CHBrCl}_2 < \text{CHBr}_2\text{Cl} < \text{CHBr}_3$.

Our hypothesis presumes the magnitude of the physiological response of crop plants may be correlated to the increasing order of bromination or chlorination in the trihalomethane molecule. Such data could contribute to the growing body of knowledge on

the mechanisms of plant response to organo chlorinated and organo-brominated water based pollutants.

Physiological measurements of plant performance are often based on plant growth or growth inhibition. Several organo-chlorinated and polycyclic aromatic hydrocarbons (PAH's) have been known to affect plant growth (Ahammed et al. 2013; Desalme et al. 2013; Wang et al. 2014). The determination of the concentration levels of essential nutrients required for plant growth is an important physiological parameter.

Many of the oxidative stress mechanisms activated by different environmental stimuli are also activated by organic pollutants. The mechanisms involved in oxidative stress are important since organo-chlorines may cause reactive oxygen species to form (Faure et al. 2012; San Miguel et al. 2013). Reactive oxygen species (ROS) such as the superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) have been identified as key signalling molecules in controlling a broad range of physiological processes such as cellular growth and development (Kärkönen and Kuchitsu 2015). In addition, ROS production may lead to protein oxidation, DNA damage, lipid peroxidation and oxidative destruction of key cellular components. Plants possess a large array of antioxidant molecules and enzymes able to maintain intracellular ROS levels and oxidative stress is the result of the imbalance between the production of ROS and their removal by antioxidants (Phung and Jung 2015; Talbi et al. 2015).

Techniques have been developed to measure oxidative stress tolerance in plants exposed to abiotic factors. Non-enzymatic responses include the measurement of total phenolic content, the ferric reducing antioxidant power of plant extracts (FRAP), the measurement of the general peroxidation of aggregated lipids in biological samples using thiobarbituric acid reacting substances (TBARS) and the oxygen radical absorbance capacity of plant extracts (ORAC). Non-structural phenolic compounds are known to perform a wide variety of functions in plants including acting as antioxidants (Ainsworth and Gillespie 2007). The FRAP assay constitutes a putative index of antioxidant, or reducing, potential of biological fluids (Benzie and Strain 1996). The ORAC assay is based on the measurement of the inhibition of free radical damages to a fluorescent probe by antioxidants. The delay in the degradation of the fluorescent probe

suggests the ability of the pre-existing antioxidants in the sample to scavenge the free radicals (Prior et al. 2003). General lipid peroxidation in the homogenized plant extracts can be determined in terms of malondialdehyde (MDA) content using thiobarbituric acid reaction (TBARS) to evaluate oxidative stress, antioxidant efficiency and lipid oxidizability in different physiological states (Chakraborty et al. 2009).

Typical enzymatic responses for the scavenging of reactive oxygen species (ROS) in plants include the measurement of the activity of antioxidant enzymes such as ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and superoxide dismutase (SOD) (Jebara et al. 2005). Superoxide dismutase (SOD; EC 1.15.1.1) is an enzyme that catalyses the dismutation of the toxic superoxide (O_2^-) radicals into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Barondeau et al. 2004). Ascorbate peroxidase (APX; EC 1.11.1.11) is the enzyme that detoxifies peroxide (H_2O_2) using ascorbate as a substrate. The reaction they catalyze is the transfer of electrons from ascorbate to peroxide, producing dehydroascorbate and water as products (Pang and Wang 2010). Guaiacol peroxidase (GPX; EC 1.11.1.7) is an important enzyme in the peroxidase group that detoxifies peroxide (H_2O_2) using guaiacol (2-methoxyphenol) as its reducing substrate (Mika and Lüthje 2003).

Lipid profiling has also been utilized to identify compositional changes in lipids associated with stress response (Welti and Wang 2004). Such changes may occur as an increased or decreased expression of essential sterols, fatty acids and esters or the production of novel lipid-metabolic species (Gajewska et al. 2012; Larson and Graham 2001; Wallis and Browse 2002).

The main aim of the present work was to assess physiological and defense system responses in two closely related tomato cultivars, experimentally exposed to the four trihalomethane chemical species. *Solanum lycopersicum* cultivars were selected based on their popularity, high yields and stability in the climatic regions of Cape Town South Africa where this study was conducted. Tomato plants have also been recently reported as able to uptake organochlorine compounds (Zhang et al. 2015). With an aim to contribute to the better understanding of the physiological response of plants to these water-based disinfectant by-products, we theorized that the magnitude of physiological response may be correlated to

the trihalomethane chlorination or bromination order. To confirm our assumptions we focused on the concentration levels of 12 essential plant nutrients, enzymatic and non enzymatic antioxidant responses, lipid peroxidation, fatty acids and tocopherol concentrations in tissues of tomato plants exposed to a known concentration of each trihalomethane compound.

2 Materials and methods

2.1 Chemicals

The chemicals used in this study were bromodichloromethane ($CHBrCl_2$), bromoform ($CHBr_3$), chloroform ($CHCl_3$) and dibromochloromethane ($CHBr_2Cl$). All chemicals were purchased from Sigma-Aldrich (Germany) as pure standards (99 % of purity). They were all provided directly solubilized in methanol.

2.2 Plant materials and treatments

This experiment was established as a randomized, complete block design with four replications per treatment. The experiment commenced with the design of a 40 pot irrigation system fitted with timers and connected to 4 separate 68 L rough tote plastic reservoirs. Four 6 m long square plastic gutters containing 8 pots each were arranged on an elevated table in the greenhouse. The 12.5 cm plastic square pots were filled with Starke-Ayres 3:1:5 organic potting soils. Each pot was connected to 4 L per hour button dripper extended from 20 mm low density polyethylene (LDPE) irrigation pipe. Each 68 L plastic reservoir was fitted with a 1400 L per hour submersible pump. Holes with diameters of 21 mm were made on each lid allowing the LDPE pipes connected to the submersible pumps to pass through. The reservoir lids remained closed to prevent loss of trihalomethanes to evaporation due to their classification as semi-volatile compounds. The irrigation timers were set to water the plants twice a day from 8.00 am to 8.30 am and 8.00 pm to 8.30 pm approximately giving each pot 4 L of water per day. To eliminate nutrient stress for all treatments, de-ionized water pre-treated with granular activated carbon to remove pre-existing organics was mixed with Starke-Ayres nutrified K2025 water-

soluble fertilizer in the reservoirs for the entire duration of the experiment. Seeds of the F1 hybrid tomato star (TS) and tomato moneymaker (TMM) cultivars were purchased from Starke-Ayres Garden Centre and soaked in nanopure water for 1 h to remove all preservatives. The seeds were planted 2 cm deep in the potting soil with two seeds per pot. The first four pots in each plastic gutter contained seeds of the variety (TMM) and the last four pots contained seeds of the hybrid variety (TS). All seeds germinated within a 14 day period and the weaker seedling was uprooted leaving one seedling per pot. The treatments began 16 days after germination when all plants were within the range of 30–35 cm in height. The 40 pot plants were divided into five groups each containing 8 pot plants. The 1st group was exposed to a concentration of 10 mg L^{-1} of bromodichloromethane (CHBrCl_2), the 2nd group to 10 mg L^{-1} of bromoform (CHBr_3), the 3rd group to 10 mg L^{-1} of chloroform (CHCl_3), the 4th group to 10 mg L^{-1} of dibromochloromethane (CHBr_2Cl) and the last group (control) was exposed to de-ionized water containing plant nutrients only.

The required total volume of 65 L of the designated trihalomethane for each gutter was prepared in the laboratory every alternate day and transferred to the reservoir. The treatment was terminated after a 30-day exposure of the plants to the trihalomethanes. The leaves of each plant were cut from the stem and immediately dipped in liquid nitrogen and stored in the Nuair glacier ultra low minus $86 \text{ }^\circ\text{C}$ freezer for oxidative stress and lipid profile determination. The rest of the plants were washed with de-ionized water to remove all soil particles, put in paper bags and dried at $65 \text{ }^\circ\text{C}$ for 72 h to stop enzymatic activity in accordance to Havlin et al. (2012).

The gain in biomass was determined by the difference in dry mass of seedlings exposed to 3 days of treatment to seedlings exposed to 14-days of treatments. Seedlings ($n = 25$) of the tomato moneymaker (TMM) and tomato star 9003 (TS) cultivars were exposed to 10 mg L^{-1} of chloroform, bromodichloromethane, dibromochloromethane, and bromoform. All seeds used in the experiment were first germinated on a double layer of Whatman No. 1 filter paper in 12 cm Petri dishes containing nanopure water only. Each Petri dish contained 25 seeds with four replicates for each treatment including control. The treatments commenced 5 days after germination in all Petri dishes. The filter papers of the seedlings were

moistened with 10 mL of trihalomethane chemical species in each Petri-dish. The Petri dishes were then kept in an incubator at room temperature. The filter papers of the Petri dishes were re-moistened every day due to the volatile nature of trihalomethane species. The control treatments were moistened with nanopure water only. The dry weight of each seedling was determined by placing them inside a furnace and dried to a constant weight at $608 \text{ }^\circ\text{C}$ for 72 h (Buczek and Burzyński 2015). This was performed on the 3rd and the 14th day of the treatments. The data from this experiment was analyzed using the software of STATISTICA program 2010 (StatSoft Inc., Tulsa, OK, USA). When significant differences were detected by the analysis of variance (ANOVA), mean values of dry weight of seedlings were used to compare treatment means at $P \leq 0.05$ according to FSD (Fischer's Least significant difference).

2.3 Determination of nutrient concentration in plant dry matter

Changes in the primary macronutrients nitrogen (N), phosphorus (P), potassium (K), the three secondary macronutrients calcium (Ca), sulfur (S), magnesium (Mg), and the micronutrients boron (B), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), and sodium (Na) concentrations of plant dry matter yields were investigated. Homogenization of the dried plant material was achieved using the micro plant grinder model FZ102. The homogenized plant powders were weighed and stored in 10 cm long airtight cylindrical glass vials. The air blower was used to clean the grinder before the next sample was homogenized so as to prevent sample mixing. The samples were sent off to Bemblab analytical laboratory where they were ashed at $480 \text{ }^\circ\text{C}$, shaken and digested with a 50:50 32 % HCL/water solution for extraction through filter paper (Campbell and Plank 1998). The primary and secondary macronutrients with the micronutrients content of the extract were measured with a Varian ICP-OES optical emission spectrometer with the exception of nitrogen (Bressy et al. 2013). Total nitrogen content of the homogenized plant material was determined by total combustion in a Leco N-analyzer (Sweeney and Rexroad 1986). Nutrient concentrations in sample extracts were expressed in mg kg^{-1} .

2.4 Non-enzymatic antioxidant extraction

The fresh leaf samples were taken from the freezer and lyophilized for 16 h at minus 86 °C using the Vir-tis genesis 35 EL freeze dryer (SP Industries, Gardiner, NY, USA). The lyophilized leaf samples were then homogenized into a fine powder using the micro plant grinder and stored in 2 mL vials at minus 40 °C. Then 20–25 mg of lyophilized plant samples were weighed into a 15 mL screw-cap tube and 5 mL of 70 % methanol in nanopure water was used to extract plant samples as described (Lapornik et al. 2005). The samples were then loaded on the Intelli-mixer RM2 rotator/mixer (ELMI Ltd, Latvia) for 24 h and centrifuged at 2200×g for 5 min using the Eppendorf centrifuge 5810 R (Eppendorf, AG, Hamburg, Germany). The supernatant was then used directly for analysis after suitable dilution method was developed.

2.4.1 Determination of total polyphenol content

Total polyphenols in plant extracts were determined by the Folin-Ciocalteu procedure as described (Meyer et al. 1997). Total polyphenols were expressed in mg L⁻¹ gallic acid equivalent (GAE).

2.4.2 Determination of the ferric reducing antioxidant power (FRAP)

The ferric reducing ability of the sample extracts was determined as described by (Benzie and Strain 1996). The FRAP reagent was prepared by mixing 30 mL acetate buffer pH 3.6 (300 mM), 3 mL of 2, 4, 6, tripyridyl-*s*-triazine (TPTZ) (10 mM) prepared in 40 mM hydrochloric acid, 3 mL FeCl₃ (20 mM), and 6.6 mL distilled water (dH₂O). 10 μL of sample was mixed with 300 μL of FRAP reagent in a 96-well plate and incubated at 37 °C for 30 min. The change in absorbance due to the redox reaction occurring was monitored in a Thermo Scientific multiscan spectrum spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at 593 nm. FRAP were expressed in mg L⁻¹ ascorbic acid equivalent (AAE).

2.4.3 Determination of the oxygen radical absorbance capacity (ORAC)

The oxygen radical absorbance capacities of plant extracts were determined as described (Prior et al.

2003). The reaction is initiated by the thermal decomposition at 37 °C of the azo-compound 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH) which serves as the source of peroxy radicals. Control mixtures are then prepared using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solution. All reagents and standards were prepared in phosphate buffer (75 mM, pH 7.4). 138 μL of fluorescence and 12 μL of the sample were mixed in a black Nunclon 96-well plate. 50 μL of AAPH was then added last to the plate and fluorescence readings were taken using a Thermo Scientific fluoroskan ascent 2.5 plate reader (Thermo Fisher Scientific, Waltham, USA) at 485 and 583 nm respectively. Antioxidant activity was expressed in Trolox equivalents.

2.5 Antioxidant enzyme extractions

Enzyme extraction of plant samples was performed as described (Zhou et al. 2004) with some modifications. The extraction buffer contained 25 mM of 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid (HEPES) at pH 7.8, 0.2 mM of ethylenediaminetetraacetic acid (EDTA), 2 % (w/v) polyvinylpyrrolidone (PVP), 1 mL of triton X-100, and 200 mM of potassium chloride (KCl) in 200 mL of nanopure water. Lyophilized samples weighing 30–35 mg were put into 15 ml screw cap tubes and 2 mL of extraction buffer were added to each tube. The tubes were loaded on the Intelli-mixer RM2 rotator/mixer for a period of 17 h allowing thorough homogenization. The homogenates were centrifuged for 20 min at 13700×g using a bench top centrifuge Eppendorf 5402 (Eppendorf, AG, Hamburg, Germany) and the supernatants obtained were used for enzyme analysis. All operations were performed at 0–4 °C.

2.5.1 Determination of superoxide dismutase activity

The superoxide dismutase (SOD; EC 1.15.1.1) activity of plant extracts was determined as described (Ellerby and Bredesen 2000) with some modifications. 170 μL of diethylenetriaminepentaacetic acid (DETAPAC) solution were added in triplicates to a visible 96-well plate. Then 12 μL of sample extract was added to each well with 18 μL of SOD buffer (50 mM of NaPO₄-buffer at pH 7.4 without triton X-100). Finally, 15 μL of 6-hydroxydopamine (6-HD) was added to each well and immediately the auto-oxidation was recorded at

490 nm for 4 min with 1 min intervals. The activity of SOD was calculated from a linear calibration curve and SOD activity was expressed as Units mg^{-1} of protein (U mg^{-1}).

2.5.2 Determination of ascorbate peroxidase activity

Ascorbate peroxidase (APX; EC 1.11.1.11) activity in plant extract was determined as previously described (Nakano and Asada 1981) with some modifications. 180 μL of 50 mM K-PO_4 buffer (pH 7.0), 30 μL of EDTA, 30 μL of 5 mM ascorbate and 30 μL of homogenized plant extract were added in triplicates to an ultra violet 96-well plate. The reaction was initiated by finally adding 30 μL of 0.1 mM hydrogen peroxide (H_2O_2) to each well and the reduction in ascorbate concentration was read by measuring the absorbance at 290 nm continuously for 180 s. The ascorbate oxidized was evaluated based on the extinction coefficient of ascorbate (E) = $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ and the results expressed as APX U mg^{-1} of protein ($\text{U} = 1 \text{ mM}$ of ascorbate oxidized per min at 25°C).

2.5.3 Determination of guaiacol peroxidase activity

Guaiacol peroxidase (GPX; EC 1.11.1.7) activity was determined as previously described (Bergmeyer et al. 1974) with some modifications. 180 μL of 0.1 M potassium phosphate buffer KH_2PO_4 (pH 7.0), 30 μL of guaiacol and 60 μL of homogenized plant extract were added in triplicates to a visible 96-well plate. The reaction was initiated by adding 30 μL of 0.1 mL of 30 % hydrogen peroxide (H_2O_2) in 120 mL of nanopure water to each well and the rate of increase in absorbance at 436 nm was recorded using a linear portion of the curve with GPX activity expressed in U mg^{-1} of protein (U mg^{-1}).

2.6 Lipid extraction by methyl-*tert*-butyl-ether (MTBE)

Lipid extraction was determined as described by Matyash et al. (2008) with some modifications. 80–100 mg of lyophilized plant homogenates were weighed into 15 mL screw cap tubes and 0.77 mL of methanol was added to each tube. The tubes were vortexed for 20 s and 2.56 mL of MTBE solvent was added to each tube. The tubes were loaded on the Intelli-mixer RM2 rotator/mixer for 1 h at room

temperature after which phase separation was induced by adding 0.64 mL of nanopure water. After 10 min of incubation at room temperature, samples were centrifuged at $13,750\times g$ for 4 min and the supernatant was removed with 2 mL disposable syringes and filtered using a 0.45 μm syringe filter. The filtrates were collected in 2 mL vials where 1–2 g of sodium sulfite was added to each vial to remove all traces of water and a known concentration of 10 μL of a mixture of PC 18:0/18:0, PE 17:0/17:0, phosphatidylinositol (PI) 17:0/17:0, C24:1 β -D-galactosylceramide, and diacylglycerol 16:0/18 were used as internal standard.

2.6.1 Determination of lipid peroxidation (TBARS)

Lipid peroxidation in the homogenized extract was determined in terms of malondialdehyde (MDA) content by thiobarbituric acid reaction (TBARS) as described (Heath and Packer 1968) with some modifications. 100 μL of homogenized plant extract, 12.5 μL of 4 mM cold butylated hydroxytoluene (BHT) in ethanol, and 100 μL of 0.2 M of orthophosphoric acid were mixed and vortexed for 10 s in 2 mL vials. Then 12.5 μL of TBA reagent (0.11 M in 0.1 M NaOH) was added to each vial and vortexed for another 10 s. The vials were heated at 90°C for one 1 h and cooled on ice for two min with further cooling at room temperature for 5 min. 1000 μL of *n*-butanol with 100 μL of saturated NaCl were added to the vials for better phase separation. The vials were then centrifuged at $19800\times g$ for 2 min at 4°C then 300 μL from each vial was put in triplicates into a visible 96-well plate. MDA equivalent was calculated from the difference in absorbance at 532 and 572 nm using extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.6.2 Gas chromatographic profiling of lipidomes

The lipid profile of the plant sample extracts was determined using the Agilent 6890 gas chromatographic mass selective detector (Agilent Technologies, Little Falls, DE, USA) and the method used to profile the lipids was developed from EPA method 551 (Hodgeson and Cohen 1990). The initial oven temperature at 0.00 min was 70°C to reach a maximum temperature of 320°C at 0.25 min. The total run-time was 43.25 min per sample and the injector front inlet mode was splitless with the Initial temp at 280°C and pressure at 60.2 kPa. The purge flow was

30 mL min⁻¹ with a purge time of 3 min and a total flow of 33.8 mL min⁻¹ with an injection volume of 1 µL and helium as the carrier gas. The capillary column used was model ZB 274305 (Phenomenex, Torrance, CA, USA) mainly used for semi-volatiles with a nominal length of 30.0 m × 250 µm (diameter) × 0.25 µm (film thickness). The GC transfer line was maintained at 280 °C with the mass selective detector operated at EMV mode and the resulting EM voltage to be 1800 with a maximum source temperature of 250 °C and a solvent delay of 6 min. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the WILEY229 library data of the GC/MS system and published data.

All values reported in this study are means of four replicates. The data were analyzed by two-way analysis of variance (ANOVA) and compared at $P \leq 0.05$ significance level by the Scheffe test using statistical analysis system (SAS).

3 Results

3.1 Effect of trihalomethanes on gain of biomass

The effect of trihalomethanes on the gain of biomass is shown in Fig. 1. We observed that the mean values for control in both seedling varieties were significantly different from the treatments. The gain in biomass of both tomato seedlings decreased with a higher degree of bromination in the trihalomethane molecule.

3.2 Effect of trihalomethanes on nutrient concentration

Figure 2 shows the effect of trihalomethanes on nutrient concentration in both tomato cultivars. The nutrient levels of N, P, K, S and B in plant dry matter decreased in a manner that correlated with a higher degree of chlorination in the trihalomethane molecules in the order of CHBr₂Cl > CHBrCl₂ > CHBr₃ > CHCl₃ in both tomato cultivars (Fig. 2). Nutrient levels in control plants were significantly different from treatments. The effect of increasing chlorination was not significant on the sulphur levels of both tomato cultivars and in the phosphorus levels of the TS variety only (Fig. 2).

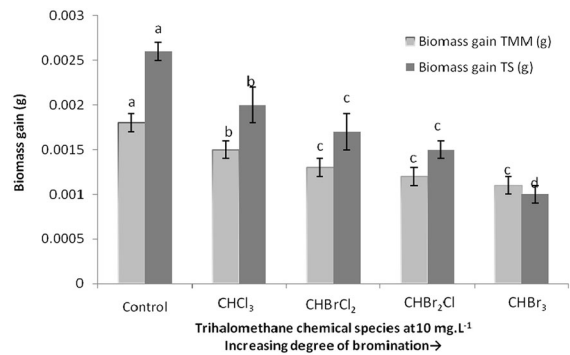


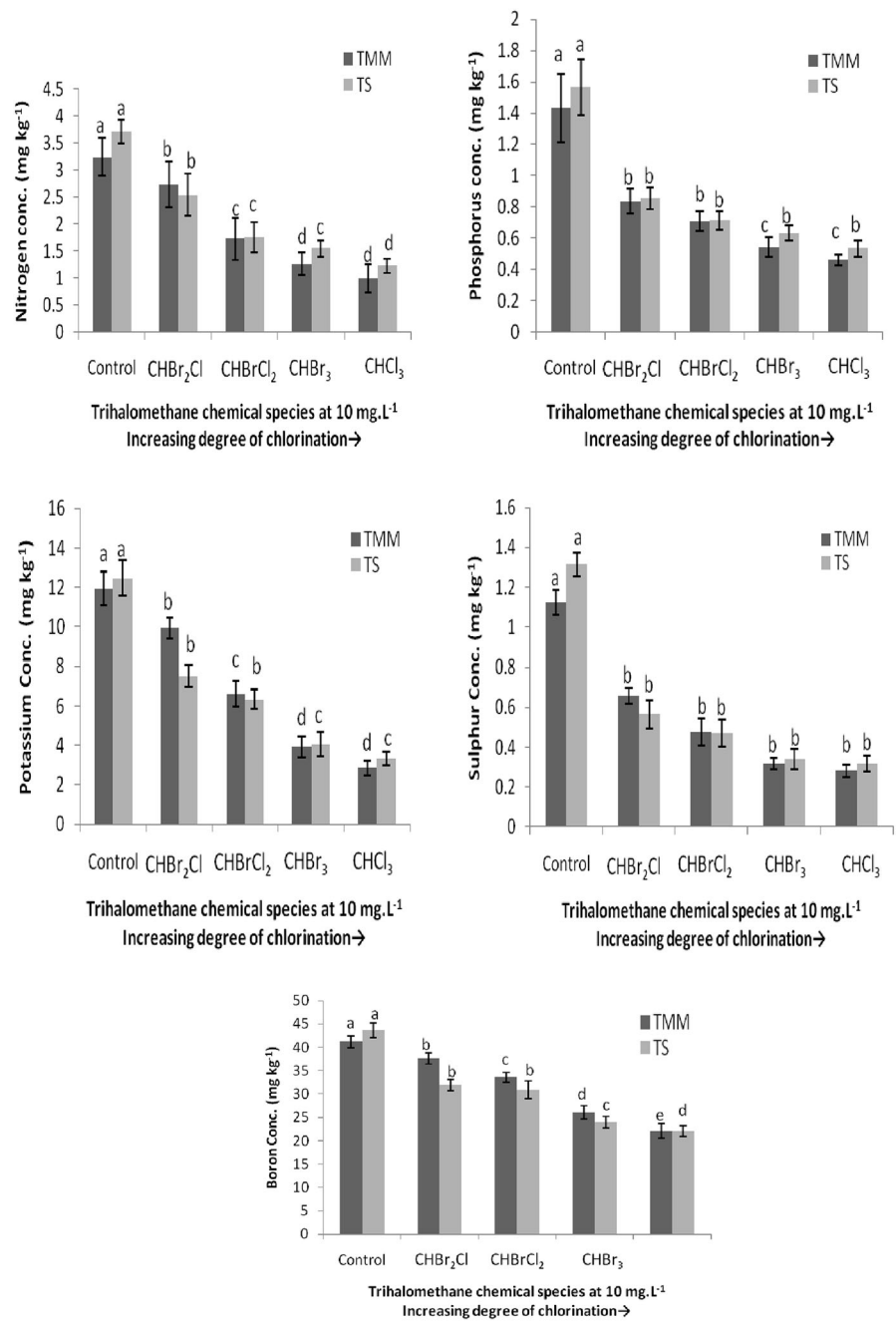
Fig. 1 Effect of *a* chloroform (CHCl₃) *b* bromodichloromethane (CHBrCl₂) *c* dibromochloromethane (CHBr₂Cl) and *d* bromoform (CHBr₃) on the gain of biomass between seedlings exposed for 3 days and seedlings exposed for 14 days. The tomato moneymaker (TMM) and tomato star (TS) seedlings were exposed to a concentration of 10 mg L⁻¹ of each trihalomethane species. Control seedlings were exposed to nanopure water only. Means followed by a similar letter in a column are not significantly different from each other at $P \leq 0.05$ according to FSD. Vertical bars represent standard error of means (n = 25)

Table 1 shows the correlation between nutrient concentration in both tomato cultivars and the increasing degree of chlorination in the trihalomethane molecule. The strongest correlations were observed in phosphorus, potassium and boron levels.

3.3 Effects of trihalomethanes on non-enzymatic biomarkers of oxidative stress

Figure 3 shows the effects of trihalomethanes on non-enzymatic biomarkers of oxidative stress in both tomato cultivars. An increase in the total phenolic content and the ferric reducing antioxidant power (FRAP) of plant extracts was observed with increasing chlorination of the trihalomethane molecule in the order of CHBr₂Cl < CHBrCl₂ < CHBr₃ < CHCl₃ in both cultivars (Fig. 3). The oxygen radical absorbance capacity (ORAC) of plant extracts increased with increasing chlorination of trihalomethane molecule with a slightly different order of CHBr₂Cl < CHBr₃ < CHBrCl₂ < CHCl₃ in both cultivars (Fig. 3). General lipid peroxidation by TBARS responded to no particular halogenation order. The di-chlorinated trihalomethane molecule (CHBrCl₂) induced the highest levels of peroxidation in lipids of both tomato cultivars (Fig. 3). Non enzymatic biomarkers in control plants were significantly different from treatments.

Fig. 2 Effect of *a* dibromochloromethane (CHBr_2Cl) *b* bromodichloromethane (CHBrCl_2) *c* bromoform (CHBr_3) and *d* chloroform (CHCl_3) on the nutrient concentration of the dry matter yields of tomato plants (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by a similar letter in a column are not significantly different from each other at $P \leq 0.05$ according to FSD. Vertical bars represent SE of means ($n = 4$)



3.4 Effects of trihalomethanes on enzymatic biomarkers of oxidative stress

In Fig. 4, we observed that the activity of the peroxidase enzymes APX and GPX increased with increasing chlorination of trihalomethane molecules in the order of $\text{CHBr}_3 < \text{CHBr}_2\text{Cl} < \text{CHBrCl}_2 < \text{CHCl}_3$ in both

cultivars (Fig. 4). Also, the activity of superoxide dismutase (SOD) responded to no particular halogenation order, although chloroform (CHCl_3) induced the most activity in both cultivars (Fig. 4). The enzymatic biomarkers in control plants were significantly different from treatments with the exception of the activity of ascorbate peroxidase (APX) in the TS cultivars.

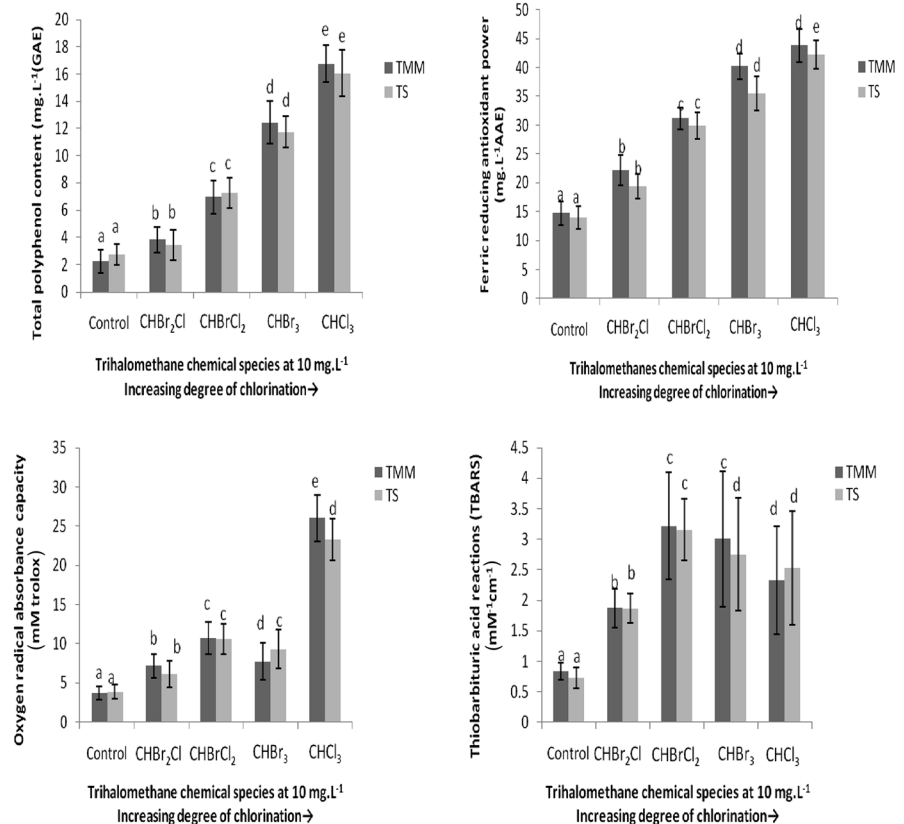
Table 1 Pearson correlation coefficient between nutrient concentration and increasing degree of chlorination

Nutrient	Correlation coefficient (R) value for nutrient concentration with increasing degree of chlorination
Nitrogen	−0.9552 at P = 0.0448
Phosphorus	−0.9964 at P = 0.0036
Potassium	−0.9834 at P = 0.0166
Sulphur	−0.9601 at P = 0.0399
Boron	−0.9804 at P = 0.0196

Table 2 shows the correlation between the oxidative biomarkers in both tomato cultivars and the increasing degree of chlorination in the trihalomethane molecule.

From Table 2, the strongest correlations were observed for the total polyphenol content, the ferric reducing antioxidant power (FRAP) and the activity of guaiacol peroxidase enzyme.

Fig. 3 Effect of *a* dibromochloromethane (CHBr_2Cl) *b* bromodichloromethane (CHBrCl_2) *c* bromoform (CHBr_3) and *d* chloroform (CHCl_3) on the non-enzymatic biomarkers of oxidative stress (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by a similar letter in a column are not significantly different from each other at $P \leq 0.05$ according to FSD. Vertical bars represent standard error of means ($n = 4$)



3.5 Effects of trihalomethanes on fatty acids and α -tocopherol concentration in membrane lipids of lyophilized leaf tissues of tomato plants

The polyunsaturated fatty acid (α -linolenic acid) increased in the leaves of the TMM cultivar with an increasing degree of chlorination in the order of $\text{CHBr}_3 < \text{CHBr}_2\text{Cl} < \text{CHBrCl}_2 < \text{CHCl}_3$ as observed in Fig. 5. The TS cultivars responded to no particular halogenation order as can be seen in Fig. 5. Similarly, the saturated fatty acid palmitic acid increased with increasing chlorination of the trihalomethane molecule in the order of $\text{CHBr}_2\text{Cl} < \text{CHBrCl}_2 < \text{CHBr}_3 < \text{CHCl}_3$ for both tomato cultivars, although the extent of accumulation was greater in the TS tomato cultivars.

The α -tocopherol concentration in both TMM and TS cultivars followed no particular halogenation order. The effects of the trihalomethane chemical varieties were significant at $P \leq 0.05$ on α -linolenic acid, palmitic acid, and α -tocopherol concentration

Fig. 4 Effect of *a* dibromochloromethane (CHBr_2Cl) *b* bromodichloromethane (CHBrCl_2) *c* bromoform (CHBr_3) and *d* chloroform (CHCl_3) on the enzymatic biomarkers of oxidative stress (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by a similar *letter* in a column are not significantly different from each other at $P \leq 0.05$ according to FSD. *Vertical bars* represent SE of means ($n = 4$)

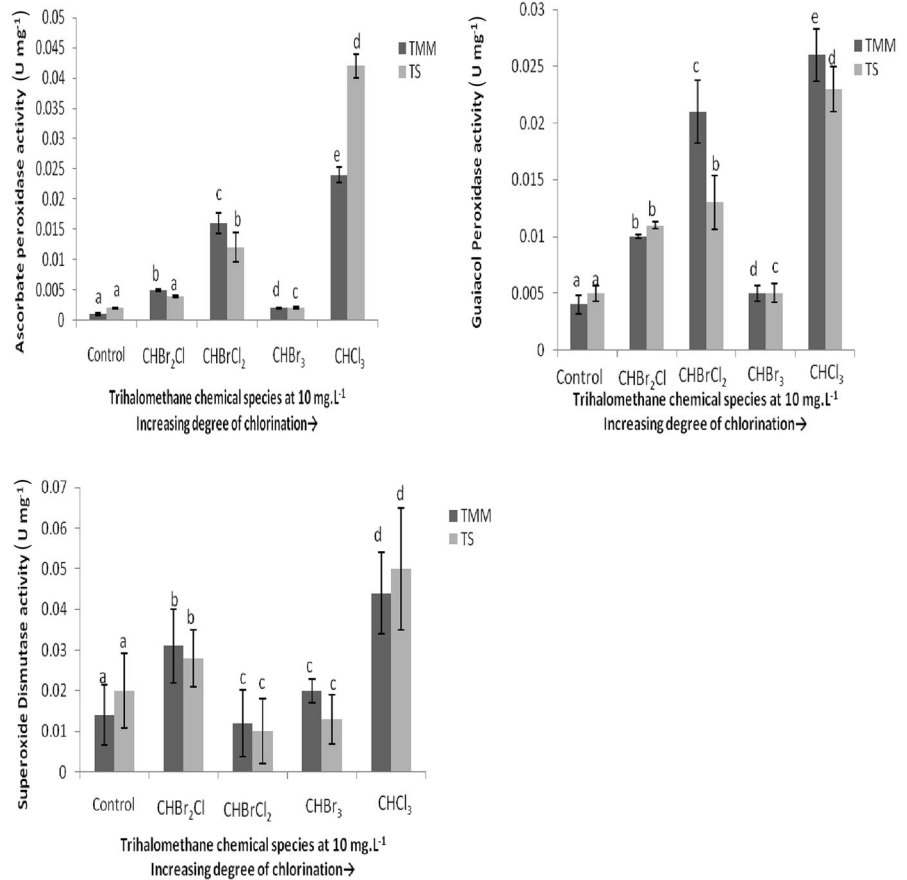


Table 2 Pearson correlation coefficient between the oxidative stress biomarkers and increasing degree of chlorination

Oxidative stress biomarkers	Correlation coefficient (R) value for stress biomarkers with increasing degree of chlorination
Total polyphenols	0.9978 at $P = 0.0022$
Ferric reducing antioxidant power (FRAP)	0.9905 at $P = 0.0095$
Oxygen radical absorbance capacity (ORAC)	0.8845 at $P = 0.1155$
Thiobarbituric acid reacting substances (TBARS)	0.3079 at $P = 0.6921$
Ascorbate peroxidase (APX)	0.9381 at $P = 0.0619$
Guaiacol peroxidase (GPX)	0.9949 at $P = 0.0051$
Superoxide dismutase (SOD)	0.4740 at $P = 0.5260$

while the effects of plant varieties were not significant at $P \leq 0.05$. The membrane lipid components measured in leaves of control plants were significantly different from treatments with the exception of α -tocopherol leaf content in TMM cultivars.

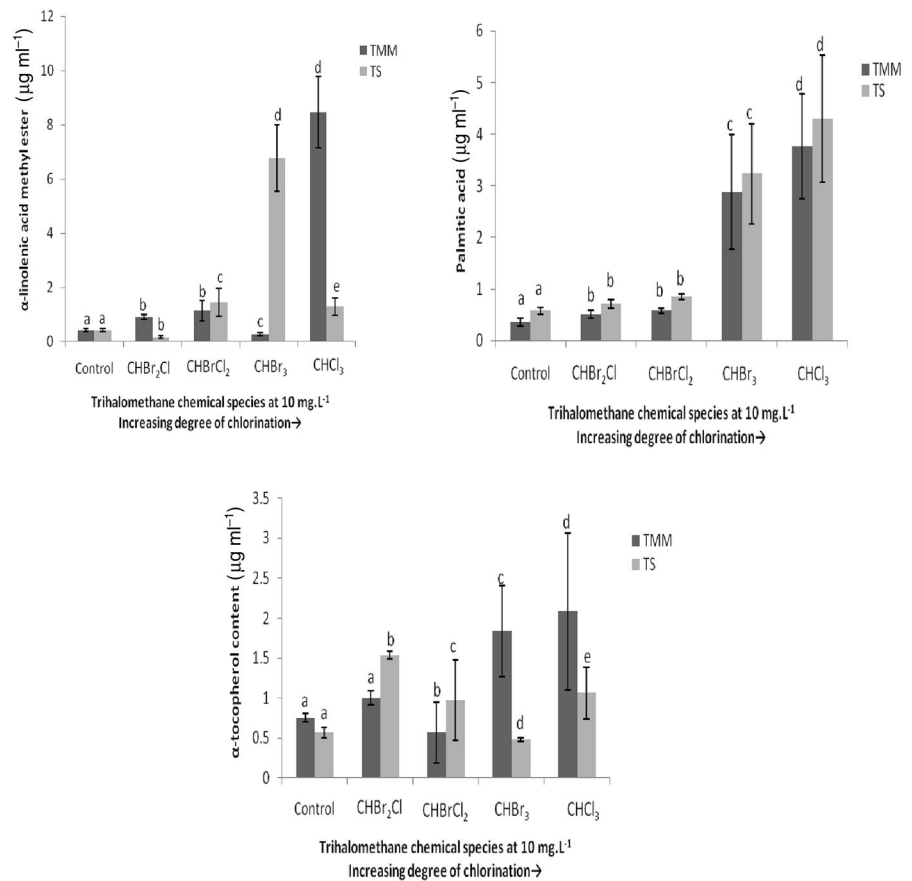
Table 3 shows the correlation between the leaf membrane lipid components measured in both tomato cultivars and the increasing degree of chlorination in the trihalomethane molecule.

The strongest correlations were observed for palmitic acid and α -linolenic acid content in leaves of the tomato cultivars.

4 Discussion

The toxicity and physiological responses of plants to organo-chlorines have become a recent focus of

Fig. 5 Effect of *a* dibromochloromethane (CHBr_2Cl) *b* bromodichloromethane (CHBrCl_2) *c* bromoform (CHBr_3) and *d* chloroform (CHCl_3) on the membrane lipids composition of leaf tissues of tomato cultivars (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by a similar letter in a column are not significantly different from each other at $P \leq 0.05$ according to FSD. Vertical bars represent standard error of means ($n = 4$)



scientific interest (Ahmed et al. 2013; Faure et al. 2012; Li et al. 2015). Plant exposure to organochlorines shows diverse physiological stress responses, of which growth inhibition is a commonly observed phenomena in all photosynthetic species reported whether phytoplankton or crop plants (Blondel et al. 2014; Gotham and Rhee 1982; Mitra and Raghu 1989; San Miguel et al. 2012).

Results from the current study reports for the first time that trihalomethanes may negatively affect the gain of biomass in tomato seedlings based on the degree of bromination (the number of bromine atoms in the trihalomethane molecule) (Fig. 1). After repeated experiments, it was concluded this unique trend could be explained by two factors, the experimental design and the volatility behaviour of trihalomethanes. While every precaution was taken to reduce volatility in the experiments on growing tomato plants, no precautions were taken to reduce volatility for the experiments on the tomato seedlings.

The volatility behaviour of the trihalomethanes indicates the more brominated trihalomethanes have higher boiling points (bromoform CHBr_3 B.P 149.1 °C) than the chlorinated trihalomethanes (chloroform CHCl_3 B.P 61.2 °C). In essence, if volatility is reduced and both bromide and chloride ions are present in solution, tomato plants tend to respond to the more reactive chloride ion (Cl^-). However, if measures are not taken to reduce the volatility of the chlorinated trihalomethanes then a larger proportion of the more brominated trihalomethanes would remain in solution, hence, only the effects of the brominated trihalomethane would be observed.

The experiments on growing plants revealed a reduction in the concentration levels of key elements required for plant growth (N, P, K, S and B) (Fig. 2) that correlated with an increasing degree of trihalomethane chlorination (Table 1). Similar relationships on the potential of increasing chlorination inhibiting plant growth have been reported for

Table 3 Pearson correlation coefficient between the leaf membrane lipid components and the increasing degree of chlorination

Leaf membrane lipid components	Correlation coefficient (R) blank;value for leaf membrane lipid components with increasing degree of chlorination
α -linolenic acid methyl ester	0.9510 at P = 0.0490
Palmitic acid	0.9846 at P = 0.0154
α -tocopherol	0.5080 at P = 0.4920

chlorobenzenes in *Zea mays* by San Miguel et al. (2012).

Reactive oxygen species (ROS) are by-products of normal cell metabolism in plants having multiple roles as diffusible signals in signal transduction pathways and also as a secondary messenger in various developmental pathways in plants (Karuppanapandian et al. 2011). However, under stress conditions the balance between ROS production and its elimination is disturbed leading to ROS damage to vital cellular organelles, inactivation of key enzymes, degradation of proteins, lipids and DNA, and destruction of membranes ultimately leading to cell death (Sharma et al. 2012). Plants possess a complex battery of enzymatic and non-enzymatic antioxidant defence systems that can protect cells from oxidative damage and scavenge harmful ROS that is produced in excess of those normally required for various metabolic reactions (Gill and Tuteja 2010).

Although the trihalomethanes are not suspected of directly generating ROS in plants, as no report was found on them stimulating Fenton-like reactions in any biological samples, exposure of both cultivars to 10 mg L⁻¹ of each trihalomethane chemical species generated a different response in both non-enzymatic and enzymatic plant defenses in the current study.

The antioxidant activity of plant phenols mainly due to their redox properties has been extensively documented in the literature (Agati et al. 2012; Dangles 2012; Martín et al. 2015). Plants are known to increase their phenolic content as a non-enzymatic response to ROS production and oxidative stress (Nogués et al. 2014). Exposure of the tomato plants to trihalomethanes in this study generated an increase in total phenolic content that correlated with the trihalomethane degree of chlorination (Table 2).

The reports of plant exposure to organo-chlorinated compounds generating excessive production of peroxide (H₂O₂) a key ROS in plants have been documented (Menone et al. 2008; Michalowicz and Duda 2009; San Miguel et al. 2012). Strong correlations have been reported in the literature for parameters that measure various non-enzymatic plant responses to stress such as total polyphenol content and the ferric reducing antioxidant power (FRAP) (Bunea et al. 2011; Ma et al. 2011; Reyes-Carmona et al. 2005). A similar trend was observed in this study as FRAP values also increased with increasing chlorination of the trihalomethane molecule in the same order with total phenolic content (Fig. 3). This indicates that phenolic compounds could be one of the main components responsible for the antioxidant capacity of plant extracts in this study.

The FRAP assay although regarded as a measure of the total antioxidant capacity of sample extracts, does not measure the SH-group containing non-enzymatic antioxidant molecules. The ORAC assay has high specificity and responds to numerous antioxidants (Cao and Prior 1998). In spite of the fact that the general trend of non-enzymatic antioxidant response with respect to increasing chlorination in total polyphenol, FRAP and ORAC were similar, the chlorination order of the ORAC plant extracts tested was slightly different. The ORAC assay in itself takes into account free peroxy radical action to completion and quantifies using an area under the curve technique, but the measured antioxidant capacity depends on which oxidant or free radical is used in the measurement (Cao and Prior 1998). In essence, our ORAC values show the total antioxidant capacity of our plant extracts to peroxy radicals, and this capacity increases with increasing chlorination of the trihalomethane molecule (Fig. 3).

The dismutation of the toxic superoxide (O₂⁻) radicals into molecular oxygen (O₂) and hydrogen peroxide (H₂O₂) is a function of superoxide dismutase (Baranenko 2005). Dismutation of the superoxide (O₂⁻) radicals in this study was random and responded to no particular halogenation order in our tomato cultivars. The type of ROS produced and the balance between steady state levels of different ROS is important and are determined by the interplay between different ROS producing and ROS scavenging mechanisms (Mittler 2002). Plant exposure to

trihalomethanes appears to trigger the peroxy producing pro-oxidant pathways in plants.

The enzymatic antioxidant response of tomato cultivars in this study was an increase in the activities of the peroxidase scavenging enzymes ascorbate & guaiacol peroxidases (Fig. 4) that correlated with an increasing order of chlorination in the trihalomethane molecule (Table 2). Our results are in consonance with San Miguel et al. (2012) who reported the same effects of mono, di and tri-chlorinated benzenes on peroxidase activities in *Zea mays* suggesting that the most chlorinated compounds produced the highest amount of H₂O₂ induced peroxidase scavenging enzymes more rapidly and intensively than the least chlorinated compounds.

There was no observed chlorination or bromination order in the general peroxidation of lipids (TBARS) in the plants exposed to trihalomethanes in this study (Fig. 3). Although San Miguel et al. (2012) did report a correlation between the chlorination degree of chlorobenzenes and the extent of lipid peroxidation, however, duration of exposure, chemical structure and plant species may play a crucial role in explaining the different responses we are reporting. Nevertheless, the di-chlorinated trihalomethane molecule bromodichloromethane (CHBrCl₂) induced the highest levels of lipid peroxidation in both tomato cultivars in this study. It is interesting to note that although no other plant studies could be found to corroborate this trend, bromodichloromethane-enhanced lipid peroxidation has been documented in experimental animal studies (Das et al. 2013; Seth et al. 2013).

Plants adjusting to stress have been known to remodel membrane fluidity with the release of α -linolenic acid (9,12,15-octadecatrienoic acid 18:3) from membrane lipids a function regulated by the fatty acid desaturases (Upchurch 2008). Adjustment of membrane fluidity maintains an environment suitable for the function of critical integral proteins during stress and the release of α -linolenic acid by regulated lipase activity is the precursor molecule for phyto-oxylipin biosynthesis (Blée 2002; Gardner 1995). Evidence of the increase in α -linolenic acid that correlated to the degree of chlorination in trihalomethanes could only be seen in the TMM tomato cultivars (Table 3). Free α -linolenic acid in itself is a stress signal and its conversion to phyto-oxylipin leading to the synthesis of jasmonic acid a key signaling molecule that mediates plant stress responses to several abiotic factors has been documented in many abiotic

tolerant plant species (De Domenico et al. 2012; Wasternack 2014; Wasternack and Hause 2013; Zhao et al. 2014). This indicates that the TMM cultivars may be more tolerant to trihalomethane chemical action as compared to the TS cultivars. The accumulation of the saturated fat palmitic acid (Hexadecanoic acid 16:0) increased in a manner that correlated with the trihalomethane degree of chlorination in both tomato cultivars (Table 3). However, the extent of accumulation was greater in the TS cultivars as compared to the TMM cultivars.

The accumulation of palmitic acid is a phenomenon that has been reported in abiotic sensitive genotypes of rice plants (Cruz et al. 2010).

The concentrations of α -tocopherol in membrane lipids of both tomato cultivars did not correlate with any particular halogenation order (Fig. 5). This was surprising at first since α -tocopherol is well documented as a lipid soluble peroxy radical scavenger (Niki 2014). Since no particular halogenation order was observed when measuring lipid peroxidation (TBARS), it should be expected that no halogenation order will be evident in the lipophilic antioxidant response with the release of α -tocopherol. This may be due to the efficient scavenging of the peroxy radicals by the peroxidase group of enzymes limiting the chlorination order effects on lipid peroxidation and α -tocopherol concentrations in plant lipids.

Trihalomethane exposure caused a general activation of the tomato plants' non-enzymatic and enzymatic antioxidant systems in response to oxidative stress, especially the peroxy radical scavenging antioxidant defences which mostly correlated with the trihalomethane degree of chlorination. Antioxidant responses of plants exposed to organo-chlorine compounds have been previously described in literature (Monferran et al. 2007; San Miguel et al. 2013; Sinha 2002). The ROS production appears to be efficiently scavenged by the peroxidase group of enzymes as no particular chlorination order effects of trihalomethanes were observed in lipid peroxidation (Fig. 3).

In this 30-day exposure, trihalomethanes did not induce sufficient oxidative damage to cause cell or plant death at the concentration level of 10 mg L⁻¹, which is higher than the international standards of 0.1 mg L⁻¹ for trihalomethanes in drinking water. The trihalomethanes were chosen based on their reputation as the most frequently detected disinfection by-products in chlorinated waters and also their

molecular structure displayed both an increasing order of bromination and chlorination. Overall, the chlorine atoms appear to induce oxidative stress and this can occur through several processes. The prevailing theory suggests that during the detoxification of chlorinated compounds by peroxidases (Park et al. 2000; Talano et al. 2012), monooxygenases P450 enzymes become actively involved in the dehalogenation process leading to the loss of Cl^- atoms (Shimazu et al. 2010; Siminszky 2006).

Also, ROS species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) can displace chloride ions from chlorinated compounds generating secondary radicals of variable reactivity (Pennathur et al. 2010; Sinha 2002). Based on the reactivity series of halogens, chlorine atoms have a greater attraction for electrons than bromine atoms and hence are more reactive. Therefore, the free chlorine radicals produced from the dehalogenation processes in plants are likely to induce a greater measure of oxidative stress than the free bromine radicals. These ROS and free chlorine radicals generated from exposure to chlorinated compounds in plants may explain the relationships between oxidative stress and the degree of chlorination of trihalomethanes. The present study demonstrates that tomato plants, when exposed to trihalomethane levels normally found in finished waters, are unlikely to show any significant oxidative damage even though chlorine produced oxidants cause deleterious effects reported to appear at concentrations as low as 0.1 mg L^{-1} , about two orders of magnitude lower than the actual concentrations in chlorinated effluents (Abarnou and Miossec 1992).

Based on the data in this present study, it can be concluded that trihalomethanes have the potential to inhibit plant growth based on the decreasing levels of key nutrients required for plant growth that correlated with the number of chlorine atoms in the trihalomethane molecule which has not been reported previously. Oxidative stress was also exerted on the tomato cultivars based on the increase in total phenolic content, FRAP and ORAC values, and the induction of the peroxidase antioxidant enzymes (APX and GPX). Aromatic chlorinated compounds have been reported to induce oxidative stress with increasing degree of chlorination. It can be concluded that in general plant response to halogenated compounds with multiple types of halogens may follow the reactivity series of

the halogens in question. α -linolenic acid stress signaling in TMM cultivars that correlated with the degree of chlorination in trihalomethane molecules suggests that this variety may be more tolerant to chemical action. This also provides evidence that trihalomethanes do possess the ability to alter fatty acid compositions in tomato plant lipids which have not been previously reported.

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