Cape Peninsula
University of Technology

Disinfection by-products and their biological influence on radicle development, biomass accumulation, nutrient concentration, oxidative response and lipid composition of two tomato (*Solanum lycopersicum*) cultivars

by

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Dissertation submitted in partial fulfillment of the requirements for the degree

Doctor of Technology: Environmental Health

in the Faculty of Applied Sciences

at the Cape Peninsula University of Technology

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Cape Town
Date submitted (January 2016)

DECLARATION

I, Babatunde Cornelius Akande, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

Trihalomethanes are disinfection byproducts of chlorinated waters, and there is a growing interest to understand plant responses to organohalogens. This study investigates the effects of increasing trihalomethane dose on the physiology of tomato (*Solanum lycopersicum*) and determines whether the extent of physiological impacts of trihalomethane exposure on seedling radicle length, biomass accumulation, concentration levels of 12 key nutrients, oxidative stress, fatty acids and α-tocopherol content in membrane lipids of tomato correlated with either the number of bromine or chlorine atoms in the trihalomethane molecules. The 2 x 4 x 5 factorial experiment was laid out in CRD with four replications. Two cultivars of tomato were exposed to 4 levels of trihalomethanes (bromodichloromethane, bromoform, chloroform and dibromochloromethane) and 5 levels of concentration (0.0, 2.5, 5.0, 7.5, and 10.0 $mg.L^{-1}$) in a green house. The decrease in seedling biomass and the inhibition of radicle growth increased with increasing trihalomethane concentrations in a dose dependent manner. Also, both these parameters decreased in response to an increase in the number of bromine atoms in the trihalomethane molecule. However, in growing plants the decrease in concentration levels of seven essential nutrients namely nitrogen (N), phosphorus (P), potassium (K), sulphur (S), copper (Cu), zinc (Zn) & boron (B) correlated to an increase in the number of chlorine atoms. Increase in trihalomethane dose also induced a decrease in all the above mentioned nutrients with the addition of manganese (Mn), although the decrease in P and S were not significant at $P \leq 0.05$. The increase in trihalomethane dose induced an increase in oxidative stress parameters such as the total phenolic content, ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and lipid peroxidation. The increase in the above parameters correlated to an increase in the number of chlorine atoms, however, no such correlations were observed in superoxide dismutase (SOD) activity, general lipid peroxidation, α-tocopherol content and total

soluble proteins. In plant membrane lipids, increase in the saturated fat hexadecanoic acid was observed in both tomato cultivars that correlated to 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. Membrane lipids adjustments in tomato plants exposed to increasing trihalomethane dose were based on two factors; first the adjustments of membrane fluidity with the increase in plant sterols and fatty acids content and secondly, the increase in lipophyllic antioxidants such as phenols, quinones and α-tocopherol content. The phenolic lipophyllic antioxidant was tentatively identified to be 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl] phenol. In conclusion, the magnitude of plant responses to trihalomethanes is more dependent on the halogenation number of the molecule and less on its concentration.

ACKNOWLEDGEMENTS

I wish to thank:

- Prof Fatoki, Prof Odendaal, Prof Marnewick and Prof Ndakidemi, my supervisory team, for their guidance and support.
- Fanie Rautenbach, Berenice Ayeleso and the entire oxidative stress research unit for the invaluable training given to me.
- My parents, Prof and Mrs. Akande, without their emotional and financial support I would not have been able to complete this study.
- Finally, I would like to thank Lucky Mokwena of Stellenbosch University for lipid analysis, Eric Jondiko with his help on the statistics, Monde in helping construct the irrigation system used, Davies in his assistance with sample collection and Cape Peninsula University of Technology Research Funds for providing the necessary funding to complete this project.

DEDICATION

This study is dedicated to God. Without his grace nothing can be accomplished.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

APX Ascorbate peroxidase

BCA Pierce bicinchoninic acid

$CHBrCl_2$ Bromodichloromethane

$CHBr_2Cl$ Dibromochloromethane

DBPs Disinfection by products

FRAP Ferric reducing antioxidant power

GCMSD Gas chromatographic mass selective detector

GPX Guaiacol peroxidase

HAN Haloacetonitriles

HKs Haloketones

LDPE Low density polyethylene

MDA Malondialdehyde

ORAC Oxygen radical absorbance capacity

PAL Phenyl analine ammonia-lyase

PBT Persistent bioaccumulative toxic chemicals

PPO Polyphenol oxidase

PUFA Poly-unsaturated fatty acids

ROS Reactive oxygen species

SOD Superoxide dismutase

TBARS Thiobarbituric acid reactions

THMs Trihalomethanes

TMM Tomato money maker

TNM Trihalonitromethanes

TS Tomato Star

CHAPTER ONE
INTRODUCTION AND BACKGROUND

1.1 Introduction

It was estimated in 1995 that about 2.3 billion people (41% of the world's population at that time) resided in river basins considered to be water stressed and this value was predicted to increase to 3.5 billion by 2025 (48% of the expected world population) (Hamilton et al., 2007). Approximately, 70% of world water use including all the water diverted from rivers and pumped from underground are used for agricultural irrigation (Pedrero et al., 2010). Therefore, the reuse of treated municipal wastewater for purposes such as agricultural and landscape irrigation reduces the amount of water that needs to be extracted from natural water sources as well as reducing discharge of wastewater to the environment. This necessitated the need to implement strategies to ease the pressures of demand on potable water. Hence, wastewater use in agriculture became a viable economic alternative (Anderson, 2003).

There have been obstacles in the definition of wastewater and the lack of data on this subject has made it complex to arrive at a strong figure for global wastewater reuse for irrigation. Nevertheless, attempts were made and probably the best known estimate in 2001 was 20 million hectares of land which were irrigated with wastewater partially diluted or undiluted (Scott et al., 2004). In general, municipal wastewater is made up of domestic wastewater, industrial wastewater, storm water and groundwater seepage entering the municipal sewage network (Al Salem, 1996).

Wastewaters of municipal and industrial origin are used to irrigate a wide variety of crops and landscapes across the world (Hamilton et al., 2005). In the early nineties, a report was compiled based on the case studies of wastewater use in agriculture in six

countries, namely USA, Jordan, Kuwait, Tunisia, Mexico and India (Pescod, 1992). The report focused on the quality of wastewater, its heavy metal content, faecal coliforms, viral load, and the organic pollutant content of wastewater used in these respective countries. Organic compounds found in these wastewaters by gas chromatography mass spectroscopy ranged from fatty acids, resin acids, clofibric acid, alkylphenol polyethoxylate carboxylic acids (APECs), trimethylbenzene sulphonic acid, steroids, n-alkanes, caffeine, diazinon, alkylphenol polyethoxylates (APEs) and trialkylphosphates. In developing countries such as Pakistan, it was reported that 26% of the total domestic vegetable production was cultivated with wastewater (Ensink et al., 2004). Concerns on the need to reduce environmental pollution and risks from agricultural use of wastewater have been raised in Ghana (Keraita et al., 2003). Some developing countries have attempted to reuse untreated wastewaters such as the case in Eritrea where heavy contamination of the vegetables by faecal coliforms as well as Giardia cysts were reported (Srikanth and Naik, 2004). The use and disposal of grey-waters in non sewered areas in South Africa, in terms of environmental and health problems have already been investigated (Carden et al., 2007).

The increasing extent and diversity of wastewater use, even without the appropriate treatment presents public health risks (Keraita et al., 2015). Recently, it was reported that *Ascaris lumbricoides* and *Trichuris trichiura* helminth infections were associated with wastewater use in Vietnam (Pham-Duc et al., 2013). 66% of the study participants were infected with helminths (*Ascaris lumbricoides* 24%, *Trichuris trichiura* 40% and hookworm 2%). However, infections with intestinal protozoa were rare (i.e. *Entamoeba histolytica* 6%, *Entamoeba coli* 2%, *Giardia lamblia* 2%, *Cryptosporidium parvum* 5% and *Cyclospora cayetanensis* 1%).

In view of the potential health risks through bacterial, viral and helminth infections associated with “untreated” grey-waters, the practice of wastewater treatment before reuse in agriculture became a norm. Chlorine is a widely utilised disinfectant and as such is a leading contender for disinfection of grey water intended for reuse. Adequate disinfection of grey water prior to reuse is important to prevent the potential transmission of disease-causing microorganisms (Winward et al., 2008).

However, the consequences of chlorine use led to the reaction between chlorine and humic substances, a major component of natural organic matter, which led to the production of disinfection by-products during wastewater treatment (Adin et al., 1991). Humic and fulvic acids show a high reactivity towards chlorine and constitute 50 - 90% of the total dissolved oxygen content in river and lake waters (Gallard and von Gunten, 2002). Most chlorine disinfection by-products (DBPs) are formed through oxidation and substitution reactions. Trihalomethanes (THMs) are disinfection by-products from the chlorination of water and have the general formula CHX_3, where X can be Cl^- or Br^- (Xie, 2004). 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).

The general focus of research has been on the mode of delivery to humans and the carcinogenic or mutagenic potential of disinfection by-products and trihalomethanes in potable waters (Shafiee et al., 2012, Grellier et al., 2015). Since scientific concern has been unevenly focused on the human perspective in terms of trihalomethane exposure in the past, knowledge is lacking on physiological impacts trihalomethanes may induce on growing crops exposed during wastewater irrigation.

Recently, the focus shifted towards the physiological and phytotoxic effects of organo-chlorines on plants (San Miguel et al., 2012, Ahammed et al., 2013, Guittonny-Philippe et al., 2015, Li et al., 2015). Chlorine produced oxidants' first signs of deleterious effects have been reported at concentrations as low as 0.1 mgL^{-1}, about two orders of magnitude lower than the actual concentrations in chlorinated effluents (Abarnou and Miossec, 1992). Other studies have reported plants which have the ability to stimulate the removal of chlorinated organic pollutants through plant uptake, phytovolatilization and/or phytodegradation (Imfeld et al., 2009, Doucette et al., 2013, Chen et al., 2014).

Despite the widespread detection of trihalomethanes in almost every surface water body, trihalomethanes are interesting because their molecular structure shows an increasing order of both bromination and chlorination. With chlorination an increasing order of $CHBr_3$ < $CHBr_2Cl$ < $CHBrCl_2$ < $CHCl_3$ is observed, while the order of bromination increases as thus $CHCl_3$ < $CHBrCl_2$ < $CHBr_2Cl$ < $CHBr_3$. 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. The data could also aid in the development of models that could be used as a tool to estimate the physiological impacts of different levels of trihalomethane chemical species in wastewater on crop physiology.

Since the case studies of the biological responses of maize (*Zea mays*) plants exposed to increasing concentrations of chlorobenzenes and *Arabidopsis* to trichlorophenol revealed growth inhibition and induction of oxidative stress (San Miguel et al., 2012, Li et al., 2015), we focused our studies on the physiological response of tomato (*Solanum*

lycopersicum) to increasing concentrations of trihalomethanes, which has not been examined previously.

Root elongation tests in plant seedlings have been used to evaluate or assess toxicity of both organic and inorganic compounds with the inhibition of radicle and plumule growth suggesting toxicity (Di Salvatore et al., 2008). Recently, toxicity of brominated and chlorinated pesticides such as deltamethrin, alpha cypremithrin and polybrominated diphenyls have been tested on the radicle length of maize, mangrove and tomato seedlings (Chadid et al., 2013, Wang et al., 2014, Duran et al., 2015). The effects of trihalomethanes on the radicle length of two varieties of tomato seedlings are reported in the present study.

Ahammed et al. (2013) evaluated the effects of polychlorinated bi-phenyls on biomass accumulation in tomato by measuring dry weight. The present study reports the effects of trihalomethanes on biomass accumulation by measuring fresh and dry weights of tomato seedlings and the variations on nutrient concentration with increasing trihalomethane dose in 12 essential nutrients required by growing plants after a 30 day exposure. 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).

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). However, under stress conditions the balance between the production of ROS and its elimination is disturbed (Karuppanapandian et al., 2011).

Reactive oxygen species (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). A majority of the antioxidant defenses in plants stimulated by abiotic factors such as light stress and drought are also stimulated by organic pollutants (Wang et al., 2008, Yang et al., 2008, Faure et al., 2012).

Methods have been developed to measure oxidative stress tolerance in plants exposed to abiotic factors. Non-enzymatic antioxidant responses can be measured using techniques such as the determinations of total phenolic content, ferric reducing antioxidant power (FRAP) and the oxygen radical absorbance capacity (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 offers 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).

The oxidation of lipids is particularly dangerous because it propagates the production of free radicals through so-called ‘chain reactions’. Lipid oxidation (also known as lipid peroxidation) is widely considered as a “hallmark” of oxidative stress (Farmer and Mueller, 2013). Severe lipid peroxidation leads to the damage of membranes, collapse of their barrier function, followed by disintegration of organelles, oxidation and dysfunction of proteins, DNA and RNA (Gutteridge and Halliwell, 2000, Farmer and Mueller, 2013). The terminal products of lipid peroxidation are ‘aggressive’ substances, such as aldehydic secondary products (malondialdehyde, 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and acrolein), which are markers of oxidative stress (Lykkesfeldt, 2007, Farmer and Mueller, 2013). They are easy to measure by using the thiobarbituric acid assay for malondialdehyde (Hodges et al., 1999).

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 lipid sterols, fatty acids and esters or the production of novel lipid-metabolic species (Larson and Graham, 2001, Wallis and Browse, 2002, Gajewska et al., 2012).

The main aim of this present work was to assess physiological and defense system responses in two closely related tomato cultivars, experimentally exposed to the four trihalomethane chemical species. With an aim to contribute to the better understanding of the physiological response of plants to these water-based disinfectant by-products and organohalogens as a whole, we theorized that the magnitude of physiological response may be correlated to the trihalomethane chlorination or bromination order. We also compared this to the effect of increasing trihalomethane dose in the tomato cultivars investigated. To confirm our assumptions we focused on the concentration levels of 12 essential plant nutrients, radicle inhibition, biomass accumulation in tomato seedlings, enzymatic and non-enzymatic antioxidant responses, lipid peroxidation, fatty acids and tocopherol concentrations in tissues of tomato plants exposed to each trihalomethane compound. The *Solanum lycopersicum* cultivars were selected based on their popularity, high yields and stability in the climatic conditions of Cape Town, South Africa, where this study was conducted. The uptake of organochlorine compounds by tomato plants have been recently reported (Zhang et al., 2015).

1.2 Problem Statement

The necessity for the control of biological contamination in grey water before its intended reuse in agriculture leads to the production of disinfection by-products that may have considerable effects in the long term on plant performance, since crops are directly exposed during irrigation.

Chlorine is a cheap, cost effective disinfectant used in many countries and hence is the leading contender amongst disinfectants used in the control of biological contamination of waste water. However, the consequences of this treatment may lead to the formation of disinfection by-products (DBPs) from the reaction between organic/inorganic matter and disinfectants (Richardson et al., 2007, Sedlak and Von Gunten, 2011). The organic matter content of grey-water is usually much higher than that of potable water and as such the chlorine dose required for complete disinfection is much more (Abarnou and Miossec, 1992). The formation of trihalomethanes and disinfection by-products have been known to rise with increasing chlorine dose and organic matter content (Brown et al., 2011). Most investigators have found this rise to be a linear relationship between chlorine dose and trihalomethane production (Amy et al., 1987, Elshorbagy et al., 2000, Gallard and von Gunten, 2002). Trihalomethanes (THM's) and haloacetic acids are the two most prevalent classes of DBPs in treated wastewater (Krasner et al., 2006). 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). Apart from the trihalomethanes, generally, scientific interest has been increasing lately on the mechanisms behind the physiological and phytotoxic plant responses to organo-halogens (San Miguel et al., 2012, Ahammed et al., 2013, Guittonny-Philippe et al., 2015, Li et al., 2015).

Previously, research on the trihalomethanes was focused on the carcinogenic and mutagenic properties of these compounds in potable water and its mode of delivery or exposure to humans. The phytotoxicity and physiological responses of crop plants in direct exposure during wastewater irrigation was not examined. Though the nature of human exposure and health risks to disinfection by-products has been well documented, mechanisms of plant toxicity and physiological alterations due to trihalomethane exposure need to be investigated. The likely consequences of such exposure to trihalomethanes may lead to adverse effects that can ultimately affect plant performance, yield or plant susceptibility to pathogens.

1.3 Purpose Statement

This study will describe and compare the effects of increasing trihalomethane dose and increasing halogenation on radicle inhibition, seedling biomass, nutrient concentration, oxidative responses and changes in membrane lipid composition of two tomato (*Solanum lycopersicum)* cultivars.

1.4 Objectives of the Research

The first objective was to report the effects of increasing trihalomethane dose on physiological parameters such as radicle development, seedling biomass, nutrient concentration levels, enzymatic and non-enzymatic oxidative responses, and changes in membrane lipid compositions of the two tomato cultivars.

The second objective was to investigate whether the extent of the physiological impacts of trihalomethane exposure correlated with either an increasing number of bromine or chlorine atoms in the trihalomethane molecules with trihalomethane dose kept constant, focusing on radicle development, seedling biomass, nutrient concentration levels, oxidative stress, fatty acids and α-tocopherol content of membrane lipids in the two tomato cultivars. The aim was also to compare the results from increasing trihalomethane dose with the results of increasing number of halogen atoms on the trihalomethane molecules at a constant dose to elucidate which factor induces a greater physiological response in plants.

The final objective was to investigate the effects of varying concentrations of trihalomethanes on the tomato cultivars.

1.5 Significance of the Research

This study aims to contribute to the ever growing body of knowledge on plant responses to organohalogens. Treated wastewater for reuse in agriculture is known to contain disinfection by-products of water chlorination and other organohalogens compounds capable of altering plant physiology and performance.

1.6 Limitations of the Study

This study was conducted in a greenhouse and as such results may vary slightly from field trials and seasonal variations in results can occur. The experiments in this study though conducted in a greenhouse were designed to simulate field conditions as much as possible. The experiments were conducted within the optimum seasonal range for tomato growth in the Southern African region between the months of January and April (2013).

1.7 Research Assumptions

The study assumes that the trihalomethanes commonly found in treated wastewaters in sufficient concentrations have the potential to affect plant physiology and ultimately plant biomass with persistent exposure during wastewater irrigation practices.

The study also assumes the physiological “effect” of this group of organohalogens known as the trihalomethanes is a concentration dependent dose-response relationship and the magnitude of physiological response may correlate to an increasing number of bromine or chlorine atoms in the trihalomethane molecule.

1.8 Instruments Used

The instruments employed in this study include the nuaire glacier ultra low minus 86 ^{o}C freezer, micro plant grinder model FZ102, Varian ICP-OES optical emission spectrometer, Leco Nitrogen-analyzer, Vir-Tis genesis freeze dryer, intelli-mixer RM2 rotator/mixer, eppendorf centrifuge 5810 R, Thermo Scientific multiskan spectrum spectrophotometer, Thermo Scientific fluroskan ascent 2.5 plate reader, and the Agilent 6890 gas chromatographic mass spectroscopy (GC-MS).

1.9 Organization of the remainder of the study

The remainder of the study consists of the following;

(A) Chapter two will review the literature on trihalomethanes, its formation and occurrence in chlorinated treated wastewaters.

(ii) The possibility of trihalomethanes inducing stress on plants as organohalogens, oxidative stress and the techniques developed to measure oxidative stress tolerance in plants exposed to abiotic factors.

(iii) Stress modulated changes in nutrient concentration, radicle development, biomass accumulation and membrane lipid composition of plants.

(iv) Protein oxidation and DNA damage in plants by Reactive oxygen species (ROS).

(v) Reactive oxygen species (ROS) scavenging and antioxidant mechanisms.

(B) Chapter three will detail the research methodology of this study.

(C) Chapter four will present the data collected and propose mechanisms that explain the findings.

(D) Chapter five will present the conclusions and recommendations of this study. The reference section lists works cited in this study.

CHAPTER TWO

LITERATURE REVIEW

2.1. Trihalomethanes: occurrence and formation in chlorinated wastewater

It is scientifically accepted that the reaction between chlorine and humic substances, a major component of natural organic matter, is responsible for the production of disinfection by-products during drinking water treatment (Thurman, 1985). Humic and fulvic organic acids demonstrate a high reactivity towards chlorine (Gallard and von Gunten, 2002).

Chlorine disinfection by-products (DBPs) are formed mainly through oxidation and substitution reactions. The well known DBPs, such as trihalomethanes (THMs), haloacetic acids (HAAs), bromide, and chlorite, are regulated in the U.S. and in other countries, but other "emerging" DBPs, such as iodo-acids, halonitromethanes, haloamides, halofuranones, and nitrosamines, are not widely regulated (Richardson and Postigo, 2012). DBPs have been reported for the four major disinfectants: chlorine, chloramines, ozone, and chlorine dioxide (and their combinations), as well as for newer disinfectants, such as UV treatment with post-chlorination (Richardson and Postigo, 2012). Each disinfectant can produce its own range of by-products. Several classes of emerging DBPs are increased in formation with the use of alternative disinfectants (e.g., chloramines), including nitrogen-containing DBPs ("N-DBPs"), which are generally more genotoxic and cytotoxic than those without nitrogen.

Trihalomethanes (THMs) have the general formula CHX_3, where X can be Cl^- or Br (Xie, 2004). Urban wastewaters containing high concentrations of organic carbon and ammonia, requires higher chlorine dosage (5–20 $mg \cdot 1^{-1}$) to fulfill the disinfection

requirements (Abarnou and Miossec, 1992). The chlorine dose is a factor that affects the type and concentration of DBPs formed. The THMs level rises with increasing chlorine dose (Brown et al., 2011). Most investigators found a linear relationship between chlorine consumption and THM production, with an order of reaction greater than or equal to unity (Amy et al., 1987, Elshorbagy et al., 2000, Gallard and von Gunten, 2002).

The formation of trihalomethanes during the disinfection stage of water treatment is a cause of health concerns in many countries (Dunnick and Melnick, 1993). The disinfection of wastewater is a necessary procedure as microbial contamination is the largest cause of water-borne diseases at a global scale. However, these chemicals and their by-products in water supplies have been related to health risks when associated with long term exposure (Villanueva et al., 2014). Epidemiological studies have reported associations of trihalomethane (THM) levels in drinking water with bladder cancer at THM levels lower than the current regulations in the United States and in the European Union (80 and 100 µg/L, respectively (Villanueva et al., 2004). The *in vitro* mammalian cell toxicity for a wide range of DBPs was conducted in an attempt to identify specific DBPs responsible for genomic DNA damage (Jeong et al., 2012). They concluded that the agents responsible for the genomic DNA damage observed in the HIWATE samples may be due to unresolved associations of combinations of identified DBPs, unknown emerging DBPs that were not identified, or other toxic water contaminants. Bladder cancer has consistently been associated with DBP exposure (Cantor, 2010) and pooled analysis combining data from studies conducted in different countries have reported associations between bladder cancer and THMs at levels below the maximum contamination levels of (80 and 100 µg/L) (Villanueva et al., 2014).

A range of non-neoplastic toxic effects have been associated with short-term and long term exposure of experimental animals to high doses of THMs. The most common THMs

which include chloroform, bromodichloromethane, dibromochloromethane and bromoform have been shown to be carcinogenic or mutagenic to rodents in high dose chronic studies. Chloroform has been known to induce toxic effects in livers and kidneys via production of reactive metabolites (Sasso et al., 2012). In a corn oil gavage study of single-dose chloroform effects, an increase in renal cell proliferation was observed at doses as low as 10 mg kg^{-1} of body weight in male Osborne-Mendel rats and 90 mg kg^{-1} of body weight in male F344 rats (Templin et al., 1996). Bromodichloromethane ($CHBrCl_2$) was found to induce both hepatotoxic and nephrotoxic effects in female rats where it was administered in aqueous gavage (Thornton-Manning et al., 1994). A depression in immune function was also observed in both sexes of CD-1 mice gavaged with dibromochloromethane in an aqueous vehicle given at doses of 125 and 250 mg kg^{-1} of body weight per day (Munson et al., 1982). Amongst all the brominated THMs, bromoform is the least potent as a lethal acute oral toxicant (Fawell, 2000).

Evidently, research has focused primarily on the carcinogenic and mutagenic effects of THMs on mammalian cells, and few studies have been conducted on their phytotoxic properties since plants are directly exposed during irrigation. Recently, scientific interest has been on the rise with numerous studies on plant response to organohalogens. A review was published that summarizes the recent progress made towards understanding how the various mechanisms attributed to organic chemical removal interact to form a functioning wetland and the main degradation pathways for different groups of organohalogens (Imfeld et al., 2009). The phytoremediation of soils containing trichloroethylene was achieved through volatilization by trees primarily through the leaves at the phytoremediation sites (Doucette et al., 2013).

The removal efficiencies and the kinetics of disinfection by-products (DBPs) were studied in six greenhouse laboratory-scale subsurface flow constructed wetlands. Cattail

(*Typha latifolia*) and its litter were used as a potential phytoremediation technology and as a primary substrate, respectively, for DBP removal (Chen et al., 2014). The results reported stated that in the primary DBPs in wastewater effluents, the removal efficiencies of chloroform were higher in planted systems than in unplanted ones and plant uptake accounted for more than 23.8% of the removal.

Disinfection by-products require further investigations to precisely evaluate their potential hazard to plant life because of their persistence and mutagenic character (Meier, 1988, Stackelberg et al., 2004). Chlorine produced oxidants' first signs of adverse effects 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). More than 250 DBPs have been identified, but the behavioral profile of only approximately 20 DBPs are adequately known (Sadiq and Rodriguez, 2004).

2.2. Phytotoxic effects of disinfection by-products as organohalogens

Chlorination of wastewater effluents have been reported to produce halogenated carbon compounds such as the trihalomethanes (THMs), haloketones (HKs), nitrogenous DBPs, haloacetonitriles (HANs) and trihalonitromethanes (TNMs) (Yang et al., 2007).

Some of these compounds are carcinogenic and are consequently of health and regulatory concern (Hrudey, 2009, Krasner et al., 2009). Furthermore, some DBPs found in wastewater treatment effluents may also be toxic to aquatic organisms (Emmanuel et al., 2004). These contaminants have received much attention in recent years due to their presence in surface waters, but little attention has been paid to their occurrence in reclaimed waters meant for reuse in agriculture. This becomes important as many of these organo-halogen compounds do possess phytotoxic properties capable of stimulating an unprecedented response in plants exposed during irrigation (Calderón-Preciado et al., 2011).

A number of recent studies have attempted to shed some light on plant responses to organohalogens. A case study was investigated (San Miguel et al., 2012) where they reported the biological responses of maize (*Zea mays*) to mono, di, and trichloro-benzenes. They reported that chlorobenzene exposure resulted in an increase in oxidative stress that correlated with the compounds degree of chlorination and concentrations less than 10 mg/L did not induce sufficient oxidative damage to cause root cell death. Brassinosteroids have been reported to alleviate different abiotic stresses including organic pollutant-induced stress. Hence, studies on the effects of polychlorinated biphenyls (PCBs) and 24-epibrassinolide (EBR) on biomass accumulation, photosynthetic machinery and antioxidant system in tomato plants were conducted (Ahammed et al., 2013). They reported that PCBs (0.4, 2.0 and 10 µg/l) mist

spray significantly decreased dry weight, photosynthesis, and chlorophyll contents in a dose dependent manner.

Both stomatal and non-stomatal factors were involved in PCBs-induced photosynthetic inhibition. Applications for these plant responses to organohalogens compounds have been proposed as an ecotoxicity tool based on the morphological responses of some selected helophytes (Guittonny-Philippe et al., 2015). A detailed morphological and physiological response of *Arabidopsis* to 2,4,6-trichlorophenol, a representative TCP, was investigated (Li et al., 2015). They discovered that seed germination and seedling growth were markedly inhibited by 2,4,6-TCP. Furthermore, they performed gene expression profiling analysis upon 2,4,6-TCP treatment in *Arabidopsis* and identified 34 transcripts induced and 212 repressed more than four folds. These TCP-responsive genes are involved in various biological processes, such as secondary metabolism, biological regulation, response to stimulus and other processes related to growth and development. Tomato plants themselves were reported to be effective in the enhancement of pollutant bioavailability in rhizospheric soils (Mitton et al., 2014).

The success of crop plants exposed to organic pollutants commonly found in reclaimed waters for reuse in agriculture may be conditioned by two main factors: the concentration or bioavailability and the toxicity of these organic compounds. The impacts of organic pollutants in wastewater irrigation need to be understood in terms of mechanism of toxicity and physiological performance of crop plants during exposure. Changes in nutrient concentration, biomass accumulation, radicle inhibition, cellular oxidative state and lipid composition of exposed crop plants could prove as effective biomarkers.

Plant performance and development largely depend on mineral nutrients available in the soil. Mineral nutrients are usually obtained from the soil through plant roots, but many

factors can affect the efficiency of nutrient acquisition (Morgan and Connolly, 2013). Many of the organic xenobiotics deposited on plant surfaces are generally characterized as micro-pollutants, due to their small concentrations (Verkleij et al., 2009). However, they can exert toxicity or stress if they are able to penetrate from the root or leaf surface (Coleman et al., 2002).

There is an inherent ambivalence in plant tolerance to organic pollutants. In certain ways organic pollutants at low concentrations are beneficial to plants as secondary metabolites or signals of reactive oxygen species (ROS). However, organic xenobiotics can exert damaging effects to plants as well at similar or higher concentrations depending on specific compound and plant species/genotype (Verkleij et al., 2009). Organic pollutants cause damage after uptake and transport into plant tissues by altering enzyme function, promoting DNA adduct formation and inducing oxidative stress (Pilon-Smits, 2005, Verkleij et al., 2009, Sharma et al., 2012).

2.3 Physiological indicators of stress in plants

Root elongation tests in plant seedlings have been used to evaluate or assess toxicity of both organic and inorganic compounds with the inhibition of radicle and plumule growth suggesting toxicity (Di Salvatore et al., 2008). The effect of alpha-cypermethrin on seed germination and seedling growth of tomato has been studied based on morphological parameters and by using four dilutions of the normal concentration used in agriculture (100%, 75%, 50%, 25%) for germinating seeds, and only the normal concentration used in agriculture for growing tomato plants (Chadid et al., 2013). The results indicated that alpha-cypermethrin induced a delay of germination and growth process. The germination rate of treated seeds was generally 20% lower than the control treatment. Furthermore, the length of roots and shoots in treated seeds was significantly reduced. In this regard, shoot length of the treated seedlings was 25% and 50% reduced for the concentrations of 25% and 100%, respectively, when compared to control shoot length. The effects of polybrominated diphenyl ether congener (BDE-47) on the growth and antioxidative responses of the seedlings of *Kandelia obovata* and *Avicennia marina* were studied in an 8-week hydroponic culture spiked with different levels of BDE-47, 0.1, 1, 5 and 10 mg l^{-1} (Wang et al., 2014). They reported that the two highest BDE-47 levels significantly suppressed root growth and increased the activities of three antioxidant enzymes, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), of *Kandelia obovata* in week 1. The effects of some brominated pesticides such as deltamethrin on the biological properties of maize were recently investigated by Duran et al. (2015). Maize seeds were exposed to environmentally relevant dosages (0.01, 0.05, 0.1 and 0.5 ppm) of deltamethrin. The results indicated that all seedling growth characters were decreased with increasing deltamethrin levels. The most negative effect on the radicle length of maize was observed by the highest deltamethrin concentration with a 61% decrease ($P < 0.05$).

Radicle growth has been reported as a far more sensitive endpoint than plumule emergence for most substances and concentrations of organic compounds (Sverdrup et al., 2003). The effects of trihalomethanes on radicle development have not been reported previously.

Alterations in biomass accumulations have also been used by researchers as a response mechanism by plants to toxicity. The study conducted by Ahammed et al., (2013) mentioned previously indicated that mist sprays of PCB's decreased plant dry weight. There are several mechanisms by which organo-halogens may induce biomass loss in plants amongst which is the effect on nutrient uptake by plants. A mechanism put forward suggests that detoxifying enzymes requiring metal co-factors may cause the plant to alter the rate of mineral uptake of some macro and micro nutrients as a defense mechanism (Huber, 2010).

There are several families of proteins involved in the transport of mineral nutrient metals in plants. The largest families of transport proteins include: (i) influx transporter families such as natural resistance associated macrophage protein (NRAMP), zinc-regulated transporter (ZIP), iron-regulated transporter protein, yellow-stripe 1-like (YSL) subfamily of the OPT super family; and (ii) efflux protein families: P_{1B}ATPases, cation diffusion facilitator (CDF), cation exchanger (CAX) and ATP-binding cassette transporters (ABC) (Hall and Williams, 2003). Organic pollutants are known to have genotoxic tendencies and can inhibit to a large extent DNA repair mechanisms, gene expression and damage of transporter proteins (Kovalchuk et al., 2001).

Reactive oxygen species (ROS) are reactive molecules produced in living cells during normal vital processes (e.g. electron transport in photosynthesis, aerobic respiration, and

enzyme catalyzed reactions) and are generated in most cell compartments (Mittler et al., 2004, Del Río et al., 2006, Navrot et al., 2007). ROS consists of both free radicals ($O_2^{\bullet-}$, superoxide anion radical; OH•, hydroxyl radical; HO_2•, perhydroxy radical and RO•, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen) (Halliwell, 1987). Several reactive oxygen species (ROS) are continuously produced in plants as by-products of aerobic metabolism. Depending on the nature of the ROS, some are highly toxic and rapidly detoxified by various cellular enzymatic and non-enzymatic mechanisms (Apel and Hirt, 2004).

The balance between prooxidant and antioxidant molecules under homeostasis is maintained only where the latter provides a good protection against ROS. However, interactions between ROS and antioxidants may occur under stress conditions, e.g. exposure to radiation (Agarwal, 2007, Yang et al., 2008), temperature stress (Suzuki and Mittler, 2006, Lushchak, 2011), water stress (Pan et al., 2006, Wang et al., 2008) and exposure to inorganic or organic pollutants (Skórzyńska-Polit et al., 2004, Mobin and Khan, 2007, Dixon et al., 2010).

Disturbances in the prooxidant-antioxidant balance lead to potential damage and ROS toxicity on various cell components (Kappus, 1987, Sies, 1991). It has been estimated that 1-2% of O_2 consumption leads to the formation of ROS in plant tissues (Bhattacharjee, 2005). There are varieties of reactions where $O_2^{\bullet-}$ leads to the formation of H_2O_2, OH and other types of ROS. The accumulation of ROS as a result of various environmental stresses is a major cause of the loss of crop yield worldwide (Mittler, 2002, Gill et al., 2011). It is important to note that whether ROS will act as damaging, protective or signalling factors depends on the delicate equilibrium between ROS

production and scavenging at the proper site and time (Gratão et al., 2005). ROS can damage cells as well as initiate responses such as new gene expression.

The cell response evoked is strongly dependent on several factors. The sub cellular location for the formation of an ROS may be especially important for a highly reactive ROS, because it diffuses only a very short distance before reacting with a cellular molecule.

Plant cells use antioxidant defense systems for protection against ROS produced during oxidative stress. These defense systems which are distributed in all cell compartments (cytoplasm and organelles) are both non-enzymatic (e.g. glutathione, proline, α-tocopherol, carotenoids and flavonoids) or enzymatic (e.g. superoxide dismutase (SOD), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) (Mittler et al., 2004, Gill and Tuteja, 2010). Stress tolerance in plants may therefore be improved by the enhancement of *in vivo* levels of antioxidant enzymes. The above antioxidants are found in almost all cellular compartments, demonstrating the importance of ROS detoxification for cellular survival (Gill et al., 2011).

It has also been shown that ROS can influence the expression of a number of genes and signal transduction pathways which suggest that cells have evolved strategies to use ROS as biological stimuli and signals that activate and control various genetic stress-response programs (Dalton et al., 1999). Recently, it has become apparent that plants actively produce ROS which may control many different physiological processes such as biotic and abiotic stress-response, pathogen defense and systemic signalling (Halliwell, 2006).

The generation of O_2 may trigger the formation of more reactive ROS like OH, and more possibly $^1O_2$, that can lead to the peroxidation of membrane lipids and cellular weakening (Elstner and Osswald, 1994, Halliwell, 2006).

It has been noted that O_2 can undergo protonation to give up a strong oxidizing agent, HO_2 in negatively charged membrane surfaces, which directly attack the poly-unsaturated fatty acids (Bielski et al., 1983). Furthermore, O_2 can also donate an electron to iron (Fe^{3+}) to produce a reduced form of iron (Fe^{2+}) which can then reduce H_2O_2, produced as a result of SOD led dismutation of O_2 to OH. The reactions through which O_2, H_2O_2 and iron rapidly generate OH is called the Haber-Weiss reaction, whereas the final step which involves the oxidation of Fe^{2+} by H_2O_2 is referred to as the Fenton's reaction.

$$O_2 + Fe^{3+} \rightarrow {}^1O_2 + Fe^{2+}$$

$$2O_2 + 2H^+ \rightarrow SOD \rightarrow O_2 + H_2O_2\ Fe^{3+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH \text{ (Fenton reaction)}$$

It is well documented in literature that $^1O_2$ is efficiently quenched by β-carotene, tocopherol or plastoquinone and if not, $^1O_2$ can activate the upregulation of genes, involved in the molecular defense responses against photo-oxidative stress (Krieger-Liszkay et al., 2008).

The univalent reduction of O_2 produces H_2O_2. H_2O_2 is moderately reactive and has relatively long half-life (1 ms) whereas, other ROS such as O_2, OH and $^1O_2$, have much shorter half-life (2 - 4 μs) (Bhattacharjee, 2005). It has been well documented that an

excess of H_2O_2 in the plant cells leads to the occurrence of oxidative stress. H_2O_2 may inactivate enzymes by oxidizing their thiol groups. H_2O_2 plays a dual role in plants. In low concentrations, it acts as a signal molecule involved in acclamatory signalling triggering tolerance to various biotic and abiotic stresses and, at high concentrations, it leads to programmed cell death (Quan et al., 2008).

H_2O_2 has also been shown to act as a key regulator in a broad range of physiological processes, such as senescence (growth phase from full maturity to death), photorespiration and photosynthesis, stomatal movement, cell cycle and growth and development (Gill and Tuteja, 2010). H_2O_2 is starting to be accepted as a second messenger for signals generated by means of ROS because of its relatively long life and high permeability across membranes (Quan et al., 2008). In an interesting study the response of pre-treated citrus roots with H_2O_2 (10 mM for 8 h) or sodium nitro prusside (SNP; 100 mM for 48 h) was investigated to know the antioxidant defense responses in citrus leaves grown in the absence or presence of 150 mM NaCl for 16 d (Tanou et al., 2009). It was noted that H_2O_2 and SNP increased the activities of leaf SOD, CAT, APX and GR along with the induction of related-isoform(s) under non-NaCl-stress conditions.

Hydroxyl radicals particularly OH^- are among the most highly reactive ROS known. In the presence of suitable transitional metals, especially Fe, OH can also be produced from O_2 and H_2O_2 at neutral pH and ambient temperatures by the iron-catalyzed, O_2 driven Fenton reactions.

$$H_2O_2 + O_2^- \rightarrow Fe^{2+}, Fe^{3+} \rightarrow OH^- + O_2 + OH$$

These OH are thought to be largely responsible for mediating oxygen toxicity *in vivo*. Hydroxyl (OH) can potentially react with all biological molecules like DNA, proteins, lipids, and almost any constituent of cells and due to the absence of any enzymatic mechanism for the elimination of this highly reactive ROS, excess production of OH ultimately leads to cell death (Vranová et al., 2002).

Photosynthesizing plants are especially at the risk of oxidative damage due to their oxygenic conditions and the abundance of the photosensitizers and the poly-unsaturated fatty acids in the chloroplast envelope. In the presence of light the chloroplasts and peroxisomes are the main source of ROS generation (Foyer and Noctor, 2003). In the darkness the mitochondria appear to be the main ROS producers. It has been estimated that 1 - 5% of the O_2 consumption of isolated mitochondria results in ROS production (Møller, 2001).

Plant mitochondria are sites of energy production which are believed to be a major site of ROS production such as H_2O_2 as well as the ROS targets (Rasmusson et al., 2004). The cellular environment of plant mitochondria is also distinctive because of the presence of photosynthesis, which creates an O_2 and carbohydrate (sucrose, glucose and fructose) rich environment. The mitochondria harbours electrons with sufficient free energy to directly reduce O_2 which is considered the unavoidable primary source of mitochondrial ROS generation, a necessary accompaniment to aerobic respiration (Rhoads et al., 2006).

However, ROS production in mitochondria takes place under normal respiratory conditions but can be enhanced in response to various biotic and abiotic stress conditions. Since the O_2 consumption of isolated mitochondria leads to H_2O_2 production,

this H_2O_2 can react with reduced Fe^{2+} and Cu^{+} to produce highly toxic OH, and these uncharged OH can penetrate membranes and leave the mitochondrion (Sweetlove and Foyer, 2004, Rhoads et al., 2006). The peroxidation of mitochondrial membrane polyunsaturated fatty acids is initiated by the removal of a hydrogen atom by ROS, especially by OH. This leads to the formation of cytotoxic lipid aldehydes, alkenals, and hydroxyalkenals, such as the much investigated 4-hydroxy-2-nonenal and malondialdehyde (MDA). Once formed, lipid peroxidation products can cause cellular damage by reacting with proteins, other lipids, and nucleic acids. Key oxylipins and smaller, lipid-derived reactive electrophile species may also be produced from lipid oxidation products (Halliwell, 2006).

Abiotic stresses have been known to affect the plant cell bioenergetics. Plant mitochondria may control ROS generation by means of energy-dissipating systems. Therefore, mitochondria may play a central role in cell adaptation to abiotic stresses, which are known to induce oxidative stress at cellular level. To investigate the effects of ROS on plant mitochondria, a ROS producing system consisting of xanthine and xanthine oxidase was used to determine the rate of membrane potential generation due to either succinate or NADH addition to durum wheat mitochondria (Pastore et al., 2002). The results showed that the early ROS production inhibits the succinate dependent, but not the NADH-dependent generation and O_2 uptake. It was found that early generation of ROS can affect plant mitochondria by impairing metabolite transport, thus preventing further substrate oxidation, generation and consequent large-scale ROS production.

The chloroplasts of higher plants and algae contain a highly organized thylakoid membrane system that harbours all components of the light-capturing photosynthetic apparatus. This provides all structural properties for optimal light harvesting (Temple et

al., 2005). Oxygen generated in the chloroplasts during photosynthesis can accept electrons passing through the photosystems, thus results in the formation of O_2. Therefore, the presence of ROS producing centres such as triplet chlorophyll, electron transport chain in photosystems I and II make chloroplasts a major site of ROS (O_2^-, $^1O_2$ and H_2O_2) production. Various abiotic stresses such as drought, salt stress, excess light and CO_2 limiting conditions enhance the production of ROS in chloroplasts.

Chloroplast-produced ROS have been shown to be capable of transmitting the spread of wound induced programmed cell death through maize tissue (Gray et al., 2002). The induced accumulation of ROS caused by chilling in cucumber resulted in decreased net photosynthetic rate and alterations to the cytochrome respiratory pathway. The effects of chilling also caused an enhancement of the protective mechanisms such as thermal dissipation, alternative respiratory pathway, and ROS scavenging mechanisms (SODs and APXs) in chloroplasts and mitochondria (Hu et al., 2008).

Peroxisomes are small spherical microbodies bound by a single lipid bilayer membrane. Peroxisomes are sub-cellular organelles with an oxidative type of metabolism and are probably the major sites of intracellular ROS production. Similar to mitochondria and chloroplasts, peroxisomes produce O_2 radicals as a consequence of their normal metabolism. Two sites of O_2 generation are established in peroxisomes (Luis et al., 2002). The first one is in the organelle matrix, where xanthine oxidase (XOD) catalyzes the oxidation of xanthine and hypoxanthine to uric acid (Corpas et al., 2001). The second site is in the peroxisome membranes dependent on NADPH where a small electron transport chain is composed of a flavo-protein NADH and cytochrome b, and here O_2 is produced by the peroxisome by the electron transport chain. Monodehydroascorbate reductase participates in O_2 production by peroxisome membranes (Luis et al., 2002).

The main metabolic processes responsible for the generation of H_2O_2 in different types of peroxisomes are the photorespiratory glycolate oxidase reaction, the fatty acid β-oxidation, the enzymatic reaction of flavin oxidases, and the disproportionation of O_2 radicals (Corpas et al., 2001). Plant peroxisomes also play a significant role in photomorpho-genesis degradation of branched amino acids, biosynthesis of the plant hormones jasmonic acid and auxin, and the production of glycine betaine (Hu, 2007).

On the one hand increased production of H_2O_2 and O_2 in the peroxisomes lead to oxidative damage and possibly cell death (Luis et al., 2002) but on the other hand it has also been shown that small levels of H_2O_2 and O_2 work as signal molecules which mediate pathogen-induced programmed cell death in plants (Grant et al., 2000, McDowell and Dangl, 2000). Therefore, it has been suggested that peroxisomes should be considered as cellular compartments with the capacity to generate and release important signal molecules such as O_2, H_2O_2 and NO into the cytosol, which can contribute to a more integrated communication system among cell compartments (Corpas et al., 2001).

Other important sources of ROS production in plants that have received little attention are detoxification reactions catalysed by cytochrome P450 in cytoplasm and the endoplasmic reticulum (Dybing et al., 1976). ROS are also generated at plasma membrane level or extracellularly in apoplast in plants. pH-dependent cell wall-peroxidases, germin-like oxalate oxidases and amine oxidases have been proposed as a source of H_2O_2 in the apoplast of plant cells (Bolwell and Wojtaszek, 1997).

Tolerance of plants depends at least partially on their ability to cope with ROS. Enzyme activities and proteins such as superoxide dismutase (Cu/Zn SOD, Mn SOD, Fe SOD)

(Skórzyńska-Polit et al., 2004, Eyidogan and Öz, 2007, Mobin and Khan, 2007, Wang et al., 2008), ascorbate peroxidase (APX) (Aravind and Prasad, 2003, Khan et al., 2007, Mobin and Khan, 2007, Singh et al., 2008) and guaiacol peroxidase (GPX) (Kronfuss et al., 1996, Mika and Lüthje, 2003, Jebara et al., 2005) influence the tolerance of organisms exposed to abiotic stress. The field of ecotoxicology has used the activities of defense enzymes as “biomarkers” to assess the effects of stress induced by xenobiotics. For example, activity measures of SOD in *Pisum sativum* L exposed to cadmium and copper (Chaoui and El Ferjani, 2005). The measurements of oxidative enzymes activities were studied as biomarkers to the abiotic stress induced by trihalomethanes in irrigation water.

For more than a decade, developments in molecular biology led to a better understanding of changes occurring at transcriptional level and allowed acquisition of a large number of very useful genomic and transcriptomic data. Therefore, it is now possible to analyze in detail expression levels of genes belonging to the same physiological function under stress conditions induced by contaminants (Dubrovina et al., 2013, Fan et al., 2013, Li et al., 2014, Liu et al., 2014).

Environmental stress factors severely limit crop productivity; hence improvement of crop plants with traits that confer tolerance to these stresses is a primary focus in traditional and modern breeding (Sreenivasulu et al., 2007). Tolerance of hybrid plants after exposure to organic xenobiotics has been reported (Siehl et al., 1996, Seppänen et al., 2000, Dhankher et al., 2002, Abhilash et al., 2009). Generally, hybrid plants are engineered to be resistant to a specific group of persistent organic pollutants in the water environment, but this may increase their susceptibility to non-target organic pollutants. The mechanism associated with this conferred resistance is usually due to an over-

expression of key protective enzymes (Gullner et al., 2001, Karavangeli et al., 2005, Schröder et al., 2008).

Lipid profiling has also been utilized to identify compositional changes in lipids associated with stress response (Welti et al., 2002). Increasing evidence in plants has demonstrated the role of lipids in diverse cellular processes that include photosynthesis, signal transduction, protein transport and secretion (Bruce, 1998, Siegenthaler and Murata, 1998, Kunst and Samuels, 2003, Wang, 2004). In addition, lipids are required for growth and developmental processes such as seed germination, organ differentiation, pollination and response to biotic and abiotic stress (Wolters-Arts et al., 1998, Beisson et al., 2003, Pracharoenwattana et al., 2005, Wasternack, 2007).

Lipid membrane composition changes in plants as a response to internal and external cues. These cues could be altered hormone levels, developmental cues, and cues resulting from environmental stress or attack by pathogens or insects (Wang, 2002, Farmer et al., 2003, Meijer and Munnik, 2003). Recognition of the various cellular roles of lipids has raised interests in identifying the genes responsible for lipid alterations in cellular functions as a response to internal and external cues.

Several recent and exciting advances in lipid analysis have provided tools for identifying and characterizing plants that have genetic alterations as a result of changes in lipid metabolism. Such changes may occur as an increased or decreased expression of essential lipid sterols, fatty acids and esters or the production of novel lipid-metabolic varieties (Wallis and Browse, 2002, German et al., 2007).

Oxidative stress is known to induce reversible or irreversible (causing complete loss of some physiological activites) modifications of bio-molecules such as proteins,

polynucleic acids, carbohydrates and lipids (Sies et al., 1985, Møller et al., 2007, Farmer and Mueller, 2013). As such, oxidation of lipids is particularly dangerous because it propagates the production of free radicals through so-called 'chain reactions'. Lipid oxidation (also known as lipid peroxidation) is widely considered as a "hallmark" of oxidative stress (Farmer and Mueller, 2013).

Lipid peroxidation consists of three sequential stages: initiation, propagation and finally termination (Catalá, 2009, Farmer and Mueller, 2013). The initiation stage is the step in which lipid radicals are produced. This can be caused by hydroxyl, alkoxyl, peroxyl radicals as well as peroxynitrite but not by hydrogen peroxide or superoxide (Gutteridge and Halliwell, 2000). Phospholipids (most abundant membrane lipids) are susceptible to radicals and peroxidation because the double bonds in the fatty acids weaken the C-H bond and facilitates H^+ subtraction.

The lipid radical –*CH- or (L*) formed from the methylene group (-CH_2-) can activate O_2 and form an oxygen centered "lipid peroxyl radical" (LOO*) which in turn is capable of removing H^+ from a neighboring fatty acid. This produces a lipid hydroperoxide (LOOH) and a second lipid radical (L*) giving rise to the propagation phase (Catalá, 2006). LOOH can undergo 'reductive cleavage' by reduced transition metals (mainly Fe^{2+} or Cu^+) and form lipid alkoxyl radical ($LO^{\bullet}$), which is also reactive and induces further removal of H^+ from neighboring fatty acid. Another important mechanism of lipid peroxidation is via direct reaction of double bonds with singlet oxygen from PSII reaction centre, which gives LOOH (Krieger-Liszkay et al., 2008, Przybyla et al., 2008, Farmer and Mueller, 2013). Singlet oxygen can also be formed from the reaction of two LOO* molecules.

Severe lipid peroxidation leads to the damage of membranes, collapse of their barrier function, followed by disintegration of organelles, oxidation and dysfunction of proteins,

DNA and RNA (Gutteridge and Halliwell, 2000, Farmer and Mueller, 2013). The terminal products of lipid peroxidation are 'aggressive' substances, such as aldehydic secondary products (malondialdehyde, 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and acrolein), which are markers of oxidative stress (Lykkesfeldt, 2007, Farmer and Mueller, 2013). They are easy to measure by using the thiobarbituric acid assay for malondialdehyde (Hodges et al., 1999) or more modern sensitive mass spectrometry-based techniques that can identify individual lipid varieties targeted by peroxidation (Shulaev and Oliver, 2006, Farmer and Mueller, 2013).

In order to prevent the propagation of the lipid radical (L*) in lipid membranes, plants produce a group of lipophyllic antioxidants. α-Tocopherol has been defined as a radical-chain breaker (Barclay and Ingold, 1981), which, due to its hydrophobic nature, operates in a lipid environment. The effects of α-tocopherol as an antioxidant are thus restricted to its direct effects on membranes and lipoprotein domains (Azzi, 2007).

Hence, levels of α-tocopherol in plants exposed to trihalomethanes from irrigation water could prove to be an effective bio-indicator of stress.

2.4 Protein oxidation and DNA damage in plants by ROS

Protein oxidation is defined as the covalent modification of a protein induced by ROS or by oxidative stress. Most types of protein oxidations are essentially irreversible, whereas, a few involving sulfur-containing amino acids are reversible (Ghezzi and Bonetto, 2003). The oxidation of a number of protein amino acids particularly Arg, His, Lys, Pro, Thr and Trp give free carbonyl groups which may inhibit or alter their activities and increase susceptibility towards proteolytic attack (Møller et al., 2007). Protein carbonylation may occur due to direct oxidation of amino acid side chains (e.g.proline and arginine to γ-glutamyl semialdehyde, lysine to amino adipic semialdehyde, and threonine to amino keto butyrate) (Shringarpure and Davies, 2002). Whatever the location of ROS synthesis and action, ROS are likely to target proteins that contain sulfur-containing amino acids and thiol groups. Cys and Met are quite reactive especially with $^1O_2$ and OH. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges (Hancock et al., 2006).

It has been found that various stresses lead to the carbonylation of proteins in tissues. Protein carbonylation was found to be higher in the mitochondria than in chloroplasts and peroxisomes in wheat leaves which suggest that the mitochondria are more susceptible to oxidative damage. A number of carbonylated proteins in a soluble fraction from green rice leaf mitochondria have been identified (Kristensen et al., 2004). Proteins can be damaged in oxidative conditions by their reactions with lipid peroxidation products, such as 4-hydroxy-2-nonenal (HNE).

Treatment of mitochondria with 4-hydroxy-2-nonenal (HNE) or paraquat (which causes superoxide formation in chloroplasts and mitochondria) or cold or drought treatment of plants leads to formation of a covalent HNE-derived adduct of the lipoic acid moiety of

several mitochondrial enzymes, including Gly decarboxylase (an enzyme in the photorespiratory pathway), 2-oxoglutarate dehydrogenase (a TCA cycle enzyme), and pyruvate decarboxylase (Millar and Leaver, 2000, Taylor et al., 2005).

Though the plant genome is very stable but its DNA might get damaged due to the exposure to biotic and abiotic stress factors which might damage the DNA, and thereby exerts genotoxic stress (Tuteja et al., 2009). Endogenously generated damage to DNA is known as ‘‘spontaneous DNA damage” which is produced by reactive metabolites (OH, O_2 and NO). High levels of ROS can cause damage to cell structures, nucleic acids, lipids and proteins (Valko et al., 2006). It has been reported that OH^- is the most reactive and cause damage to all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Gutteridge and Halliwell, 2000). $^1O_2$ primarily attacks guanine, and H_2O_2 and O_2 do not react at all (Wiseman and Halliwell, 1996). ROS is capable of inducing damage to almost all cellular macromolecules including DNA which includes base deletion, pyrimidine dimers, cross-links, strand breaks and base modification, such as alkylation and oxidation (Tuteja et al., 2001). DNA damage results in various physiological effects, such as reduced protein synthesis, cell membrane destruction and damage to photosynthetic proteins, which affects growth and development of the whole organism (Britt, 1999). DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors, cell membrane destruction and genomic instability (Cooke et al., 2003).

2.5 ROS scavenging antioxidant defense mechanisms

Exposure of plants to unfavourable environmental conditions such as temperature extremes, heavy metals, drought, water availability, air pollutants, nutrient deficiency, or salt stress can increase the production of ROS, e.g., $^1O_2$, O_2, H_2O_2 and OH. To protect themselves against these toxic oxygen intermediates, plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ antioxidant defense systems. A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against various stresses (Mittler, 2002, Yaghubi et al., 2014). The components of antioxidant defence systems are both enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include SOD, catalase (CAT), APX, Monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) and non-enzymatic antioxidants are ascorbic acid, reduced glutathione (GSH) (both water soluble) carotenoids, phenolic compounds and tocopherols (lipid soluble) (Mittler, 2002, Gill et al., 2011).

The metallo-enzyme superoxide dismutase (SOD) is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and in all sub-cellular compartments prone to ROS mediated oxidative stress. It is well established that various environmental stresses often lead to the increased generation of ROS, where, SOD has been proposed to be important in plant stress tolerance and provide the first line of defense against the toxic effects of elevated levels of ROS. The SODs remove O_2 by catalyzing its dismutation, one O_2 being reduced to H_2O_2 and another oxidized to O_2. It removes O_2 and hence decreases the risk of OH formation via the metal catalyzed Haber-Weiss-type reaction. This reaction has a 10,000 fold faster rate than spontaneous dismutation. SODs are classified by their metal cofactors into three known types: the

copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD), which are localized in different cellular compartments (Mittler, 2002).

The sub-cellular distribution of these isozymes is also distinctive. The Mn-SOD is found in the mitochondria of eukaryotic cells and in peroxisomes; some Cu/Zn-SOD isozymes are found in the cytosolic fractions, and also in chloroplasts of higher plants (Corpas et al., 2001, Luis et al., 2003). The prokaryotic Mn-SOD and Fe-SOD, and the eukaryotic Cu/Zn-SOD enzymes are dimers, whereas Mn-SOD of mitochondria are tetramers and there are no reports of extra cellular SOD enzymes in plants. All forms of SOD are nuclear-encoded and targeted to their respective sub cellular compartments by an amino terminal targeting sequence. Several forms of SOD have been cloned from a variety of plants (Scandalios, 1990). Significant increase in SOD activity was observed under salt stress in *Lycopersicum esculentum* (Gapińska et al., 2008). Furthermore, significant increase in the activities of Cu/ZnSOD and MnSOD isozymes were observed under salt stress. Moreover, increased SOD activity has also been detected following Cd treatment in *Arabidopsis thaliana* (Hsu and Kao, 2004).

The effect of water stress on the activities of total leaf SOD and chloroplast SOD in *Trifolium repens L*. was reported by Chang-Quan and Rui-Chang (2008). They noted a significant increase in SOD activity under water stress. There have been many reports of the production of abiotic stress tolerant transgenic plants over-expressing different SODs. Protoplasts with Mn-SOD over expression showed less oxidative damage, higher H_2O_2 content and significant increase in SOD and GR activities under photo-oxidative stress (Melchiorre et al., 2009). Over-expression of a Mn-SOD in transgenic *Arabidopsis* plants also showed increased salt tolerance. Furthermore, they showed that Mn-SOD

activity as well as the activities of Cu/Zn-SOD, Fe-SOD, CAT and POD was significantly higher in transgenic *Arabidopsis* plants than in control plants (Wang et al., 2004).

Ascorbate peroxidase (APX) is thought to play an essential role in scavenging ROS and protecting cells in higher plants, algae, euglena and other organisms. APX is involved in scavenging of H_2O_2 in water-water and ASH-GSH cycles and utilizes ASH as the electron donor. The APX family consists of at least five different isoforms including thylakoid (tAPX) and glyoxisome membrane forms (gmAPX), as well as chloroplast stromal soluble form (sAPX), cytosolic form (cAPX) (Noctor and Foyer, 1998). APX has a higher affinity for H_2O_2 (µM range) than catalase (CAT) and peroxidase (POD) (mM range) and it may have a more crucial role in the management of ROS during stress. Enhanced expression of APX in plants has been demonstrated during different stress conditions. Increased leaf APX activity under Cd stress has been reported in *Ceratophyllum demersum* (Aravind and Prasad, 2003*)*. The pre-treatment of *Oryza sativa* seedlings with H_2O_2 under non-heat shock conditions resulted in an increase in APX activity and protected rice seedlings from subsequent Cd stress (Hsu and Kao, 2007). Transgenic *Arabidopsis* plants over-expressing *OsAPXa* or *OsAPXb* genes exhibited increased salt tolerance. It was found that the overproduction of *OsAPXb* enhanced and maintained APX activity to a much higher extent than *OsAPXa* in transgenic plants under different NaCl concentrations (Lu et al., 2007). Over-expression of *Capsicum annuum* APX-like 1 gene (*CAPOA1*) in transgenic tobacco plants exhibited increased tolerance to oxidative stress. It was suggested that the overproduction of APX increased the peroxidase POD activity which strengthens the ROS scavenging system and leads to oxidative stress tolerance and oomycete pathogen resistance (Sarowar et al., 2005). Over-expression of APX in transgenic plants has been known to confer abiotic stress tolerance.

Ascorbate peroxidase (APX) can be distinguished from plant-isolated guaiacol peroxidase (GPX) in terms of differences in sequences and physiological functions. GPX decomposes indole-3-acetic acid (IAA) and has a role in the biosynthesis of lignin and defence against biotic stresses by consuming H_2O_2. GPX prefers aromatic electron donors such as guaiacol and pyragallol usually oxidizing ascorbate at the rate of around 1% that of guaiacol (Asada, 1999). The activity of GPOX varies considerably depending upon plant species and stress conditions. GPX activity has been reported to increase in Cd exposed plants of *Arabidopsis thaliana* (Cho and Seo, 2005). An initial increase in GPX activity in spruce needles subjected to Cd stress was observed and subsequent Cd treatment caused a decline in the activity (Radotić et al., 2000).

Non-enzymatic antioxidants such as α-tocopherols (Vitamin E) are lipid soluble antioxidants considered as potential scavengers of ROS and lipid radicals (Holländer-Czytko et al., 2005). α-tocopherols are considered as a major antioxidant in bio-membranes, where they play both antioxidant and non-antioxidant functions. Tocopherols are considered general antioxidants for protection of membrane stability including quenching or scavenging ROS like $^1O_2$. Tocopherols are localized in plants in the thylakoid membrane of chloroplasts. Out of four isomers of tocopherols found in plants, α-tocopherol has the highest antioxidant activity due to the presence of three methyl groups in its molecular structure (Kamal-Eldin and Appelqvist, 1996). A high level of α-tocopherol has been found in the leaves of many plant species including Arabidopsis but these are low in γ-tocopherol. Tocopherols has been shown to prevent the chain propagation step in lipid auto-oxidation which makes it an effective free radical trap. Additionally it has been estimated that one molecule of a-tocopherol can scavenge up to 120 $^1O_2$ molecules by resonance energy transfer (Munné-Bosch, 2007). Recently, it has been found that oxidative stress activates the expression of genes responsible for

the synthesis of tocopherols in higher plants (Gang et al., 2007). Increased levels of α-tocopherol and ASH have been found in tomato following trizole treatment which may help in protecting membranes from oxidative damage and thus chilling tolerance in tomato plants (Shao et al., 2007).

Flavonoids occur widely in the plant kingdom, and are commonly found in leaves, floral parts, and pollens. Flavonoids usually accumulate in the plant vacuole as glycosides, but they also occur as exudates on the surface of leaves and other aerial plant parts. Flavonoid concentration in plant cells is often over 1 mM (Vierstra et al., 1982). Flavonoid scan be classified into flavonols, flavones, isoflavones, and anthocyanins based on their structure. Flavonoids are suggested to have many functions like flowers, fruits, and seed pigmentation, protection against UV light; defence against phyto-pathogens (pathogenic microorganisms, insects, animals); role in plant fertility and germination of pollen and; acting as signal molecules in plant-microbe interactions (Olsen et al., 2010). Flavonoids are among the most bioactive plant secondary metabolites. Most flavonoids outperform well-known antioxidants, such as ASH and α-tocopherol (Hernández et al., 2009). Flavonoids serve as ROS scavengers by locating and neutralizing radicals before they damage the cell thus important for plants under adverse environmental conditions (Løvdal et al., 2010). Flavonoids function by virtue of the number and arrangement of their hydroxyl groups attaches to ring structures. Their ability to act as antioxidants depends on the reduction potentials of their radicals and accessibility of the radicals. Flavonoids and other phenolic compounds absorb UV light, and plants able to synthesize these compounds were more tolerant to high UV irradiation than mutants impaired in the flavonoid pathway (Clé et al., 2008). Many flavonoid biosynthetic genes are induced under stress conditions. It has been found that there is considerable increase in flavonoid levels following biotic and abiotic stresses,

such as wounding, drought, metal toxicity and nutrient deprivation (Winkel-Shirley, 2002).

The response of crop plants to the organohalogen group known as the trihalomethanes has not been previously examined. This response will be measured based on the plant's enzymatic and non-enzymatic antioxidant machinery and several other physiological parameters discussed.

CHAPTER 3

RESEARCH DESIGN AND METHODOLOGY

This study reports the effects of increasing trihalomethane dose on the physiology of two commercial tomato varieties (*Solanum lycopersicum*) and determines whether the extent of physiological impacts of trihalomethane exposure on tomato seedling radicle length, biomass accumulation, concentration levels of 12 key nutrients, oxidative stress, fatty acids and α-tocopherol content in membrane lipids of tomato plants correlated with either the number of bromine or chlorine atoms in the trihalomethane molecules. The 2 x 4 x 5 factorial experiment was laid out in CRD with four replications. The three factors were as follows: Cultivar of tomato, four levels of trihalomethanes and five levels of concentration.

3.1. Chemicals

The chemicals used in this study, bromodichloromethane ($CHBrCl_2$), bromoform ($CHBr_3$), chloroform ($CHCl_3$) and dibromochloromethane ($CHBr_2Cl$), were purchased at Sigma-Aldrich (South Africa) as pure standards (99% of purity). They were all provided directly solubilized in methanol. The tomato seeds were purchased at Stark-Ayres.

3.2. Plant materials, exposure time and treatments for seedling tests

Uniform size seeds (n = 25) of two commercial varieties of the tomato moneymaker and the F1 hybrid cultivar known as tomato star 9003 were treated with four levels of trihalomethane (chloroform, bromodichloromethane, dibromochloromethane and bromoform) and five levels of concentration specifically (control, 2.5 $mg.L^{-1}$, 5.0 $mg.L^{-1}$, 7.5 $mg.L^{-1}$ and 10 $mg.L^{-1}$). All seeds used in the experiment were first soaked in nanopure water for an hour to remove preservatives. The soaked seeds were spread on a double layer of Whatman No. 1 filter paper in 80 well labeled 12 cm petri dishes for

each treatment. The filter papers of the seeds were then moistened with the assigned concentrations of trihalomethane solution so that the seeds just barely start to float (about 10 mL).

The petri dishes were covered and then kept in an incubator at room temperature. The filter papers of the petri dishes were re-moistened every 2 days due to the volatile nature of trihalomethane species. The control treatments were moistened with nanopure water only. Four replicates for each group including the control were performed for different concentrations of each trihalomethane compound. Root (radical) length of each seed was measured using a digital calliper 7 days after germination noting that radical emergence was the criterion for germination to be recorded (Wang, 1985).

Exactly 20 days after germination the fresh weight of each seedling was determined for each treatment. 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. The dry weight of each seedling was determined by placing them inside a furnace and dried to a constant weight at 60^0C for 72 hours (Buczek and Burzyński, 2015). This was performed on the 3rd and the 14th day of the treatments. The data from this experiment was subjected to analysis of variance (ANOVA) using the STATISTICA program 2010 (StatSoft Inc., Tulsa, OK, USA). The means of root elongation, biomass accumulation in terms of fresh weight and dry weight of seedlings were compared according to FSD (Fischer's Least significant difference).

3.3. Plant materials, exposure time and treatments for growing plants

The 2 x 4 x 5 factorial experiment was laid out in completely randomized block with four replications. The experiment commenced with the design of a 160 pot irrigation system fitted with timers and connected to 20 separate 68 L rough tote plastic reservoirs. Twenty

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 organic potting soils with a nitrogen, phosphorus and potassium (NPK) ratio of 3:1:5. Each pot was connected to 4 L per hour button dripper extended from 20 mm low density poly-ethylene (LDPE) irrigation pipe. Each 68 L plastic reservoir was fitted with a 1,400 liter 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-8.30 am and 8.00 pm-8.30 pm approximately giving each pot 4 L of water per day.

To eliminate nutrient stress, de-ionized water pre-treated with granular activated carbon to remove previously existing organic compounds was mixed with Starke-Ayres nutrifeed K2025 water soluble fertilizer in the reservoirs for the entire duration of the experiment. Seeds of the F1 hybrid (TS) and the natural (TMM) varieties were purchased from Starke-Ayres Garden Centre and soaked in nanopure water for one hour 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 emerged within 7 days after planting and the weaker seedling was uprooted leaving one seedling per pot 14 days after planting. The treatments began 16 days after emergence when all plants were within the range of 30-35 cm in height.

The 160 pot plants were divided into four groups each containing 40 pot plants. The 1st group was exposed to varying concentrations of bromodichloromethane ($CHBrCl_2$), the 2nd group to bromoform ($CHBr_3$), the 3rd group to chloroform ($CHCl_3$) and the last group to dibromochloromethane ($CHBr_2Cl$). In each group plants in the first gutter continued to receive de-ionized water and nutrient solution only (control), whereas plants in gutters 2-5 were treated differentially by varying the concentration of trihalomethanes in their reservoirs. Plants in gutters 2-5 in each group were exposed to concentrations of 2.5 $mg.L^{-1}$, 5.0 $mg.L^{-1}$, 7.5 $mg.L^{-1}$ and 10 $mg.L^{-1}$ of the designated trihalomethane diluted with de-ionized water and nutrient solution. 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 glacier ultra low minus 86 ^oC freezer (Nuaire, Plymouth, USA) 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 ^oC for 72 hours to stop enzymatic activity (Havlin et al., 2012).

3.4. Nutrient concentration determination in plants

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) content of plant extracts were investigated.

This was achieved by grinding and homogenizing the dried plant materials into fine powder using the micro plant grinder model FZ102 (Tianjin taisite instruments, Tianjin, China). 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 ground so as to prevent sample mixing. The samples were sent off to Bemlab analytical laboratory where they were ashed at 480 ^{o}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 an ICP-OES optical emission spectrometer (Varian Vista-Pro, Springvale, Australia) with the exception of nitrogen. The plasma gas of the ICP-OES was argon with a flow rate of 15 L min^{-1} and an auxiliary gas flow rate of 1.5 L min^{-1}. The viewing height (above coil) was 6 mm with a nebulizer pressure of 150 kPa. The pump rate was 3.0 mL min^{-1}. The RF generator power was 1.55 kW with the frequency of 40 MHz. The eluent was 75:25 (v/v) 1-propanol in water with an elution rate of 2.0 mL min^{-1}. The analytical wavelengths used to detect metals were P (214.912 nm), K (766.450 nm), Ca (315.880 nm), Mg (279.071 nm), B (249.672 nm), Mn (257.610 nm), Fe (259.940 nm), Zn (213.857 nm), Cu (324.754 nm) and Na (588.980 nm). Total nitrogen content of the homogenized plant leaves were determined by total combustion in a Leco N-analyzer (Leco Corp, Henderson, USA) (Sweeney and Rexroad, 1986). Nutrient concentrations in sample extracts were expressed in mg kg^{-1}.

3.5. Non-enzymatic antioxidant extraction

Non-enzymatic stress responses include the measurement of total phenolic content (Yadav et al., 2012). The “antioxidant power” of plant sample extracts can be measured using the ferric reducing ability of plasma (FRAP) assay (Kumar et al., 2010). The total

antioxidant capacity of a sample can then be estimated using the oxygen radical absorbance capacity (ORAC) assay (Cao et al., 1993).

The fresh leaf samples were taken from the freezer and lyophilized for 16 hours at minus 86 ^{o}C using the Vir-tis genesis freeze dryer (SP Industries, Gardiner, NY, USA). The lyophilized leaf samples were then homogenized into fine powder using the micro plant grinder and stored in 2 mL vials at minus 40 ^{o}C.

Then 20-25 mg of lyophilized plant samples were weighed into a 15 mL screw-cape tube and 5 mL of 70% methanol in nanopure water was used to extract plant samples (Lapornik et al., 2005). The samples were then loaded on the intelli-mixer RM2 rotator/mixer (ELMI Ltd, Latvia) for 24 hr and centrifuged at 2200 x g for 5 minutes using the eppendorf centrifuge 5810 R (Eppendorf, AG, Hamburg, Germany). The supernatant was then used directly for analysis after a suitable dilution method was developed.

3.5.1. Determination of total polyphenols

Non structural phenolic compounds are known to perform a wide variety of functions in plants including acting as antioxidants (Ainsworth and Gillespie, 2007). Total polyphenols in plant extracts were determined by the Folin-Ciocalteu procedure as described by Meyer et al. (1997). This is a microplate adapted colorimetric total phenolics assay that utilizes Folin-Ciocalteu (F-C) reagent. The F–C assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which were determined spectroscopically at 765 nm (Ainsworth and Gillespie, 2007). Total polyphenols were expressed in mg.L^{-1} gallic acid equivalent (GAE).

3.5.2. Determination of the ferric reducing antioxidant power (FRAP)

The ferric reducing/antioxidant power (FRAP) assay is a direct test of "total antioxidant power." Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in a known concentration. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form. The ferric reducing ability of the sample extracts were determined as described by (Benzie and Strain, 1996).

The FRAP reagent was prepared by mixing 30 mL acetate buffer pH 3.6 (300mM), 3 ml of 2, 4, 6, tripyridyl-s-triazine (TPTZ) (10 mM) prepared in 40 mM hydrochloric acid, 3 mL $FeCL_3$ (20 mM), and 6.6 mL distilled water (dH_20). 10 µl of sample was mixed with 300 µl of FRAP reagent in a 96-well plate and incubated at 37 ^oC for 30 minutes. The change in absorbance due to the redox reaction occurring was monitored in a Thermo Scientific multiskan spectrum spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at 593nm. FRAP were expressed in $mg.L^{-1}$ ascorbic acid equivalent (AAE).

3.5.3. Determination of oxygen radical absorbance capacity (ORAC)

The oxygen radical absorbance capacities of plant extracts were determined as described by Prior et al. (2003). 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 anti-oxidants in the sample to scavenge the free radicals.

The reaction is initiated by the thermal decomposition at 37 ^oC of the azo-compound 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH), which serves as the source of peroxyl 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 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 fluroskan ascent 2.5 plate reader (Thermo Fisher Scientific, Waltham, USA) at 485nm and 583nm respectively. Antioxidant activity was expressed in trolox equivalents.

3.6. Enzyme extraction and total protein determination

Enzyme extraction of plant samples were performed as described by Zhou et al. (2004) with some modifications. The extraction buffer contained 25 mM of 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic 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 17hrs allowing thorough homogenization. The homogenates were centrifuged for 20 min at 6,600 xg and the supernatants obtained were used for enzyme analysis. All operations were performed at 0-4 ^{o}C. An aliquot of each extract was used to determine total protein content using the Pierce bicinchoninic acid (BCA) protein assay kit according to the manufacturer's protocol (Scientific, 2011). The BCA protein assay is based on colorimetric detection and quantification of total protein. The working reagent was prepared by combining 50 mL of reagent A with 1 mL of BCA reagent B (50:1, Reagent A:B). 25 µl of the homogenized extract were transferred in triplicates into microplate wells. Then 200 µl of the working reagent was added to each well and plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37 ^{o}C for 30 minutes. The plate was then cooled to room temperature and the absorbance was measured 562 nm on a Thermo Scientific

multiskan spectrum plate reader. 1 mL ampule of 2 mg/mL of bovine serum albumin (BSA) was sufficient to prepare a set of known protein concentration standards which were assayed alongside the unknown homogenized extract.

3.6.1. Determination of superoxide dismutase (SOD)

Superoxide dismutase (SOD; EC 1.15.1.1) is an enzyme that catalyses the partitioning of the toxic superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) (Barondeau et al., 2004). Superoxide is the by-product of oxygen metabolism and can cause many types of cell damage. Hydrogen peroxide is also damaging but less reactive than the superoxide molecule and can be degraded by other enzymes. Hence, superoxide dismutase is an important defensive antioxidant enzyme in living cells exposed to oxygen (Shah et al., 2001). Superoxide dismutase activity of plant extracts were determined as described in 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 $NaPO_4$-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 concentration was expressed as Units mg^{-1} of protein (U mg^{-1}).

3.6.2. Determination of ascorbate peroxidase (APX)

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).

Ascorbate peroxidase (APX) activity in plant extract was determined (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 seconds. The ascorbate oxidized was evaluated based on the extinction coefficient of ascorbate (E) = 2.8 $mM^{-1}cm^{-1}$ and the results expressed as APX units mg^{-1} of protein (U= 1 mM of ascorbate oxidized per min at 25°C).

3.6.3. Determination of guaiacol peroxidase (GPX)

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). Guaiacol peroxidase (GPX) activity was determined with the use of a standard method first described in 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 guaicol 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 Units mg^{-1} of protein (U mg^{-1}).

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

The accurate profiling of lipidomes were obtained by MTBE extraction which allows faster and cleaner lipid recovery 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 sec 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 hr at room temperature after which phase separation was induced by adding 0.64 mL of nanopure water. After 10 min of incubation at room temp, samples were centrifuged at 13,750 xg 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 gm 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.

3.7.1. 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 by Heath and Packer (1968) with some modifications. 100 µl of homogenized plant extract, 12.5 µl of 4 mM cold butylated hydoxytoluene (BHT) in ethanol, and 100 µl of 0.2 M of ortho-phosphoric acid were mixed and vortexed for 10 sec 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 sec. The vials were heated at 90 ^{o}C for one 1 hr and cooled on ice for two min with further cooling at room temp 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 19,800 xg for 2 min at 4 ^{o}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 mM^{-1} cm^{-1}.

3.7.2. 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 ^{o}C to reach a maximum temperature of 320 ^{o}C at 0.25 min.

The total runtime was 43.25 min per sample and the injector front inlet mode was splitless with initial temperature at 280 ^{o}C and pressure at 60.2 kPa. The purge flow was 30 mL/min with a purge time of 3 min and a total flow of 33.8 mL/min 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 x 250 µm (diameter) x 0.25 µm (film thickness).

The GC transfer line was maintained at 280 ^{o}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 ^{o}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.

CHAPTER 4
RESULTS AND DISCUSSION

In this chapter the results of the data analysis are presented and the findings explained. The data were collected and processed in response to the research objectives posed in chapter 1 of this dissertation. Three fundamental objectives drove the collection of the data and the subsequent data analysis. These goals were to determine the effects of concentration responses on the two tomato cultivars to increasing trihalomethane dose, focusing on physiological parameters such as radicle inhibition, seedling biomass, nutrient concentration, enzymatic and non-enzymatic oxidative responses, and changes in membrane lipid compositions.

The second objective was to investigate whether the extent of physiological impacts of trihalomethane exposure on the two tomato cultivars correlated with either the number of bromine or chlorine atoms in the trihalomethane molecules.

Finally, we compared the effects of increasing trihalomethane dose with the effect of increasing number of halogen atoms on the trihalomethane molecules to elucidate which factor induces a greater physiological response in plants.

These objectives were achieved. The findings in this chapter demonstrate the potential for trihalomethanes to induce a physiological response in plants. All values reported are means of four replicates. The data were analyzed by three-way analysis of variance (ANOVA) and compared at $P \leq 0.05$ significance level by the Scheffe test using Statistical Analysis System (SAS).

4.1. Results

4.1.1. Effects of the increasing halogenation of trihalomethanes on the gain of biomass in the tomato seedlings

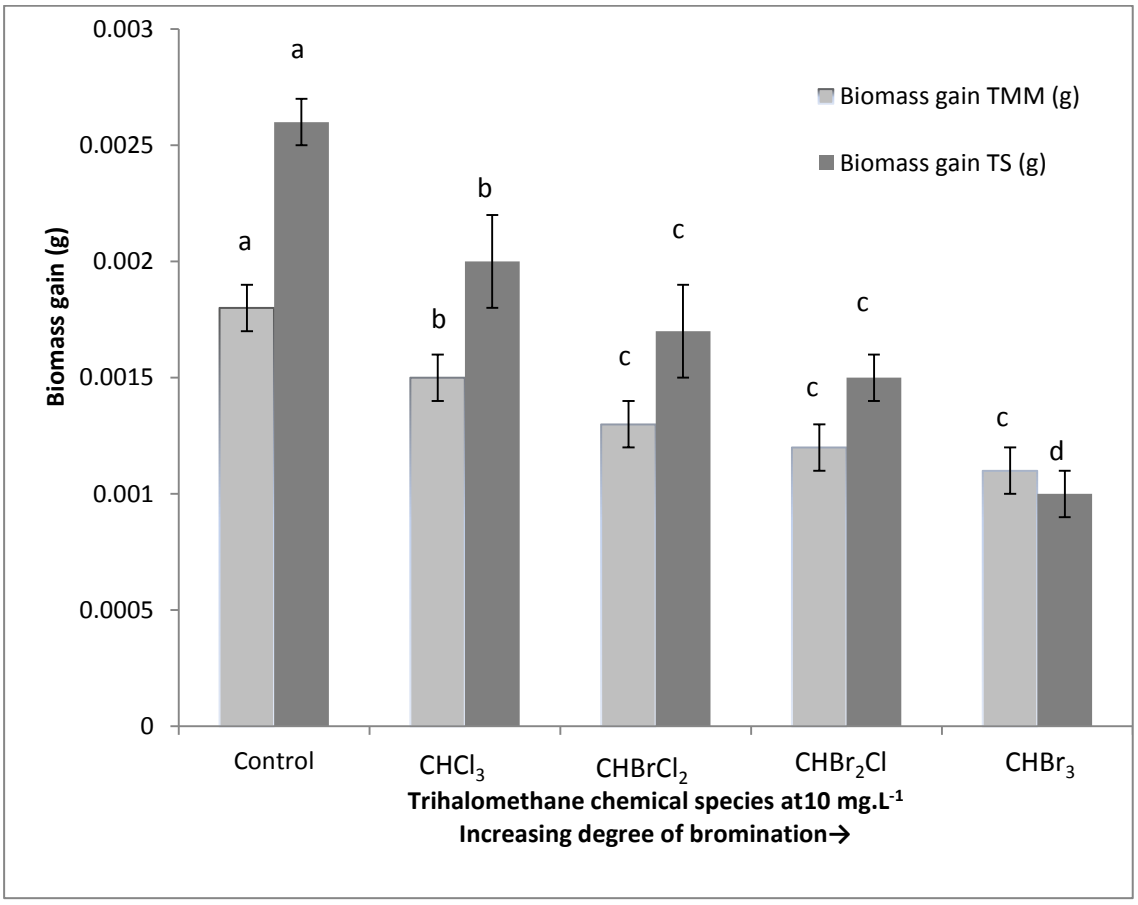


Fig 1: Effect of trihalomethanes and cultivars on the accumulation of biomass between seedlings exposed for 11 days. The tomato moneymaker (TMM) and tomato star (TS) seedlings were exposed to a concentration of 10 mg.L^{-1} of each trihalomethane species. Control seedlings were exposed to nanopure water only. Means followed by 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).

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 higher than the other treatments. The gain in biomass of both tomato seedlings decreased with a higher degree of bromination in the trihalomethane molecule.

4.1.2. Effects of the increasing halogenation of trihalomethanes on nutrient concentration

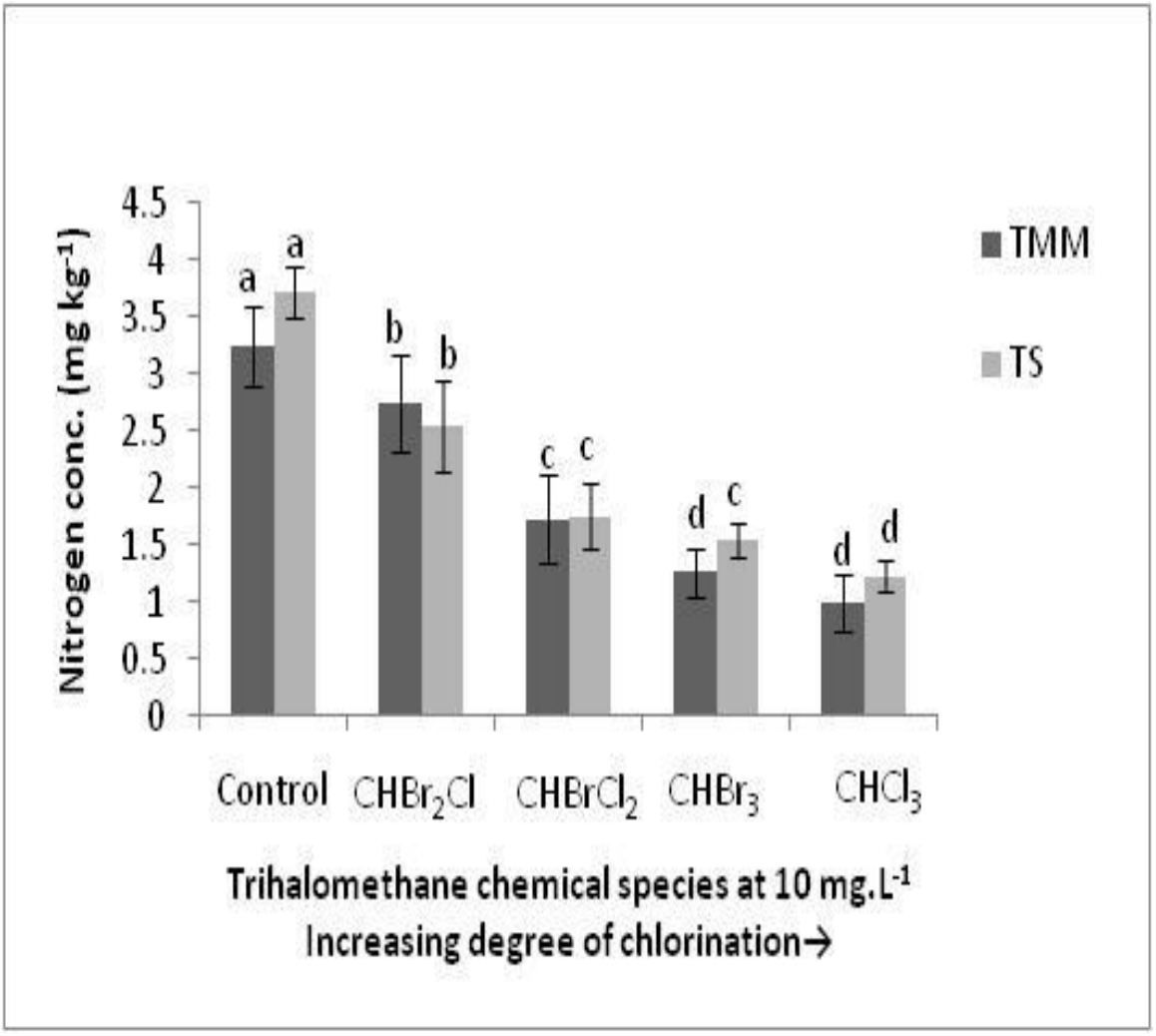


Fig 2: Effect of trihalomethanes on nitrogen concentration of the dry matter yields of tomato plants (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 $mg.L^{-1}$ of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

Figure 2 shows the effect of trihalomethanes on the nitrogen concentration in both tomato cultivars. The nitrogen levels in plant dry matter decreased in a manner that correlated with a higher degree of chlorination in the trihalomethane molecules in the order of $CHBr_2Cl > CHBrCl_2 > CHBr_3 > CHCl_3$ in both tomato cultivars (Fig 2). Nitrogen levels in control plants were significantly higher than the other treatments.

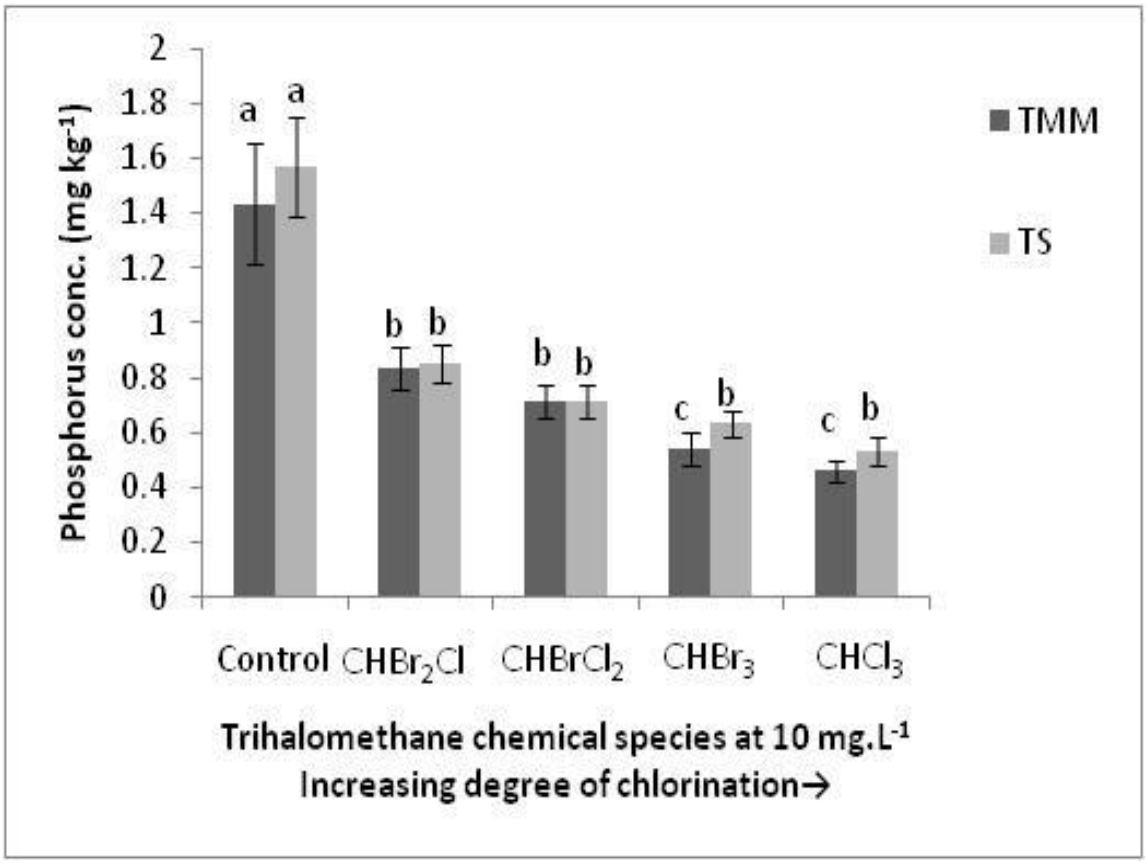


Fig 3: Effect of trihalomethanes on phosphorus concentration of the dry matter yields of tomato plants (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg.L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

Figure 3 shows the effect of trihalomethanes on the phosphorus concentration in both tomato cultivars. The nutrient levels of phosphorus in the plant dry matter of the TS cultivars did not decrease significantly with exposure to a higher degree of chlorination in the trihalomethane molecules (Fig 3). The phosphorus levels in the TMM cultivars did not decrease significantly between mono-chlorinated and di-chlorinated trihalomethanes. However, a significant decrease in phosphorus levels was observed between TMM cultivars exposed to di-chlorinated and tri-chlorinated trihalomethanes. Phosphorus levels in control plants were significantly higher than the other treatments.

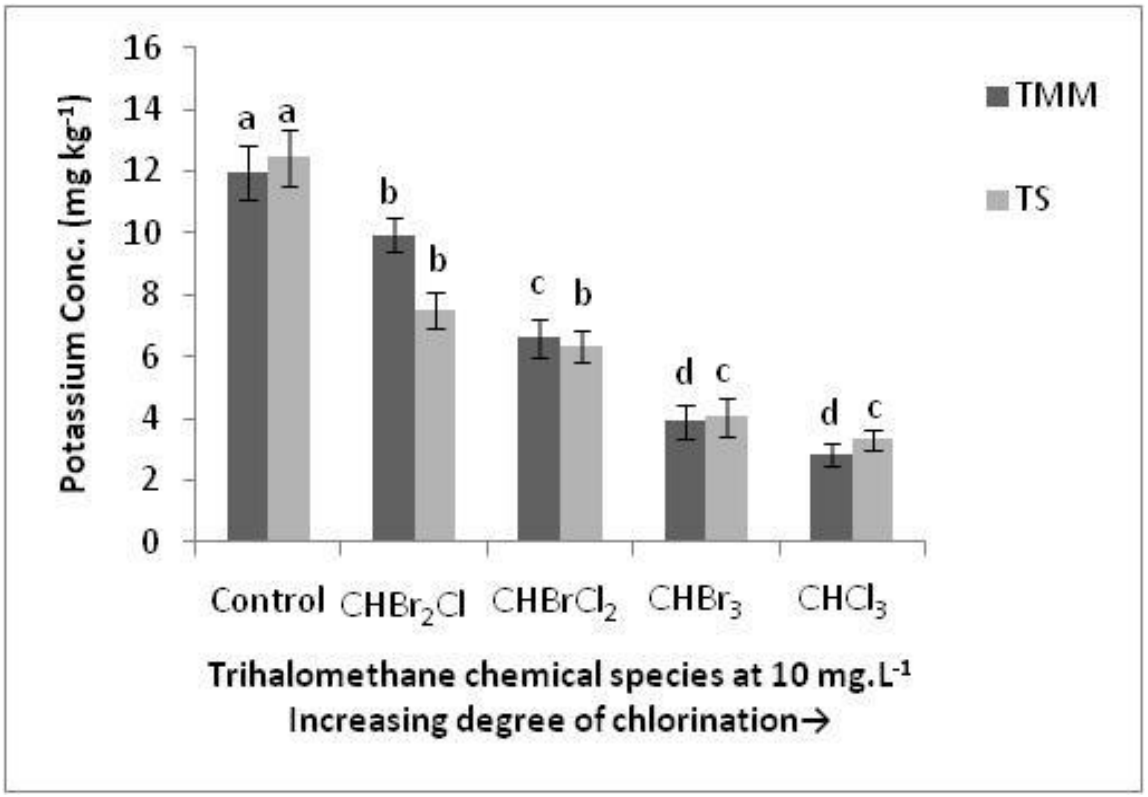


Fig 4: Effects of trihalomethanes on potassium concentration of the dry matter yields of tomato plants (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 $mg.L^{-1}$ of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

Figure 4 shows the effect of trihalomethanes on the potassium concentration in both tomato cultivars. The nutrient levels of potassium in plant dry matter decreased in a manner that correlated with a higher degree of chlorination in the trihalomethane molecules in the order of $CHBr_2Cl > CHBrCl_2 > CHBr_3 = CHCl_3$ in the TMM tomato cultivars (Fig 4). The TS cultivars exposed to mono-chlorinated and di-chlorinated trihalomethanes were not significantly different in potassium levels. A significant decrease in potassium levels was observed between TS cultivars exposed to di-chlorinated and tri-chlorinated trihalomethanes. Nutrient levels in control plants were significantly different from treatments.

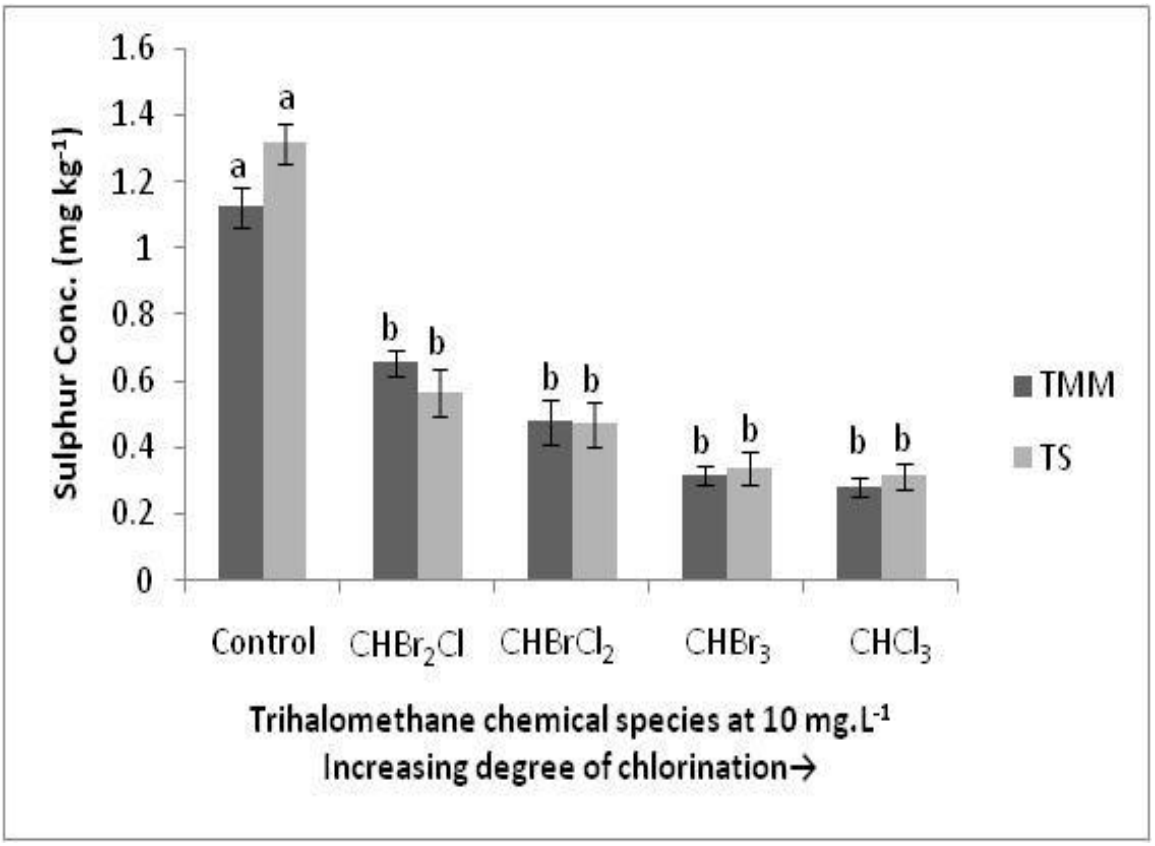


Fig 5: Effect of trihalomethanes on the sulphur concentration of the dry matter yields of tomato plants (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 $mg.L^{-1}$ of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

The effect of trihalomethanes on the sulphur concentration in both tomato cultivars is shown in fig 5. The sulphur levels in the plant dry matter of both cultivars did not decrease significantly with exposure to a higher degree of chlorination in the trihalomethane molecules (Fig 5). However, sulphur levels in control plants were significantly higher than the other treatments.

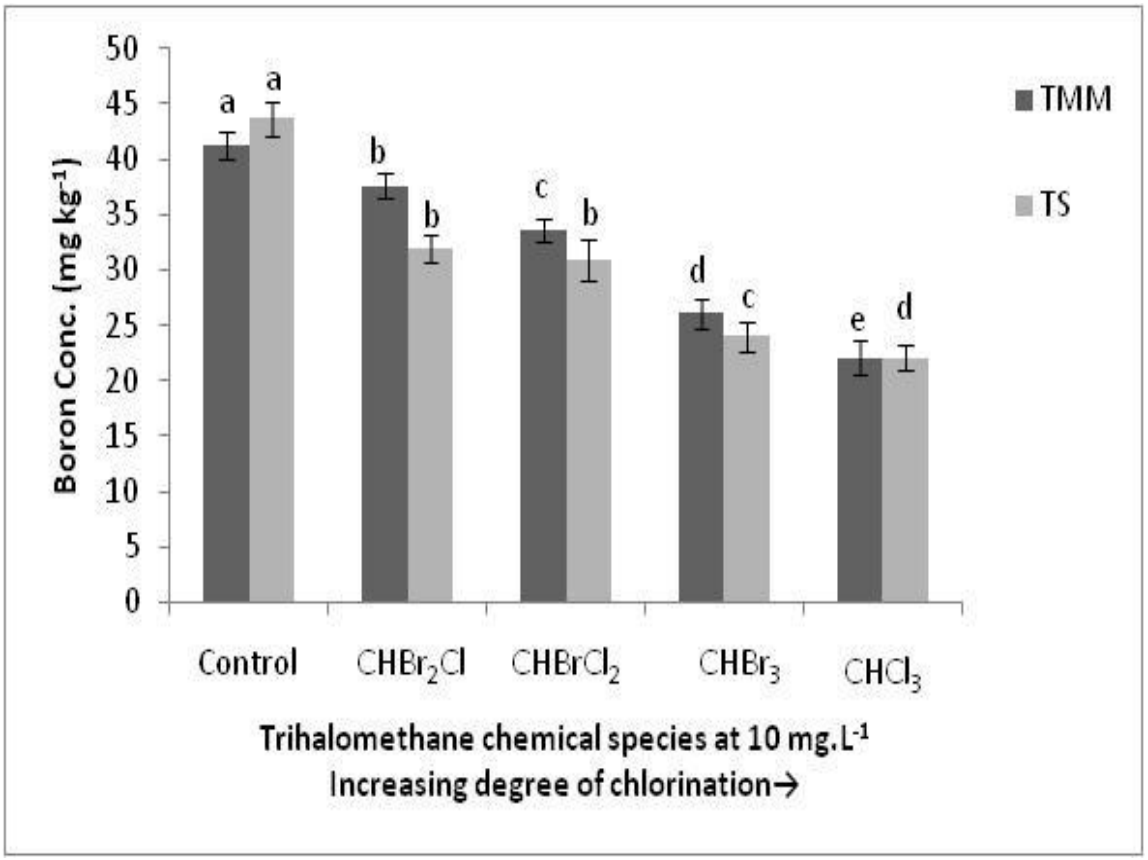


Fig 6: Effect of trihalomethanes on boron concentration of the dry matter yields of tomato plants (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 $mg.L^{-1}$ of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

Figure 6 shows the effect of trihalomethanes on the boron concentration in both tomato cultivars. The boron levels in plant dry matter decreased in a manner that correlated with a higher degree of chlorination in the trihalomethane molecules in the order of $CHBr_2Cl$ > $CHBrCl_2$ > $CHBr_3$ > $CHCl_3$ in both tomato cultivars. The TS cultivars exposed to mono-chlorinated and di-chlorinated trihalomethanes were not significantly different in boron levels. Boron levels in control plants were significantly higher than the other treatments.

The effect of trihalomethanes on the levels of all nutrients determined can be seen in Table 9 (Appendix).

The decrease in nutrient levels with increasing degree of chlorination was also observed for Mn, Cu and Zn but only in the TMM tomato cultivars. The TS cultivars exhibited no particular halogenation order with respect to Mn and Cu (Table 9). The nutrient levels of Zn in the TS cultivars however increased with increasing bromination in the order of $CHBrCl_2$ < $CHCl_3$ < $CHBr_2Cl$ < $CHBr_3$ (Table 9). The nutrient levels of Mg decreased with increasing bromination of the trihalomethane molecules in the order of $CHBrCl_2$ > $CHBr_2Cl$ > $CHBr_3$ > $CHCl_3$ in the TS tomato cultivar. No particular order was observed in the TMM tomato cultivar for Mg (Table 9). The reverse was observed with Na levels in the TMM cultivars which decreased with increasing bromination while no particular order was observed for Na in the TS tomato cultivar. The effects and interactions of trihalomethanes in both tomato cultivars proved not significant at $P \leq 0.05$ for Ca and Fe levels (Table 9).

The nutrients reported in figure 2, 3, 4, 5 and 6 were significantly proven at $P \leq 0.05$ to correlate with an increasing degree of chlorination in both tomato cultivars. Table 1 shows the correlation between nutrient levels 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. This implies that as the degree of chlorination increases the nutrient levels decreases.

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

The percentage decrease in nutrient levels from the mean effects of the mono-chlorinated trihalomethanes ($CHBr_2Cl$) to the mean effects of tri-chlorinated trihalomethanes ($CHCl_3$) was N (58.70%), P (41.07%), K (64.48%), S (51.06%), Cu (29.34%), Zn (22.67%) and B (36.26%).

4.1.3. Effects of increasing halogenation of trihalomethanes on non enzymatic biomarkers of oxidative stress

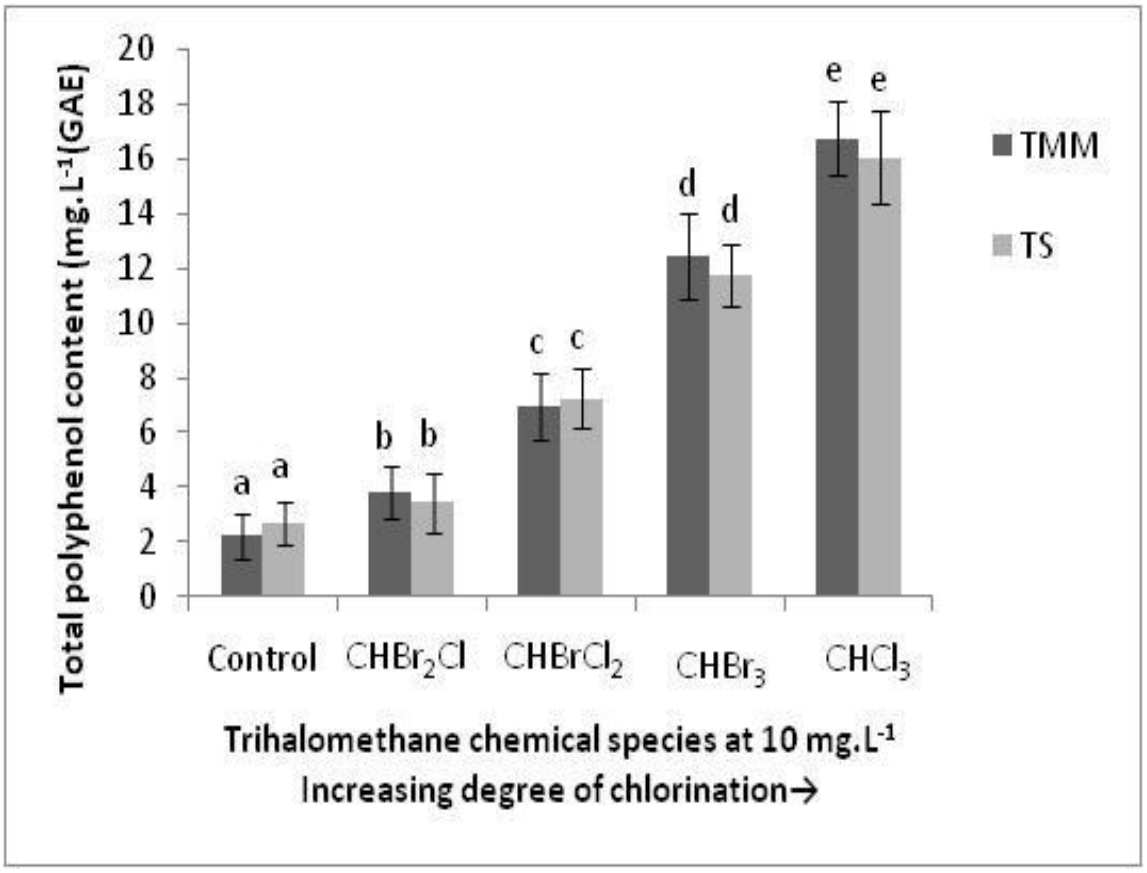


Fig 7: Effect of trihalomethanes on the total polyphenol content (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg.L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

Figure 7 shows the effects of trihalomethanes on the total polyphenol content in both tomato cultivars. An increase in the total phenolic content was observed with increasing chlorination of the trihalomethane molecule in the order of $CHBr_2Cl < CHBrCl_2 < CHBr_3 < CHCl_3$ in both cultivars (fig 7). The total polyphenol content in control plants were significantly lower than the treatments.

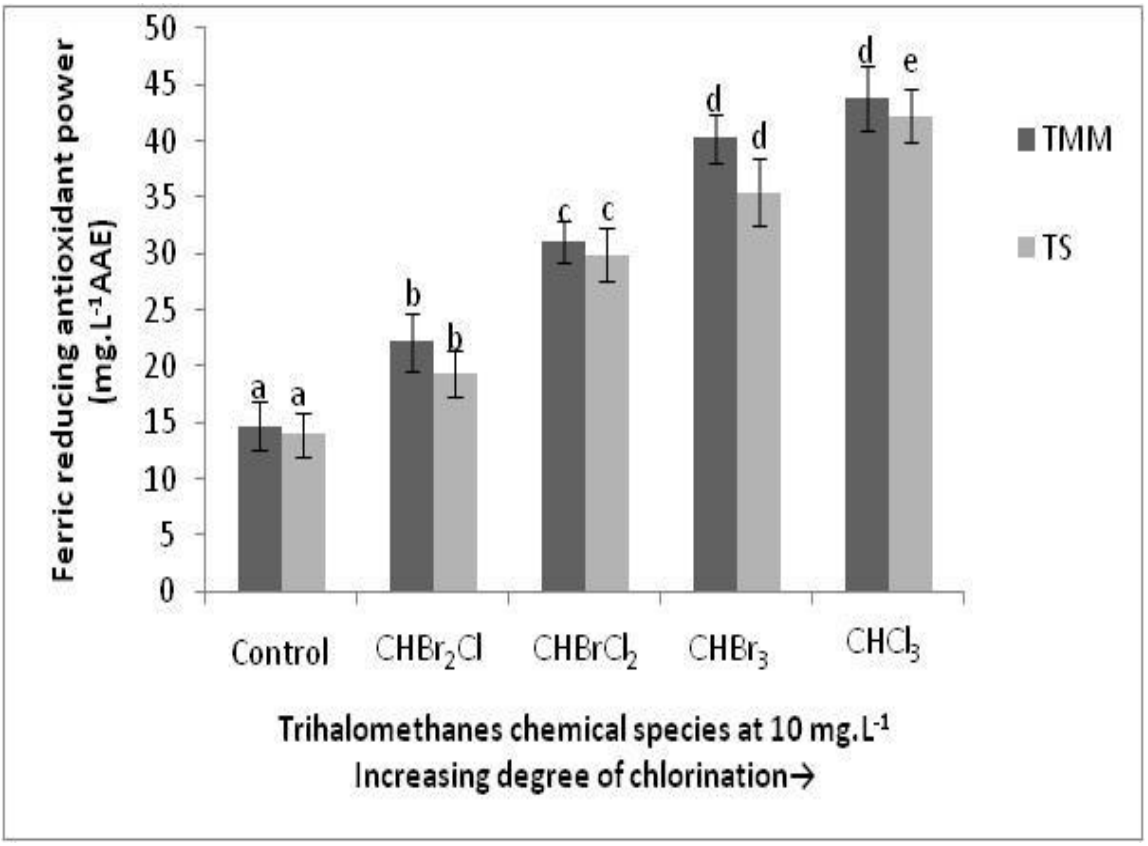


Fig 8: Effect of trihalomethanes on the ferric reducing antioxidant power of plant extracts (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 $mg.L^{-1}$ of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

In figure 8, the effects of trihalomethanes on the ferric reducing antioxidant power of the plant extracts in both tomato cultivars can be seen. An increase in the ferric reducing antioxidant power (FRAP) was observed with increasing chlorination of the trihalomethane molecule in the order of $CHBr_2Cl < CHBrCl_2 < CHBr_3 < CHCl_3$ in both cultivars (fig 8). The ferric reducing antioxidant power in control plants was significantly lower than the other treatments.

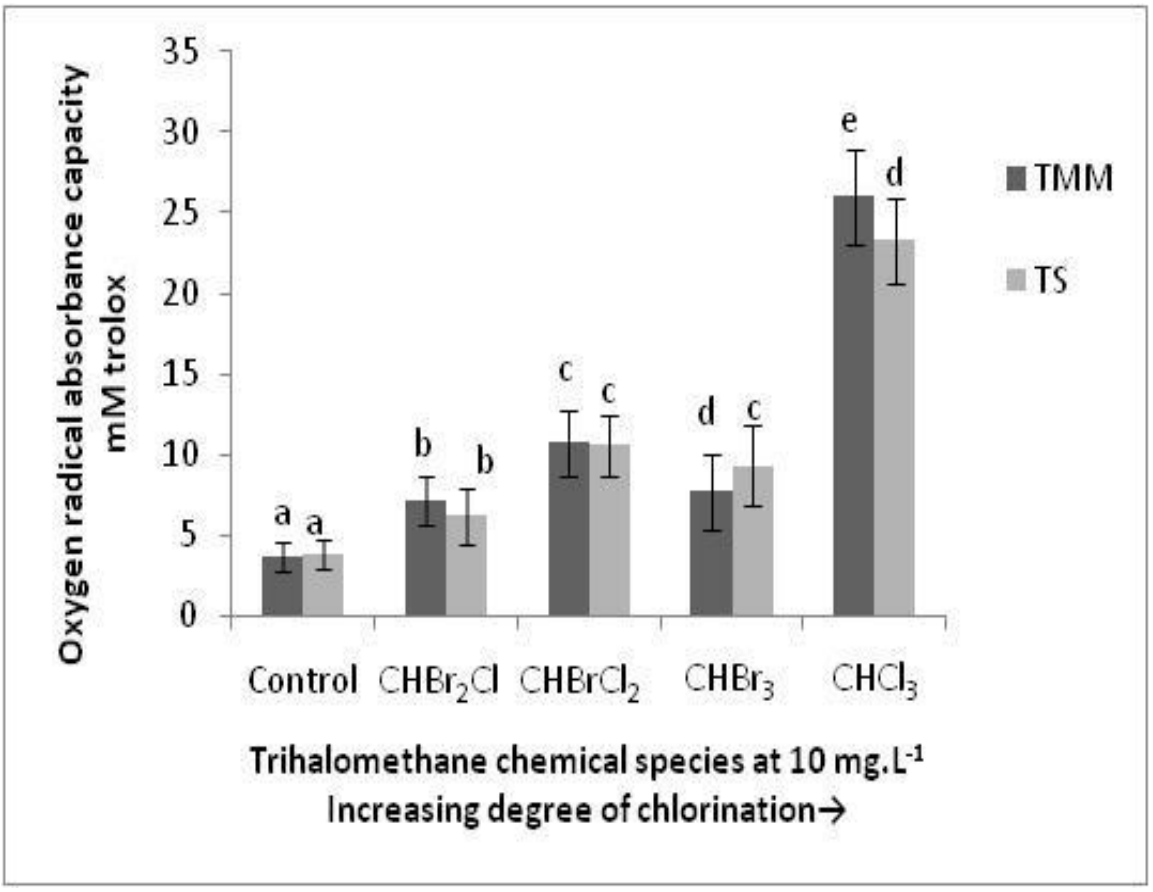


Fig 9: Effect of trihalomethanes on the oxygen radical absorbance capacity of plant extracts (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 $mg.L^{-1}$ of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

Figure 9 shows the effects of trihalomethanes on the oxygen radical absorbance capacity in both tomato cultivars. The oxygen radical absorbance capacity (ORAC) of plant extracts increased with increasing chlorination of trihalomethane molecule with a slightly different order of $CHBr_2Cl < CHBr_3 < CHBrCl_2 < CHCl_3$ in both cultivars. The oxygen radical absorbance capacity in control plants was significantly different from treatments.

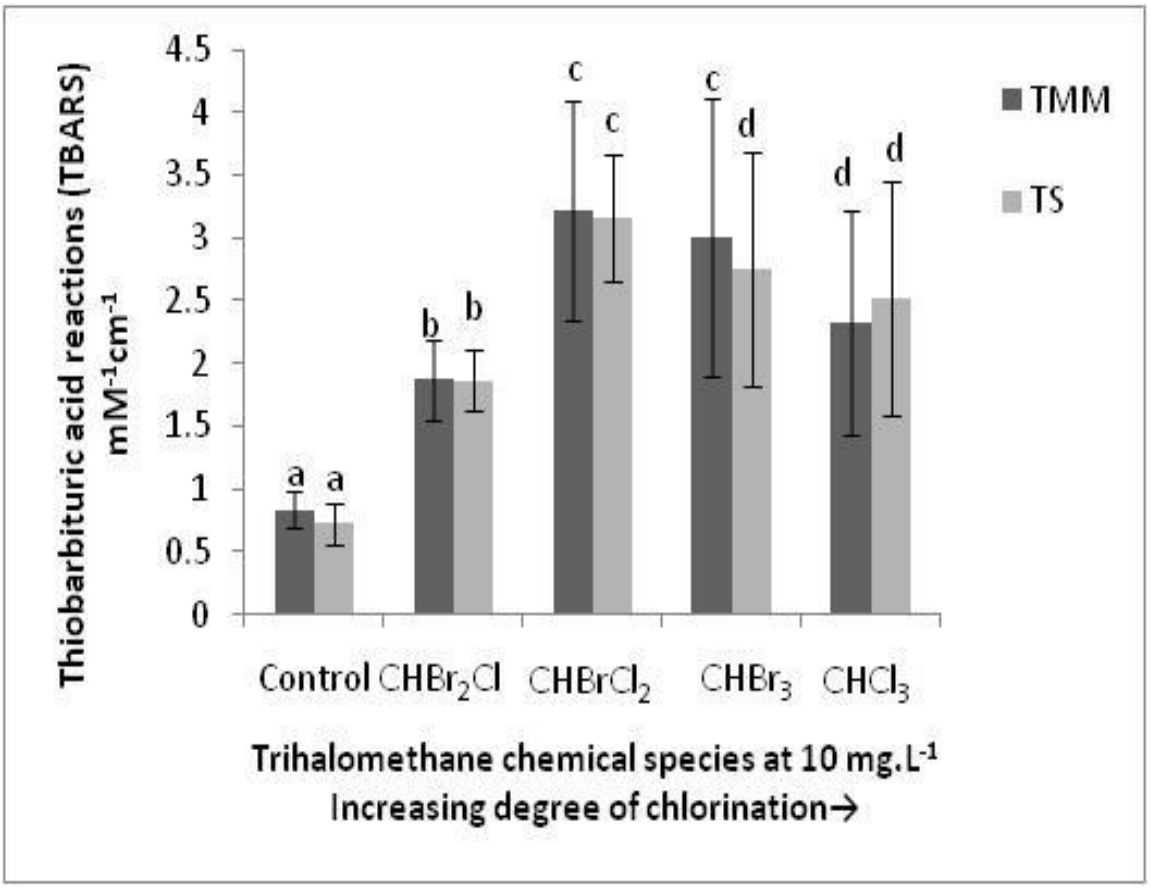


Fig 10: Effect of trihalomethanes on the thiobarbituric acid reactions of plant extracts (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 $mg.L^{-1}$ of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

The effects of trihalomethanes on the thiobarbituric acid reactions (TBARS) of plant extracts from both tomato cultivars are shown in (Fig 10). General lipid peroxidation by TBARS responded to no particular halogenation order. The di-chlorinated trihalomethane molecule ($CHBrCl_2$) induced the highest levels of peroxidation in lipids of both tomato cultivars. The thiobarbituric acid reactions of control plants were the lowest compared to other treatments.

4.1.4. Effects of the increasing halogenation of trihalomethanes on the enzymatic biomarkers of oxidative stress

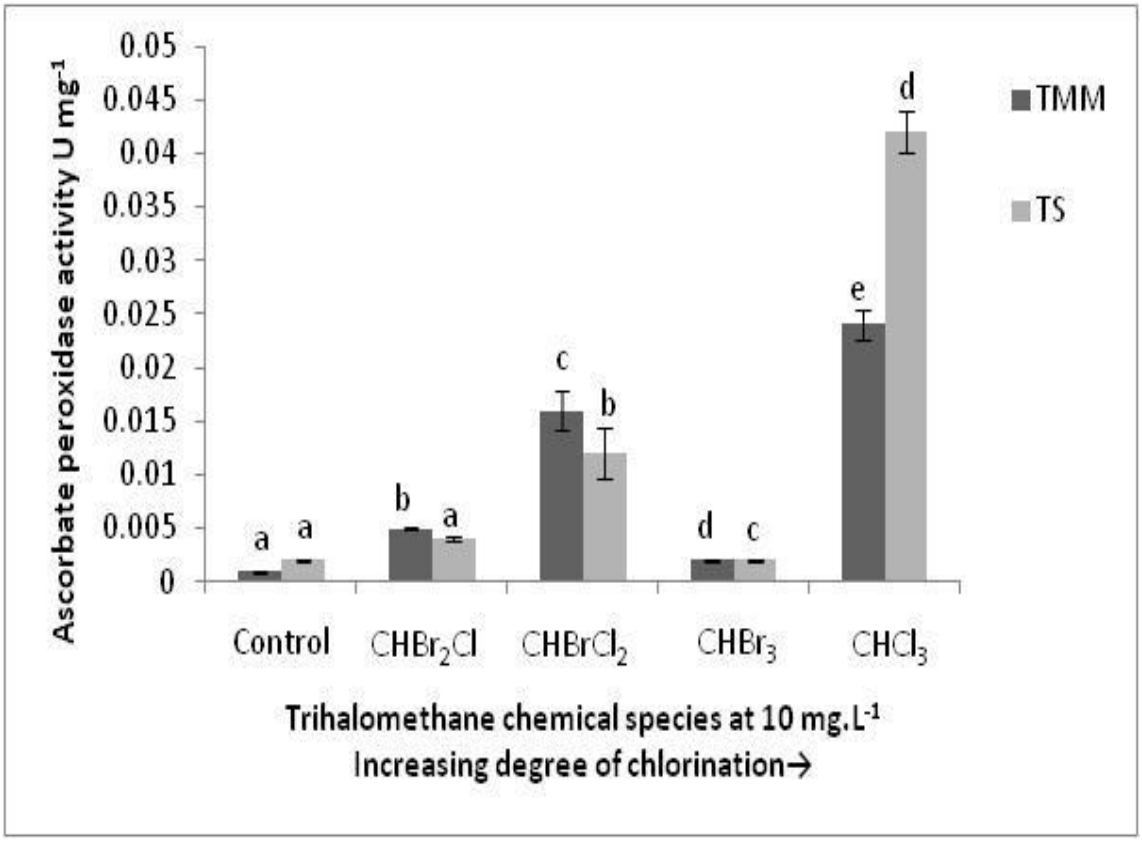


Fig 11: Effect of trihalomethanes on the ascorbate peroxidase (APX) activity of plant extracts (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 $mg.L^{-1}$ of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

In figure 11, It was observed that the activity of the peroxidase enzymes APX in plant extracts increased with increasing chlorination of trihalomethane molecules in the order of $CHBr_3$ < $CHBr_2Cl$ < $CHBrCl_2$ < $CHCl_3$ in both cultivars (fig 11). The ascorbate peroxidase activity of control plants were not significantly different from plants exposed to the mono-chlorinated trihalomethanes in the TS cultivars.

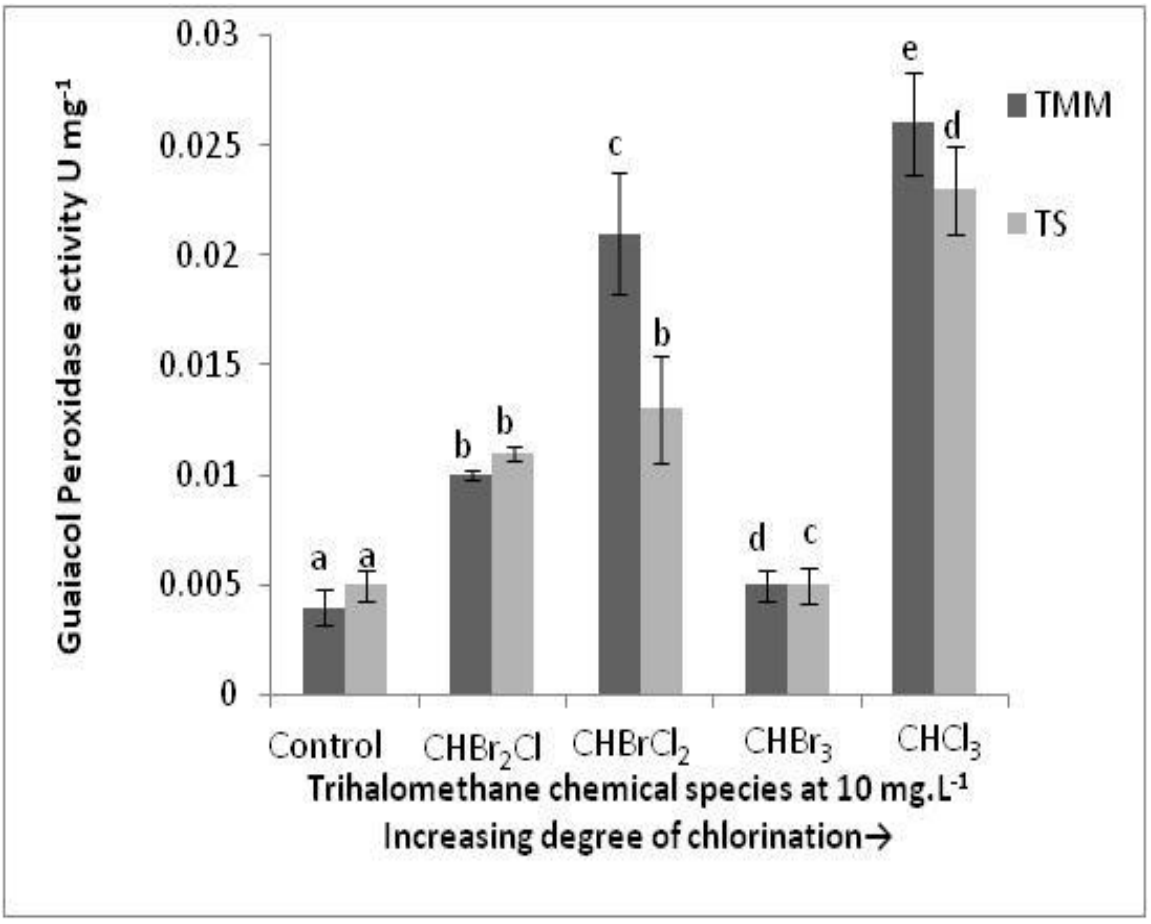


Fig 12: Effect of trihalomethanes on the guaiacol peroxidase (GPX) activity of plant extracts (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg.L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

Figure 12 shows the effects of trihalomethanes on the guaiacol peroxidase (GPX) activity of plant extracts from both tomato cultivars. It was observed that the activity of the peroxidase enzymes GPX in plant extracts increased with increasing chlorination of trihalomethane molecules in the order of $CHBr_3$ < $CHBr_2Cl$ < $CHBrCl_2$ < $CHCl_3$ in both cultivars (fig 12). The guaiacol peroxidase activity of control plants was significantly different from treatments.

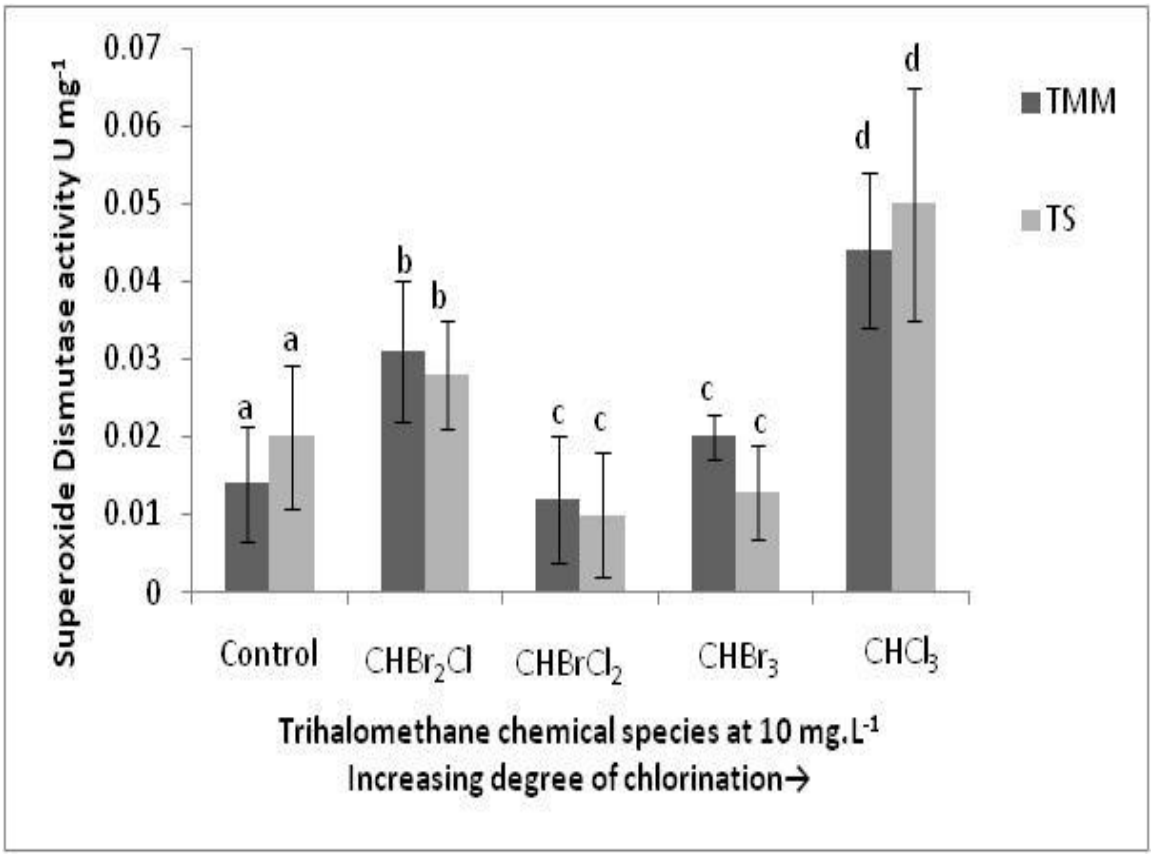


Fig 13: Effect of trihalomethanes on the superoxide dismutase (SOD) activity (data at 30 days after commencement of treatment). The tomato money-maker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 $mg.L^{-1}$ of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

In figure 13, we observed the activity of the superoxide dismutase enzymes (SOD) in both cultivars exposed to the trihalomethanes. The activity of superoxide dismutase (SOD) responded to no particular halogenation order, although chloroform ($CHCl_3$) induced the most activity in both cultivars. The superoxide dismutase activity in control plants was significantly different from treatments.

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

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

Significant correlations were observed for the total polyphenol content, the ferric reducing antioxidant power (FRAP) and the activity of guaiacol peroxidase enzyme. This implies that as the degree of chlorination increases, the oxidative stress biomarkers mentioned above increases.

The effects of the increasing halogenation of the trihalomethane molecule on all biomarkers of oxidative stress determined can be seen in Table 10 (Appendix). The total soluble proteins (BCA) of the exposed plants responded to no particular halogenation order. The tomato plants in the TMM cultivars exposed to bromoform ($CHBr_3$) had the highest soluble protein content while those exposed to chloroform ($CHCl_3$) had the lowest soluble protein content (Table 10).

The percentage increase in antioxidant parameters from the mean effects of mono-chlorinated trihalomethanes ($CHBr_2Cl$) to the mean effects of the tri-chlorinated

trihalomethanes ($CHCl_3$) was; total phenolic content (351.25%), FRAP (107.16%), ORAC (269.18%), APX activity (725%), and GPX activity (150%).

4.1.5 Effects of the increasing halogenation of trihalomethanes on fatty acids and α-tocopherol concentration in membrane lipids of lyophilized leaf tissues of tomato plants.

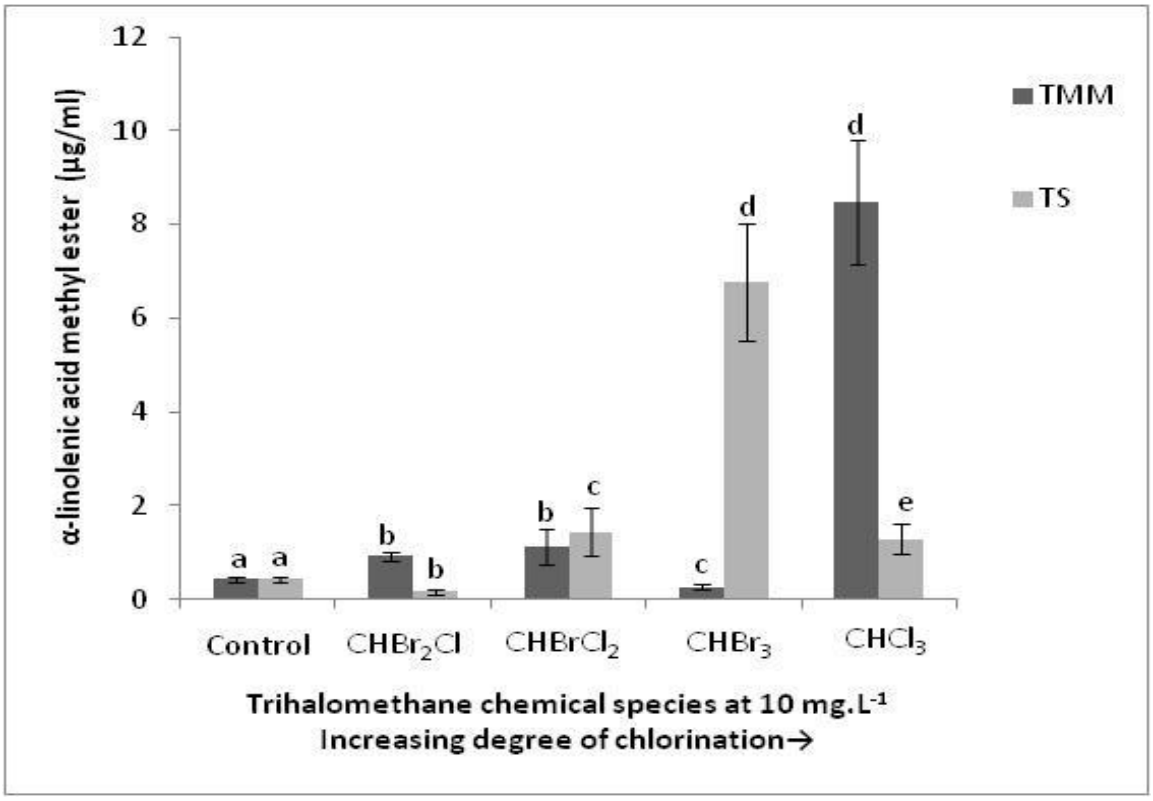


Fig 14: Effect of trihalomethanes on the α-linolenic acid content in membrane lipids of leaf tissues of tomato cultivars (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg.L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter in a column are not significantly different from each other at P ≤ 0.05 according to FSD. Vertical bars represent standard error of means (n=4).

Figure 14 shows the effects of the trihalomethanes on the α-linolenic acid content in membrane lipids of tomato leaf tissues. 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 $CHBr_3$ < $CHBr_2Cl$ < $CHBrCl_2$ < $CHCl_3$. There was no significant difference between TMM cultivars exposed to the mono-chlorinated and di-chlorinated trihalomethanes. The TS cultivars responded to no particular halogenation order as can

be seen in figure 14. The α-linolenic acid in control plants was significantly different from treatments.

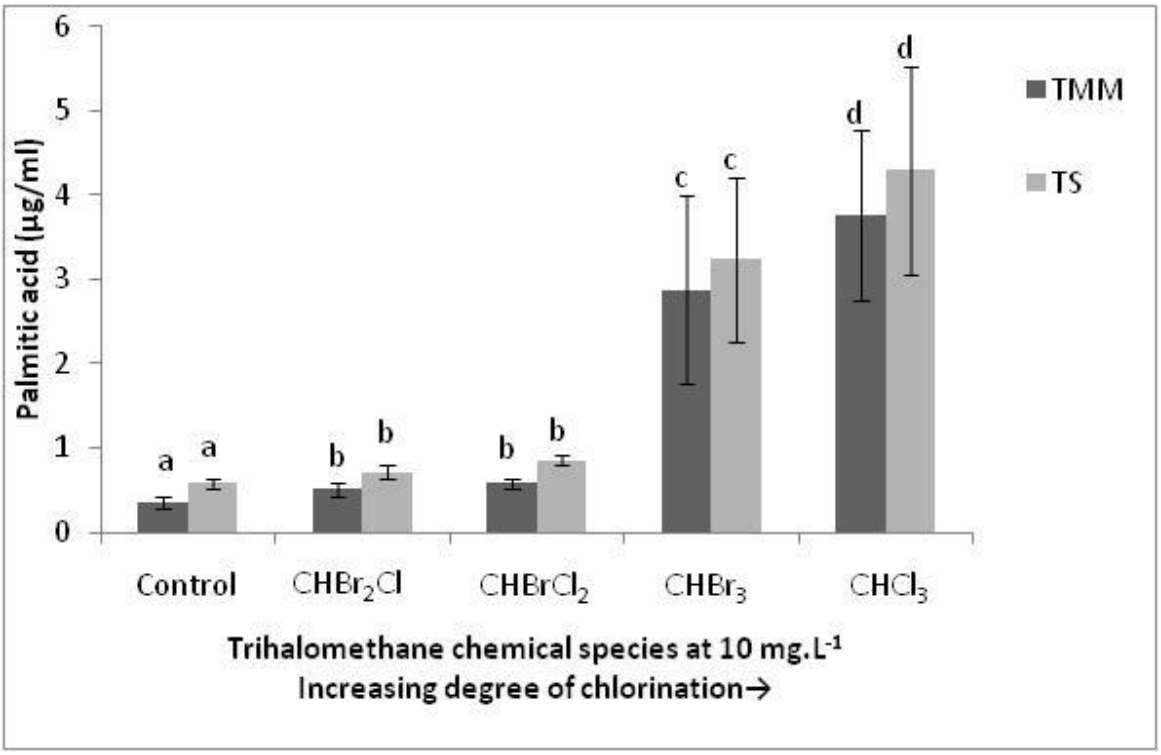


Fig 15: Effect of trihalomethanes on the palmitic acid content in membrane lipids of leaf tissues of tomato cultivars (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg.L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

In figure 15, we observed the effects of trihalomethanes on the palmitic acid content in the leaf membrane lipids of the tomato cultivars. The saturated fatty acid (palmitic acid) increased with increasing chlorination of the trihalomethane molecule in the order of $CHBr_2Cl = CHBrCl_2 < CHBr_3 < CHCl_3$ for both tomato cultivars, although the extent of accumulation was greater in the TS tomato cultivars. There was no significant difference in palmitic acid content between tomato plants exposed to mono-chlorinated and di-chlorinated trihalomethanes in both cultivars. The palmitic acid content in control plants was the lowest amongst the treatments.

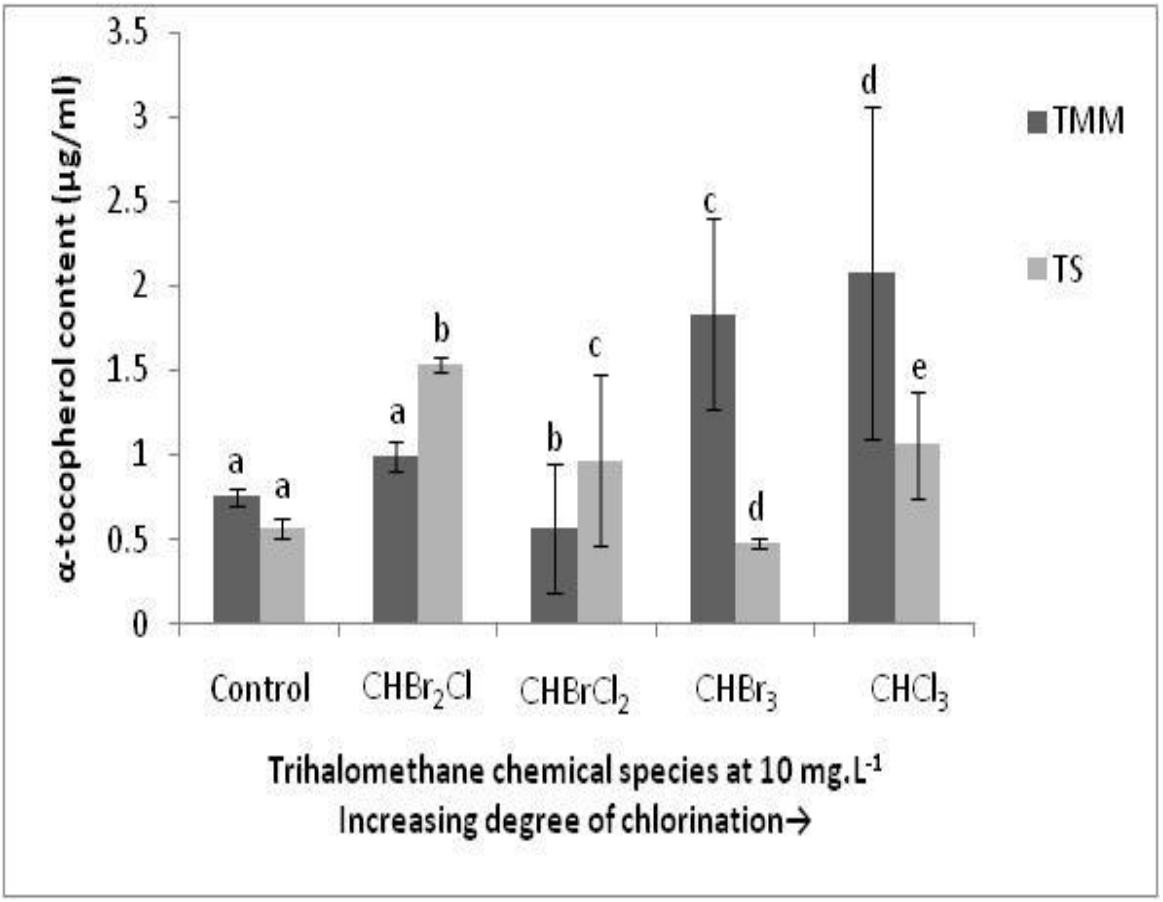


Fig 16: Effect of trihalomethanes on the α-tocopherol content in membrane lipids of leaf tissues of tomato cultivars (data at 30 days after commencement of treatment). The tomato moneymaker (TMM) and tomato star (TS) cultivars were exposed to a concentration of 10 mg.L^{-1} of each trihalomethane species. Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter in a column are not significantly different from each other at P ≤ 0.05 according to FSD. Vertical bars represent standard error of means (n=4).

Figure 16 shows the effects of trihalomethanes on the α-tocopherol content in membrane lipids of the leaf tissues of the tomato cultivars. The α-tocopherol concentration in both TMM and TS cultivars followed no particular halogenation order. There was no significant difference at P ≤ 0.05 in α-tocopherol content between control plants and plants exposed to mono-chlorinated trihalomethanes 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.

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

Leaf Membrane lipid components	Correlation coefficient (R) 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

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

4.1.6. Effect of increasing trihalomethane dose on root elongation and seedling biomass accumulation

Table 4 reports the data relative to the effects of trihalomethanes on root elongation of the seedlings of the two tomato cultivars tested on filter paper. In general, it may be inferred that the increase in trihalomethane concentrations led to a reduction in root growth of the tomato seedlings in question. The root growth inhibiting effect of the trihalomethanes appears to follow a bromination order as follows $CHCl_3$ > $CHBrCl_2$ > $CHBr_2Cl$ > $CHBr_3$ whereby increase in the bromine atoms in the trihalomethanes correlated to a decrease in root elongation.

Biomass accumulation in seedlings of both tomato cultivars decreased with increasing trihalomethane concentration in terms of fresh weight and dry weight in a dose dependant manner. The reduction in seedling biomass also correlated with an increase in the number of bromine atoms in the trihalomethane molecule.

Table 4
Effect of increasing trihalomethane dose on root elongation, fresh weight and dry weight of TMM and TS tomato seedlings.

Treatments	Root elongation	Fresh weight	Dry weight
	(mm)	(g)	
Variety			
Tomato moneymaker (TMM)	25.98± 1.65b	0.0368±0.0014a	0.0014±0.0001a
tomato star 9003 (TS)	29.61±2.30a	0.0322±0.0015b	0.0011±0.0000b
Chemical			
$CHCl_3$	32.50±2.91a	0.0405±0.0017a	0.0015±0.0001a
$CHBrCl_2$	30.47±2.00a	0.0362±0.0023b	0.0013±0.0001ab
$CHBr_2Cl$	24.79±3.06b	0.0318±0.0023c	0.0012±0.0001bc
$CHBr_3$	23.43±3.10b	0.0297±0.0016c	0.0011±0.0001c
Concentrations ($mg.L^{-1}$)			
Control	55.96±2.04a	0.0521±0.0018a	0.0018±0.0001a
2.5	28.42±1.92b	0.0378±0.0013b	0.0014±0.0001b
5.0	22.52±1.84c	0.0309±0.0016c	0.0012±0.0001c
7.5	17.99± 1.63d	0.0274±0.0014cd	0.0010±0.0001cd
10.0	14.08± 1.49e	0.0244±0.0015d	0.0010±0.0001d
3-Way ANOVA (F-statistics)			
Plant varieties	9.518**	16.065***	45.617***
Chemical Species	13.792***	17.708***	7.964***
Concentrations	159.445***	74.478***	44.780***
Plant varieties & Chemical Species	1.838ns	0.701ns	1.516ns
Plant varieties & Concentrations	6.263***	1.414ns	0.707ns
Chemical Species & Concentrations	6.300***	1.857*	1.191ns
Plant varieties, Chemical Species & Concentrations	0.925	0.987	0.291ns

Values presented are means of n = 25 ± MSE. *, **, *** = significance at P≤ 0.05, P ≤ 0.01, P ≤ 0.001 respectively; NS = not significant, MSE= Standard error of mean. Means followed by similar letter in a column are not significantly different from each other at P = 0.05 according to FSD.

4.1.7. Effects of increasing trihalomethane dose on nutrient concentration

Figure 17 to 22 shows the effects of the increasing trihalomethane dose on the nutrient levels in the dry matter yields of the mean value of both TMM and TS tomato cultivars.

After 30 days of single exposure to each trihalomethane chemical specie, eight of the twelve nutrients decreased with increasing trihalomethane concentration in both tomato cultivars. These nutrients include nitrogen (N), phosphorus (P), potassium (K), sulphur (S), manganese (Mn), copper (Cu), zinc (Zn) and boron (B) although the decrease of phosphorus and sulphur were not significant at ($P \leq 0.05$).

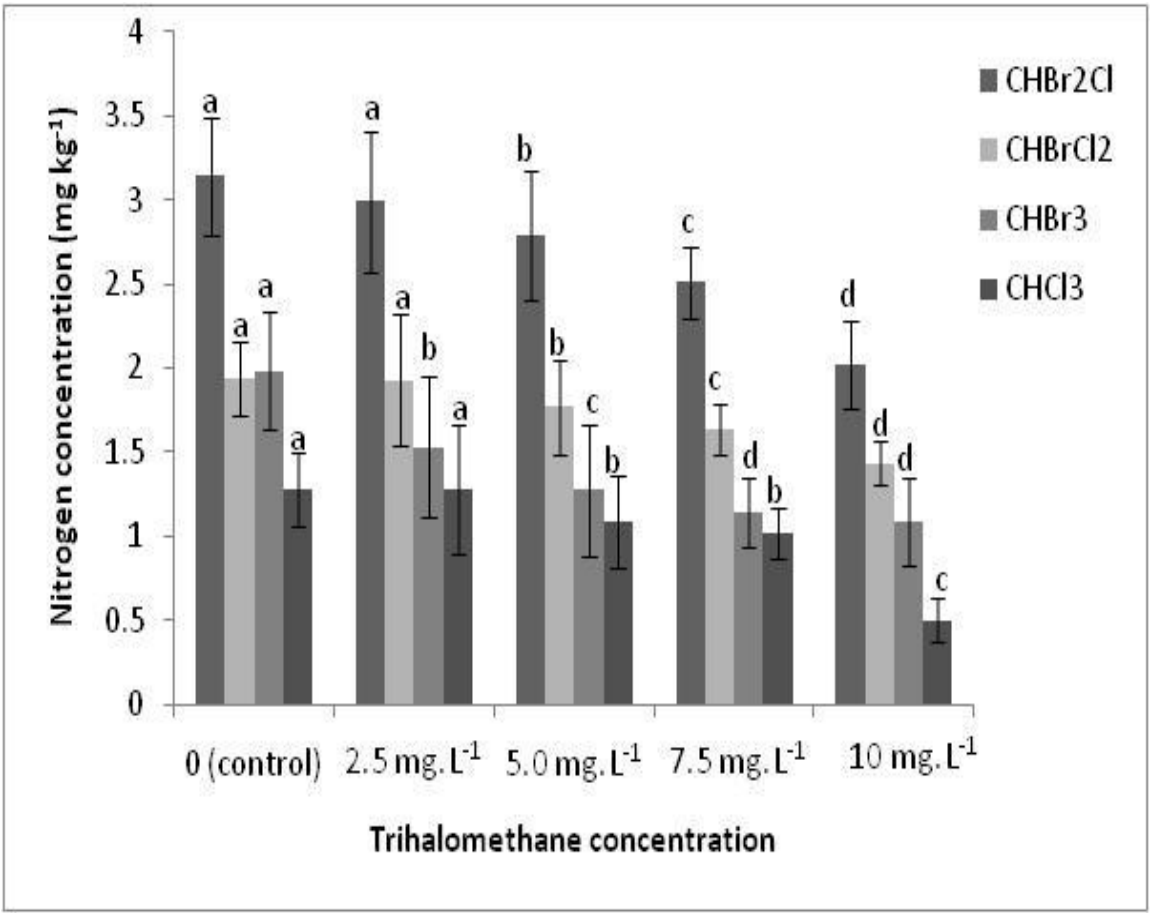


Fig 17: Effect of trihalomethane and concentration on nitrogen levels in the dry matter yields of the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

In figure 17, we observe the effects of the increasing dose of trihalomethanes on the nitrogen content in the dry matter yields of the tomato cultivars. In general, nitrogen content decreased with increasing trihalomethane dose. The tomato plants exposed to 2.5 $mg.L^{-1}$ of dibromochloromethane, bromodichloromethane and chloroform were not significantly different in nitrogen content from the control plants.

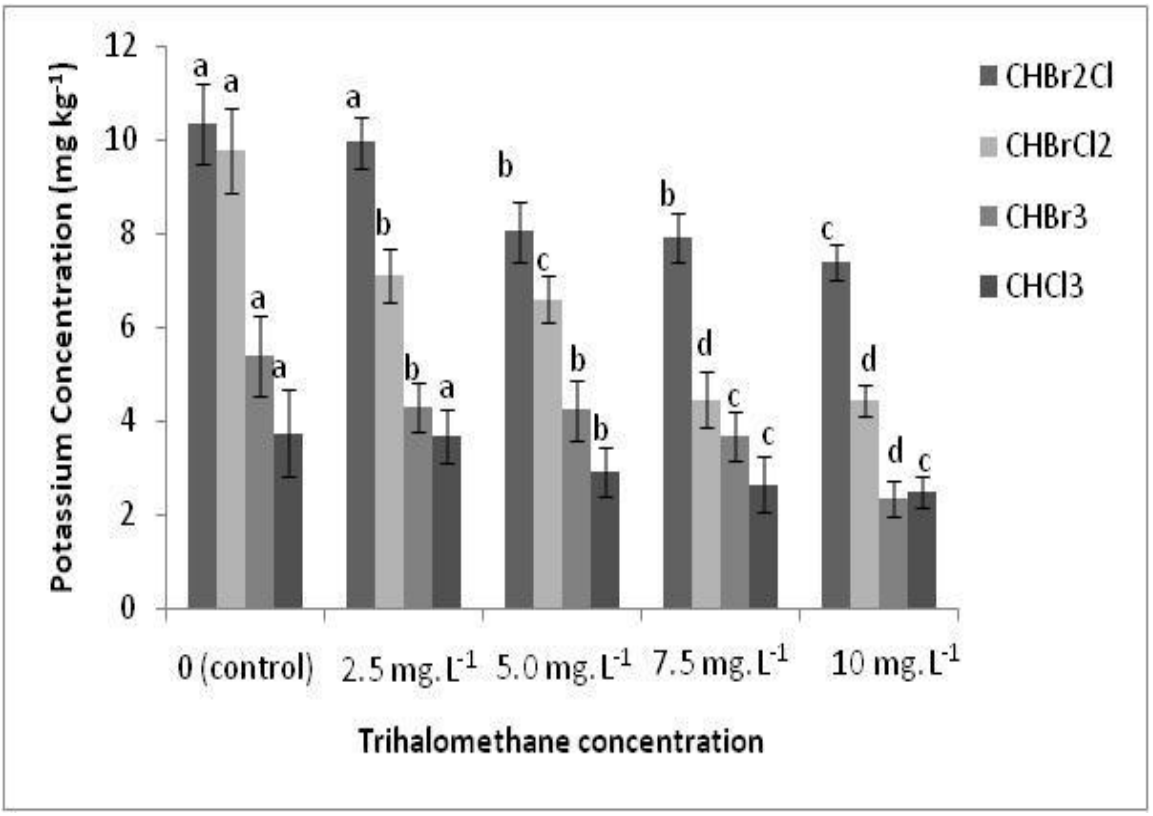


Fig 18: Effect of trihalomethane and concentration on potassium levels in the dry matter yields of the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

Figure 18 shows the effects of the increasing dose of trihalomethanes on the potassium content in the dry matter yields of the tomato cultivars. In general, potassium content decreased with increasing trihalomethane dose. The tomato plants exposed to 2.5 $mg.L^{-1}$ of dibromochloromethane and chloroform were not significantly different in potassium content from the control plants. The tomato plants exposed to 2.5 $mg.L^{-1}$ of bromoform were not significantly different in potassium content from the tomato plants exposed to

5.0 mg.L^{-1} of bromoform. The tomato plants exposed to 5.0 mg.L^{-1} of dibromochloromethane were not significantly different in potassium content from tomato plants exposed to 7.5 mg.L^{-1} of dibromochloromethane.

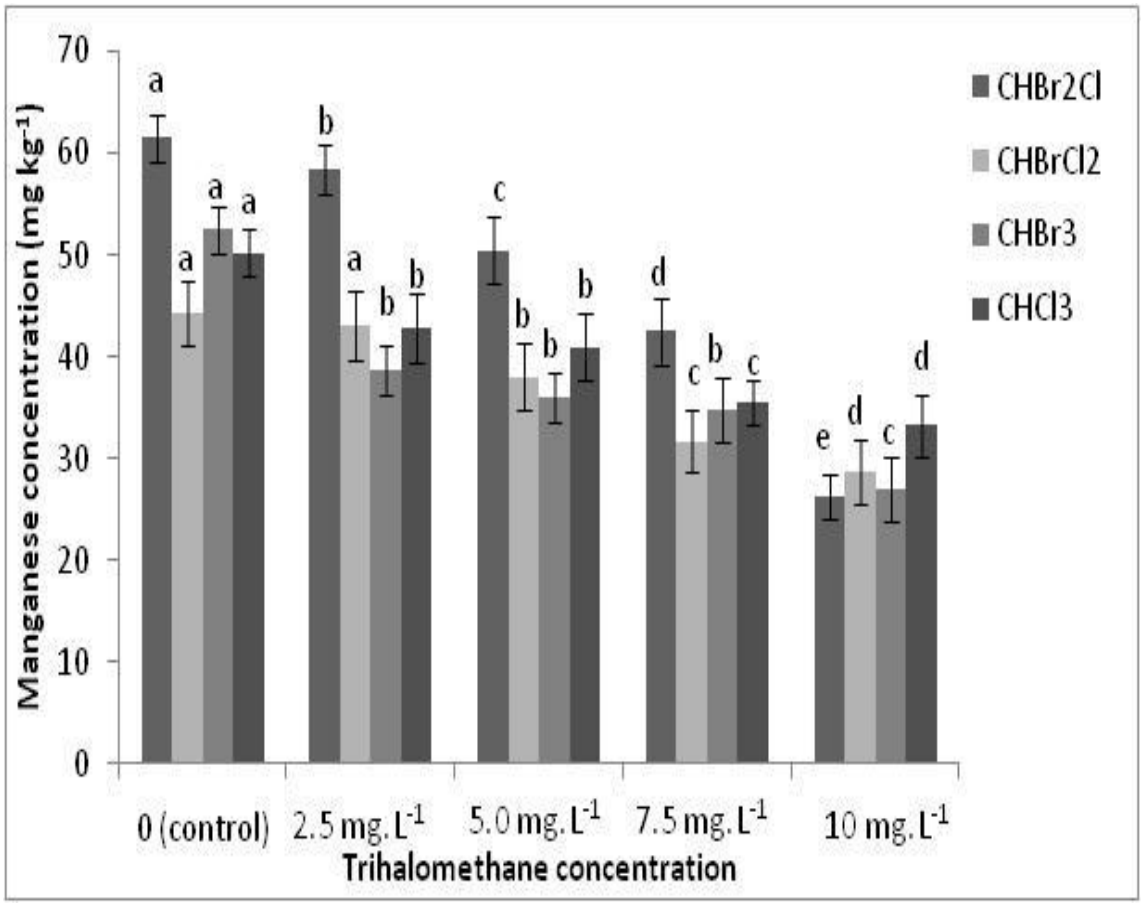


Fig 19: Effect of trihalomethane and concentration on manganese levels in the dry matter yields of the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter in a column are not significantly different from each other at P ≤ 0.05 according to FSD. Vertical bars represent standard error of means (n=4).

Figure 19 shows the effects of the increasing dose of trihalomethanes on the manganese content in the dry matter yields of the tomato cultivars. In general, manganese content decreased with increasing trihalomethane dose.

The tomato plants exposed to 2.5 mg.L^{-1} of bromodichloromethane were not significantly different in manganese content from the control plants. The tomato plants exposed to 2.5

mg.L^{-1}, 5.0 mg.L^{-1} and 7.5 mg.L^{-1} of bromoform were not significantly different in manganese content from each other.

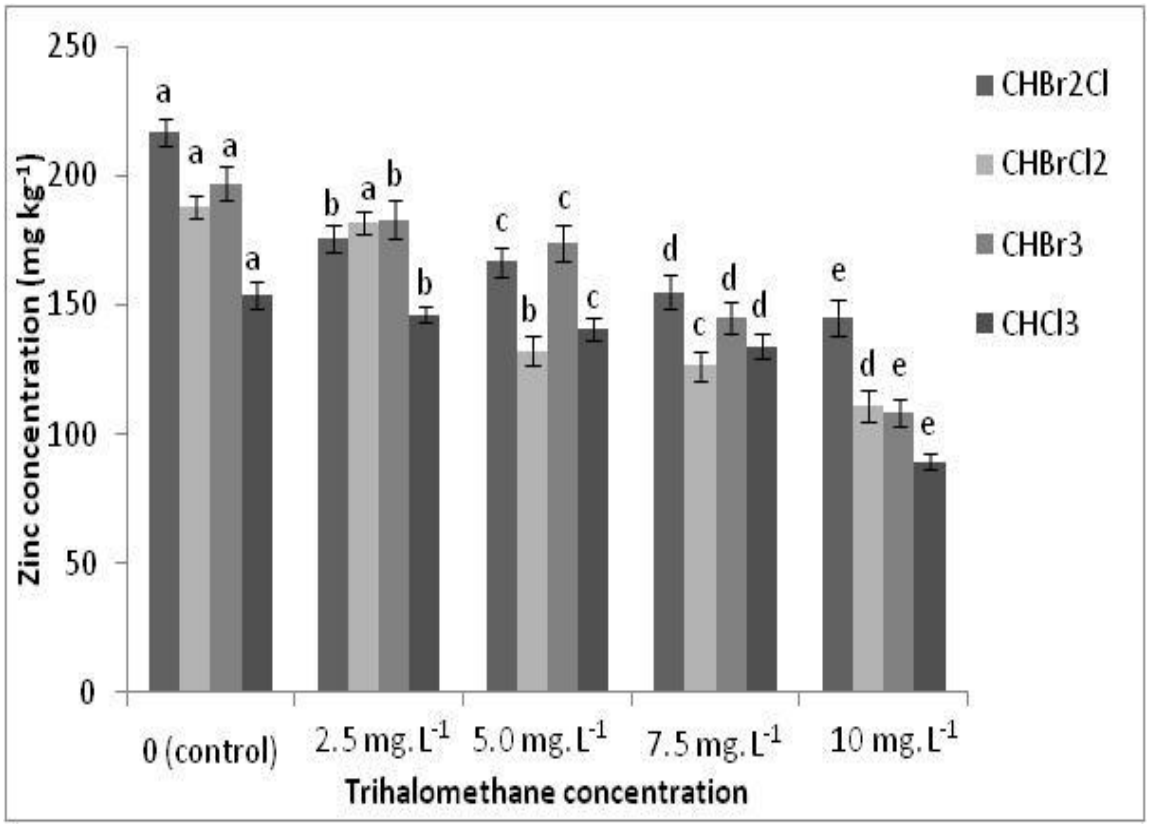


Fig 20: Effect of trihalomethane and concentration on zinc levels in the dry matter yields of the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter in a column are not significantly different from each other at P ≤ 0.05 according to FSD. Vertical bars represent standard error of means (n=4).

Figure 20 shows the effects of the increasing dose of trihalomethanes on the zinc content in the dry matter yields of the tomato cultivars. In general, zinc content decreased with increasing trihalomethane dose. The tomato plants exposed to 2.5 mg.L^{-1} of bromodichloromethane were not significantly different in zinc content from the control plants.

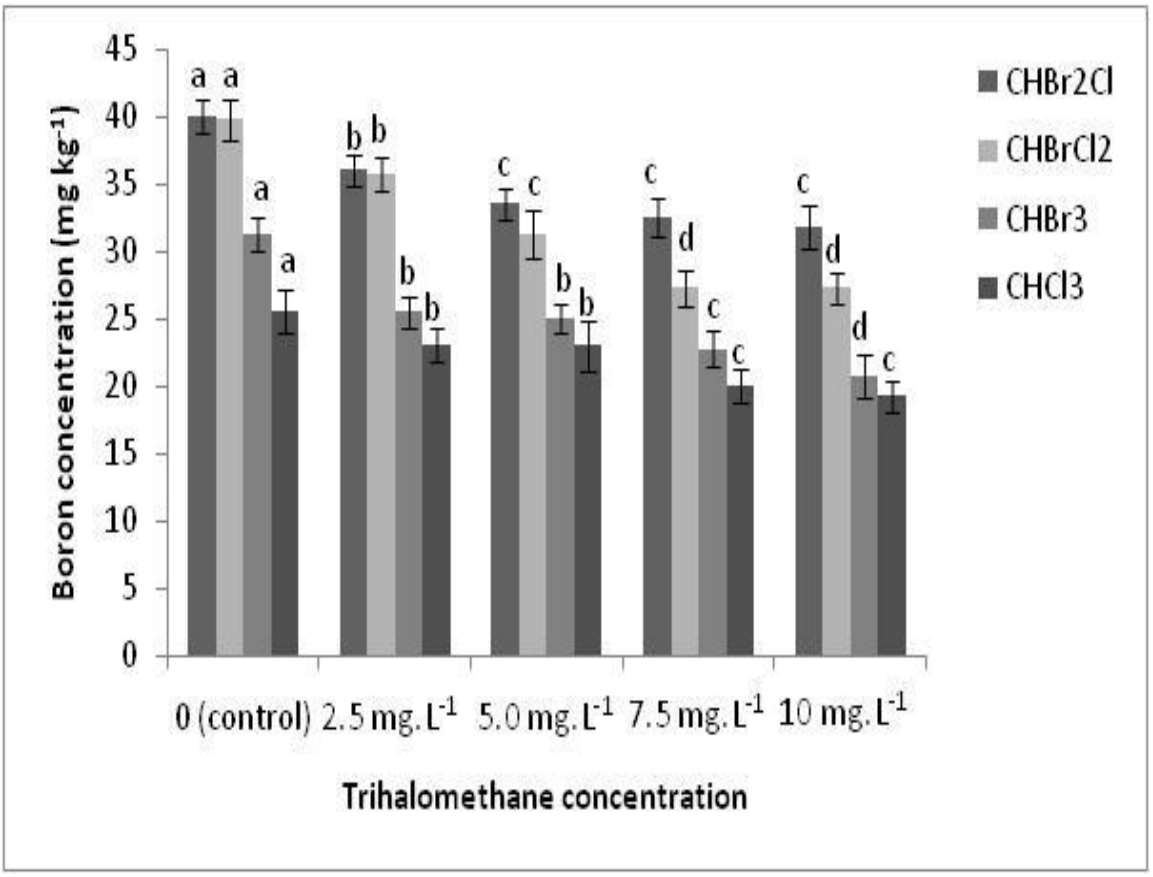


Fig 21: Effect of trihalomethane and concentration on boron levels in the dry matter yields of the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar 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).

Fig. 21 shows the effects of the increasing dose of trihalomethanes on the boron content in the dry matter yields of the tomato cultivars. In general, boron content decreased with increasing trihalomethane dose. The tomato plants exposed to 2.5 $mg.L^{-1}$ of each trihalomethanes were significantly different in boron content from the control plants. The tomato plants exposed to 2.5 $mg.L^{-1}$ of bromoform and chloroform were not significantly different in boron content from the tomato plants exposed to 5.0 $mg.L^{-1}$ of bromoform and chloroform. The tomato plants exposed to 5.0 $mg.L^{-1}$, 7.5 $mg.L^{-1}$ and 10 $mg.L^{-1}$ of dibromochloromethane were not significantly different in boron content from each other. The tomato plants exposed to 7.5 $mg.L^{-1}$ of bromodichloromethane and chloroform were

not significantly different in boron content from the tomato plants exposed to 10 mg.L^{-1} of bromodichloromethane and chloroform.

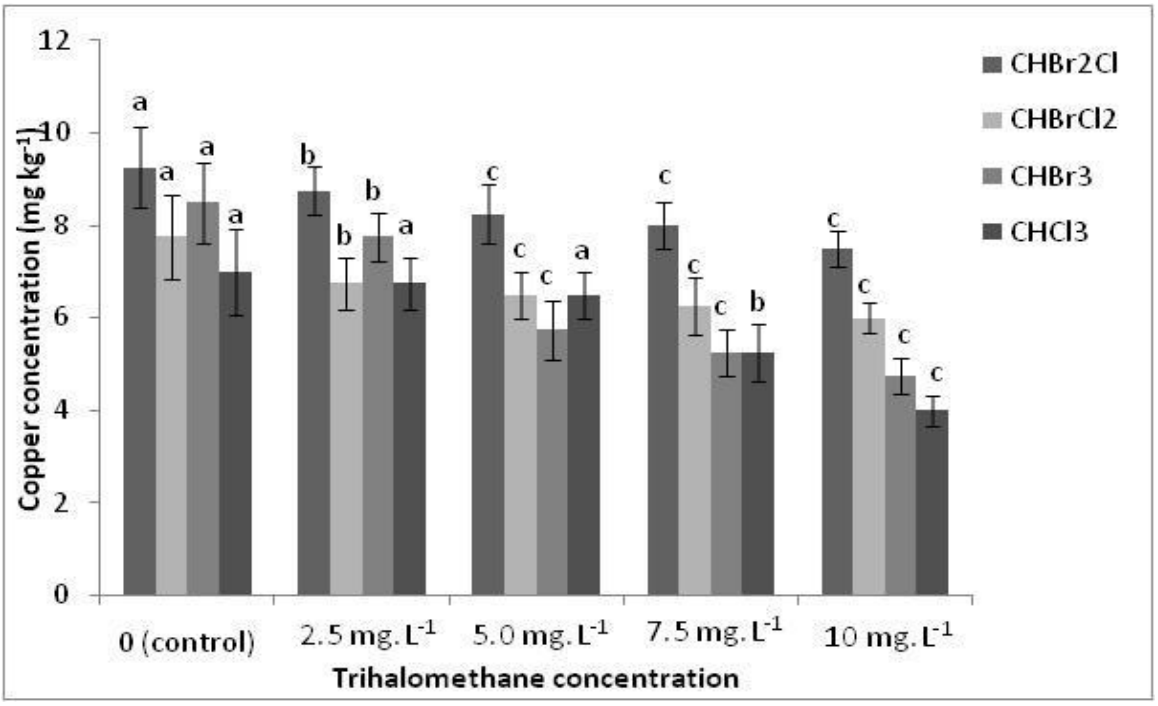


Fig 22: Effect of trihalomethane and concentration on copper levels in the dry matter yields of the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter in a column are not significantly different from each other at P ≤ 0.05 according to FSD. Vertical bars represent standard error of means (n=4).

Fig. 22 shows the effects of the increasing dose of trihalomethanes on the copper content in the dry matter yields of the tomato cultivars. In general, copper content decreased with increasing trihalomethane dose. The tomato plants exposed to 2.5 mg.L^{-1} and 5.0 mg.L^{-1} of chloroform were not significantly different in copper content from the control plants. The tomato plants exposed to 5.0 mg.L^{-1}, 7.5 mg.L^{-1} and 10 mg.L^{-1} of dibromochloromethane, bromodichloromethane and bromoform were not significantly different in copper content from each other.

The percentage decrease in nutrient levels from the overall mean value of nutrients in control plants to the mean value of nutrients in both tomato cultivars exposed to 10 mg.L^{-}

^{1} for all trihalomethane chemical species were N (34.72%), P (29.61%), K (43.02%), S (31.88%), Mn (44.72%), Cu (31.53%), Zn (39.96%) and B (27.47%).

The effects of increasing trihalomethane dose on all nutrients determined can be seen in Table 11 (Appendix). The nutrients whose concentrations were unaffected by trihalomethane dose in both tomato cultivars, displaying no particular trends, were calcium (Ca) and iron (Fe) (Table 11). Na and Mg concentrations in tomato plant dry matter were affected by trihalomethane dose although no discernable trends were observed. However, tomato plants exposed to concentrations of 5.0 $mg.L^{-1}$ and 7.5 $mg.L^{-1}$ of trihalomethanes appear to be increasing in Na and Mg (Table 11).

The correlation relationships between the nutrients exhibiting a similar behavior to increasing trihalomethane dose were tested using the Pearson correlation matrix in Table 12 (Appendix).

The strongest correlations were observed between N-K, P-K, N-S, P-B and K-B; all exhibiting a correlation value of $r > 0.8$ with increasing trihalomethane dose (Table 12). Correlation values of $r > 0.7$ were observed between N-P, P-S, K-S and N-B with increasing trihalomethane dose.

4.1.8. Effects of increasing trihalomethane dose on non-enzymatic biomarkers of oxidative stress

Figure 23 to 26 shows the effects of the increasing trihalomethane dose on the non-enzymatic biomarkers of oxidative stress on the mean value of TMM and TS tomato cultivars. The total phenolic content, ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) and the thiobarbituric reacting substances (TBARS) all increased with increasing trihalomethane dose. The non-enzymatic biomarkers in control plants were significantly different ($P \leq 0.05$) from the treatment plants with the exception of the tomato plants exposed to 2.5 $mg.L^{-1}$ of bromoform in both FRAP and ORAC tests (fig 24 & 25).

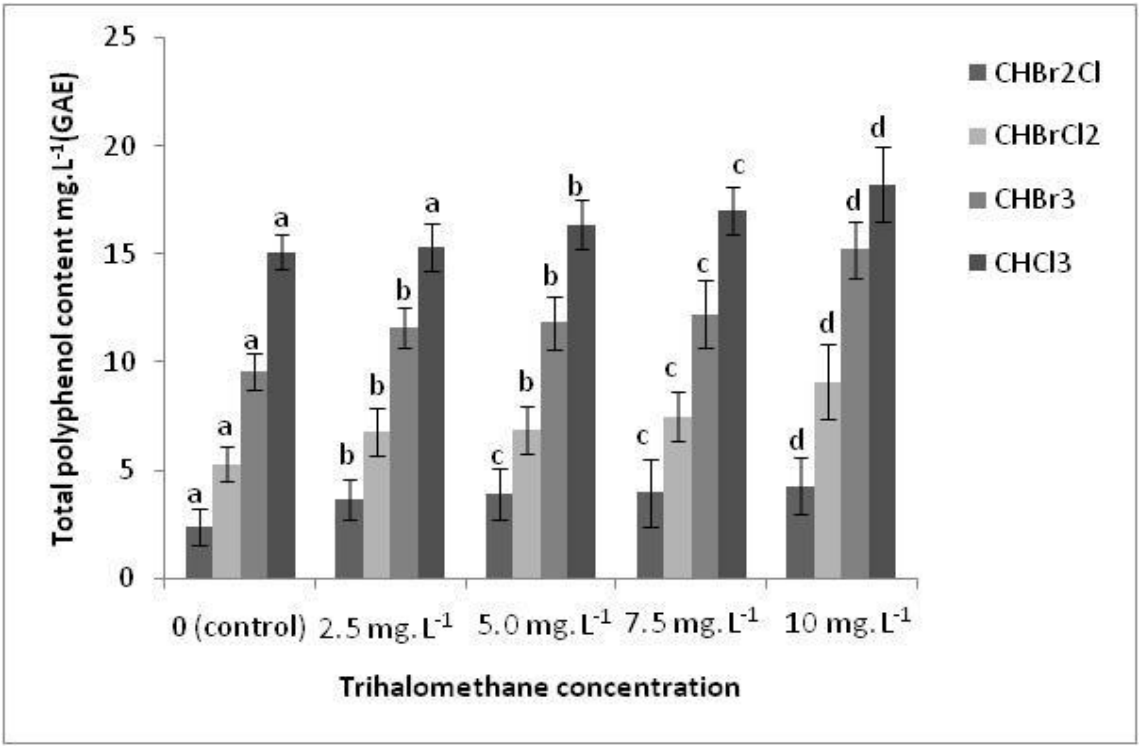


Fig 23: Effect of trihalomethane and concentration on the total polyphenol content in the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter 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).

Fig. 23 shows the effects of the increasing dose of trihalomethanes on the total polyphenol content in the leaves of the tomato cultivars. In general, the total polyphenol

content increased with increasing trihalomethane dose. The tomato plants exposed to 2.5 mg.L^{-1} of chloroform were not significantly different in total phenolic content from the control plants. The tomato plants exposed to 2.5 mg.L^{-1} of bromodichloromethane and bromoform were not significantly different in total phenolic content from the tomato plants exposed to 5.0 mg.L^{-1} of bromodichloromethane and bromoform. The tomato plants exposed to 5.0 mg.L^{-1} of dibromochloromethane were not significantly different in total phenolic content from the tomato plants exposed to 7.5 mg.L^{-1} of dibromochloromethane.

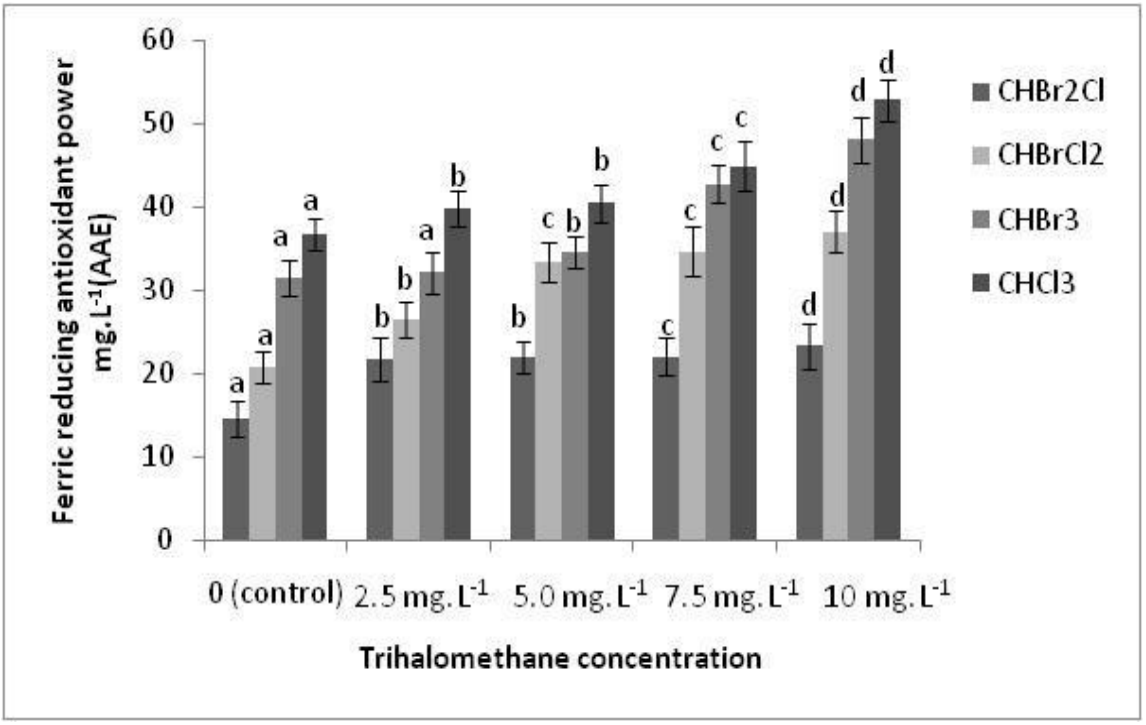


Fig 24: Effect of trihalomethane and concentration on the ferric reducing antioxidant power (FRAP) in the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter in a column are not significantly different from each other at P ≤ 0.05 according to FSD. Vertical bars represent standard error of means (n=4).

The effects of the increasing trihalomethane concentration on the ferric reducing antioxidant power (FRAP) in the extracts of the tomato plant cultivars is shown in Fig 24. The Fig. shows that in general, FRAP increased with increasing trihalomethane concentration. The tomato plants exposed to 2.5 mg.L^{-1} of bromoform were not (P ≤

0.05) different in FRAP values from the control plants. Similarly, tomato plants exposed to 2.5 $mg.L^{-1}$ of $CHBr_2Cl$ and $CHCl_3$ were not ($P \leq 0.05$) different in FRAP values from the tomato plants exposed to 5.0 $mg.L^{-1}$ of each trihalomethane compound. The tomato plants exposed to 5.0 $mg.L^{-1}$ of $CHBrCl_2$ were not ($P \leq 0.05$) different in FRAP values from the tomato plants exposed to 7.5 $mg.L^{-1}$ of $CHBrCl_2$.

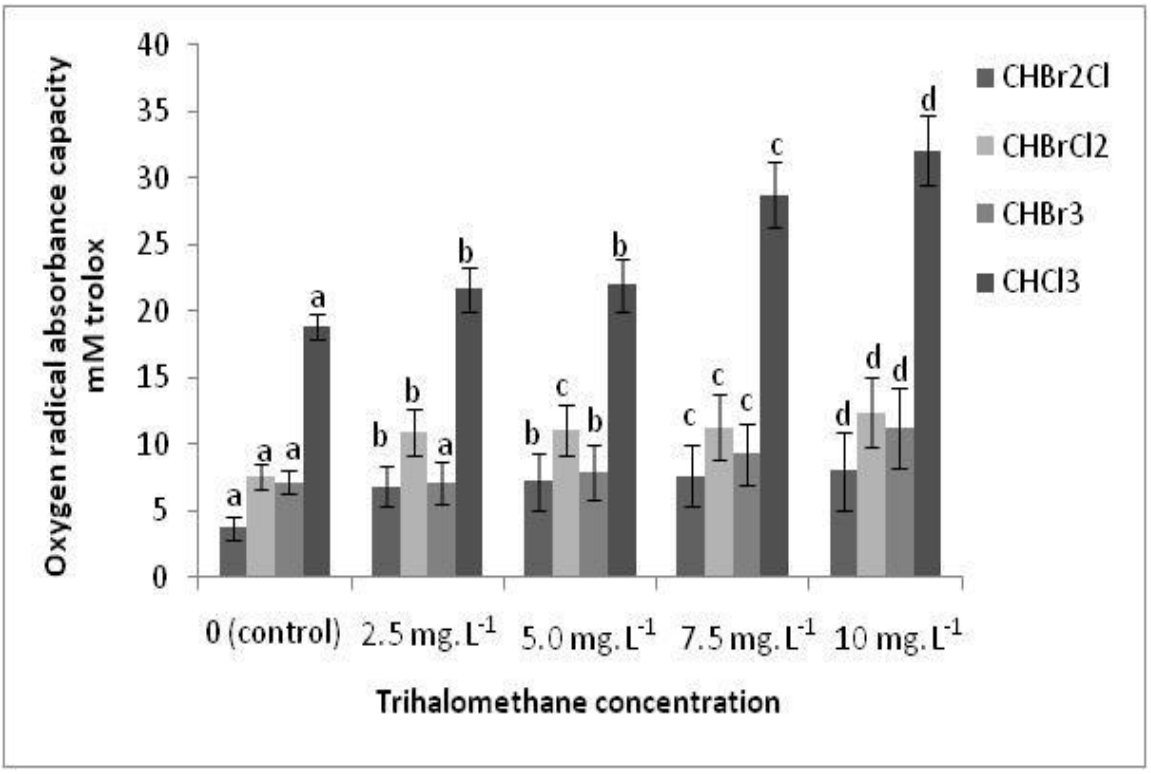


Fig 25: Effect of trihalomethane and concentration on the oxygen radical absorbance capacity (ORAC) in the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by 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).

Fig. 25 shows the effects of the increasing dose of trihalomethanes on the oxygen radical absorbance capacity in the plant leaf extracts of the tomato cultivars. In general, the oxygen radicle absorbance capacity (ORAC) increased with increasing trihalomethane dose. The tomato plants exposed to 2.5 $mg.L^{-1}$ of bromoform were not significantly different in ORAC values from the control plants. The tomato plants exposed

to 2.5 mg.L^{-1} of dibromochloromethane and chloroform were not significantly different in ORAC values from the tomato plants exposed to 5.0 mg.L^{-1} of dibromochloromethane and chloroform. The tomato plants exposed to 5.0 mg.L^{-1} of bromodichloromethane were not significantly different in ORAC values from the tomato plants exposed to 7.5 mg.L^{-1} of bromodichloromethane.

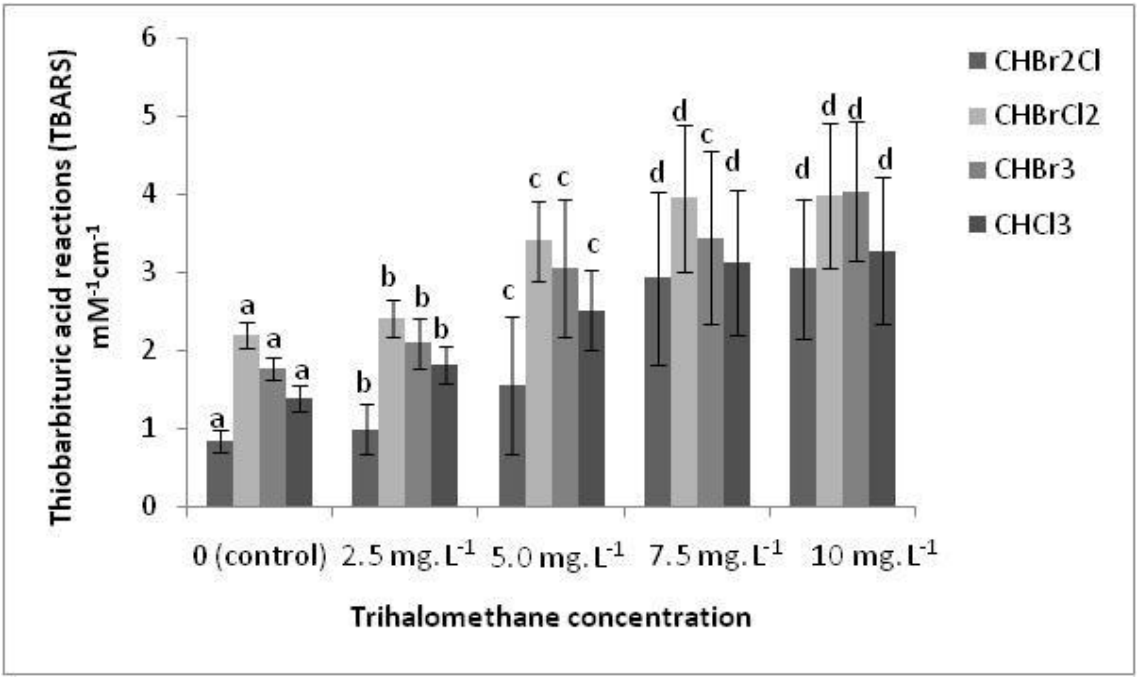


Fig 26: Effect of trihalomethane and concentration on the thiobarbituric acid reactions (TBARS) in the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter in a column are not significantly different from each other at P ≤ 0.05 according to FSD. Vertical bars represent standard error of means (n=4).

Fig 26 shows the effects of the increasing dose of trihalomethanes on the thiobarbituric acid reactions (TBARS) in plant extracts from the leaves of the tomato cultivars. The TBARS assay measuring general lipid peroxidation increased with increasing trihalomethane dose. The tomato plants exposed to 5.0 mg.L^{-1} of bromoform were not significantly different in TBARS values from the tomato plants exposed to 7.5 mg.L^{-1} of bromoform. The tomato plants exposed to 7.5 mg.L^{-1} of bromodichloromethane,

dibromochloromethane and chloroform were not significantly different in TBARS values from the tomato plants exposed to 10 $mg.L^{-1}$ of each of these trihalomethanes.

4.1.9. Effects of increasing trihalomethane dose on the enzymatic biomarkers of oxidative stress

In figures 27 and 28, we observe the effects of the increasing trihalomethane dose on the mean value of the enzymatic biomarkers of oxidative stress for both TMM and TS cultivars. The activities of ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) increased with increasing trihalomethane dose (fig 27 & 28).

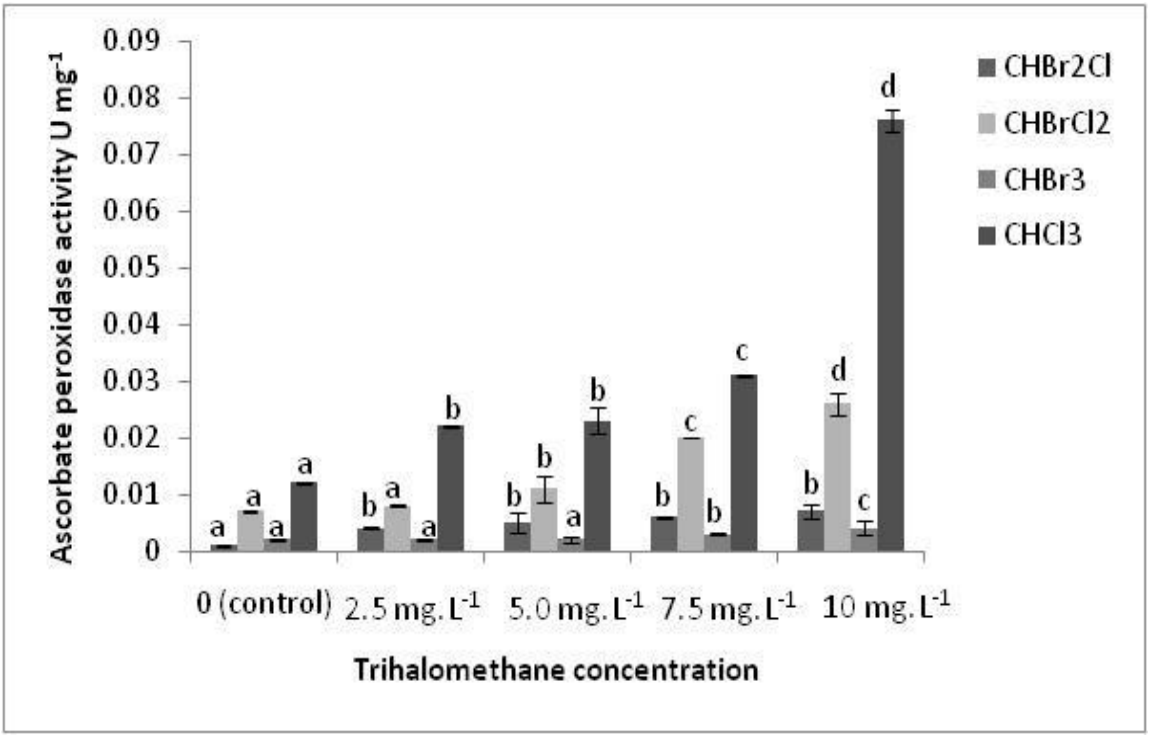


Fig 27: Effect of trihalomethane and concentration on ascorbate peroxidase activity in the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter 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).

Fig 27 shows the effects of the increasing dose of trihalomethanes on the activity of the enzyme ascorbate peroxidase (APX) in plant extracts from the leaves of the tomato cultivars. The activity of ascorbate peroxidase in general increased with increasing trihalomethane dose. The activity of ascorbate peroxidase in tomato plants exposed to 2.5 $mg.L^{-1}$, 5.0 $mg.L^{-1}$, 7.5 $mg.L^{-1}$ and 10 $mg.L^{-1}$ of dibromochloromethane were not

significantly different from each other, although the APX activity in these plants were significantly different from the control plants. The tomato plants exposed to 2.5 $mg.L^{-1}$ of bromodichloromethane were not significantly different in APX activity from the control plants. The tomato plants exposed to 2.5 $mg.L^{-1}$ and 5.0 $mg.L^{-1}$ of bromoform were not significantly different in APX activity from the control plants. The tomato plants exposed to 2.5 $mg.L^{-1}$ of chloroform were not significantly different in APX activity from the plants exposed to 5.0 $mg.L^{-1}$ of chloroform.

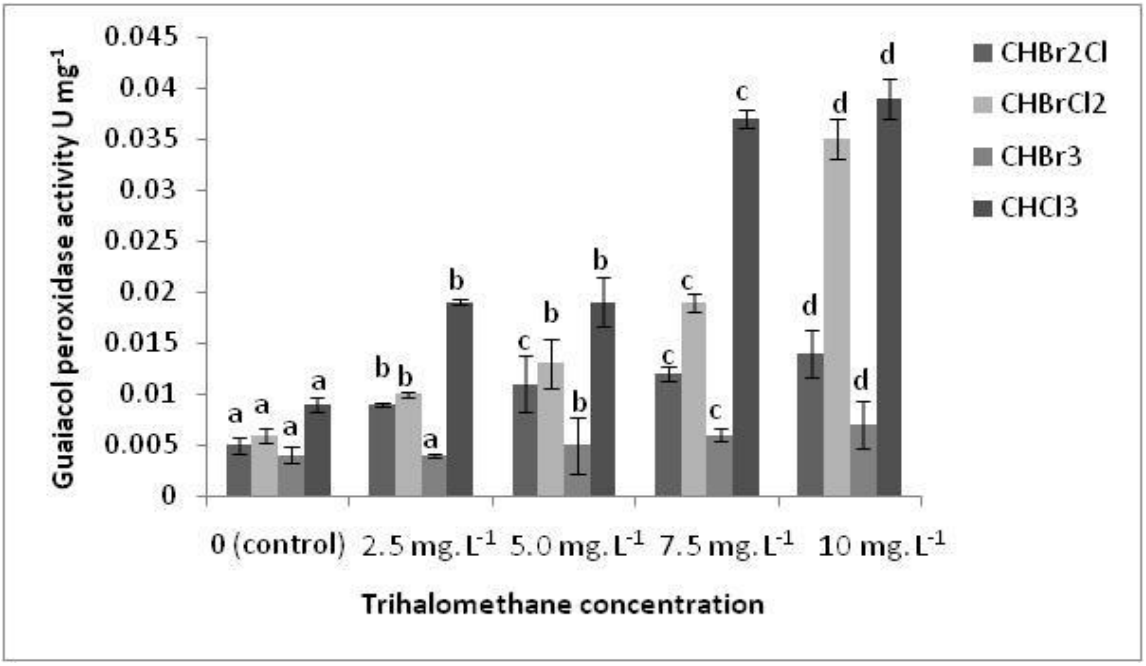


Fig 28: Effect of trihalomethane and concentration on guaiacol peroxidase activity in the overall mean value of both TMM and TS tomato plants (data at 30 days after commencement of treatment). Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter 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).

Fig 28 shows the effects of the increasing dose of trihalomethanes on the activity of the enzyme guaiacol peroxidase (GPX) in plant extracts from the leaves of the tomato cultivars.

The activity of guaiacol peroxidase increased with increasing trihalomethane dose. The activity of guaiacol peroxidase in tomato plants exposed to 2.5 $mg.L^{-1}$ of

bromodichloromethane and chloroform were not significantly different from the tomato plants exposed to 5.0 $mg.L^{-1}$ of each trihalomethane. The tomato plants exposed to 5.0 $mg.L^{-1}$ of dibromochloromethane were not significantly different in GPX activity from the tomato plants exposed to 7.5 $mg.L^{-1}$ of dibromochloromethane.

In Table 13 (appendix), the effects of all oxidative stress biomarkers determined were reported. It can be seen that no particular response to increasing trihalomethane dose were observed for the activity of superoxide dismutase (SOD) and the total soluble protein content (BCA) in both tomato cultivars (Table 13).

The correlation relationships between the biomarkers of oxidative stress in response to increasing trihalomethane dose were tested using the Pearson correlation matrix in table 14 (Appendix). The strongest correlations between oxidative stress parameters to increasing trihalomethane dose were observed between total polyphenols and FRAP exhibiting a correlation of $r > 0.8$. Correlations of $r > 0.7$ were observed between total polyphenols and ORAC. The peroxidase enzymes exhibited a relatively strong correlation amongst themselves of $r > 0.6$ (Table 14).

The percentage increase in the antioxidant parameters measured from the mean of control plants to the mean effects of plants exposed to 10 $mg.L^{-1}$ for all trihalomethane chemicals were total polyphenols (44.79%), FRAP (55.51%), ORAC (71.13%), ascorbate peroxidase (APX) activity (366.67%), guaiacol peroxidase (GPX) activity (300%) and TBARS (132.02%).

4.2. A comparison of the response to increasing trihalomethane dose with the response to increasing halogenation in nutrient concentration

A comparison between the effects of increasing trihalomethane dose and the effects of increasing chlorination on the percentage decrease in nutrient concentration can be seen in figure 9. The effects of increasing halogenation induced a greater decrease on nutrient levels with the exception of copper and zinc where the effects of increasing trihalomethane dose appear to be greater.

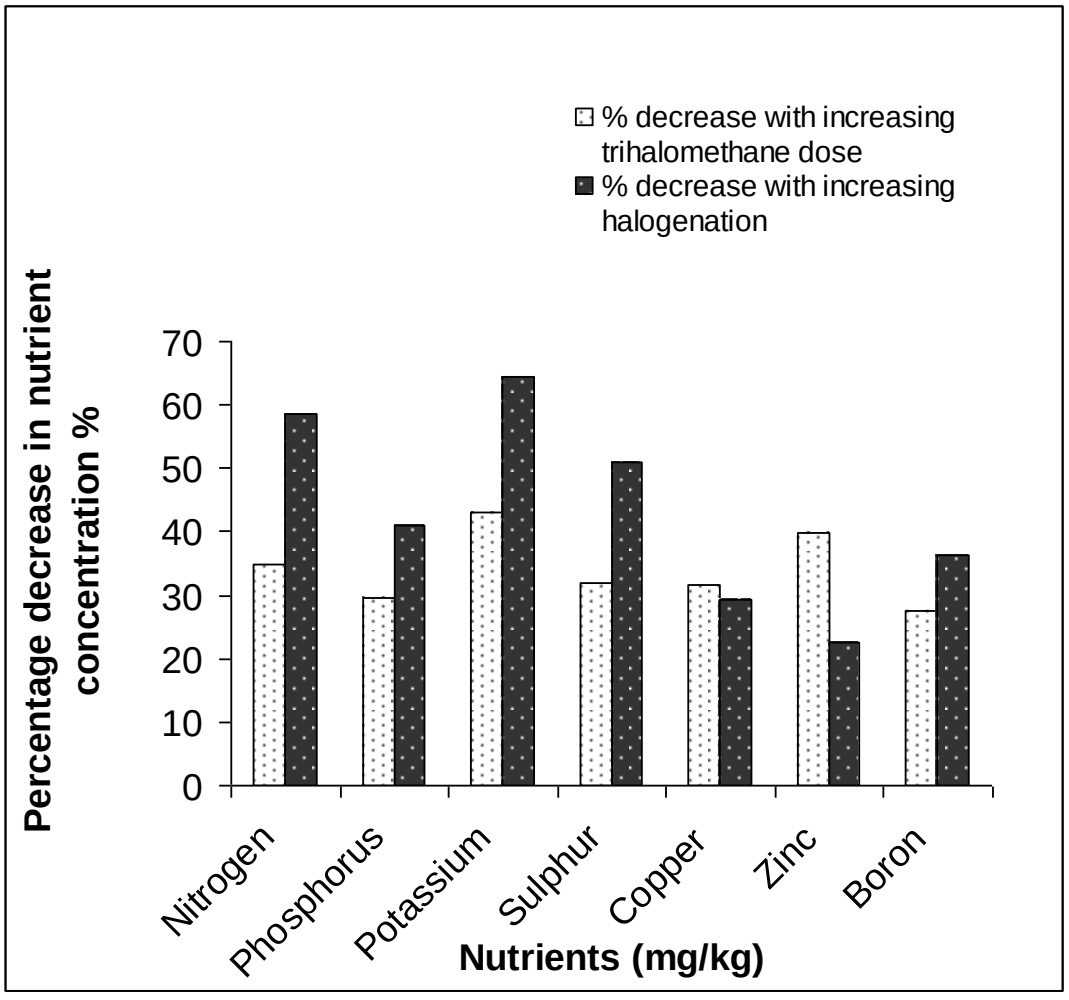


Fig 29: Comparison between the effects of increasing trihalomethane dose and increasing halogenation on nutrient concentration of the dry matter of tomato plants after a 30 day exposure.

4.2.1 A comparison between the effects of increasing trihalomethane dose and the effects of increasing halogenation on the antioxidant response of tomato plants

A comparison between the effects of increasing trihalomethane dose and the effects of increasing chlorination on the percentage increase in antioxidant parameters can be seen in figure 10. The effects of increasing halogenation induced a greater response on all antioxidant parameters with the exception of the activities of guaiacol peroxidase (GPX) that appear to be more sensitive to the effects of increasing trihalomethane dose.

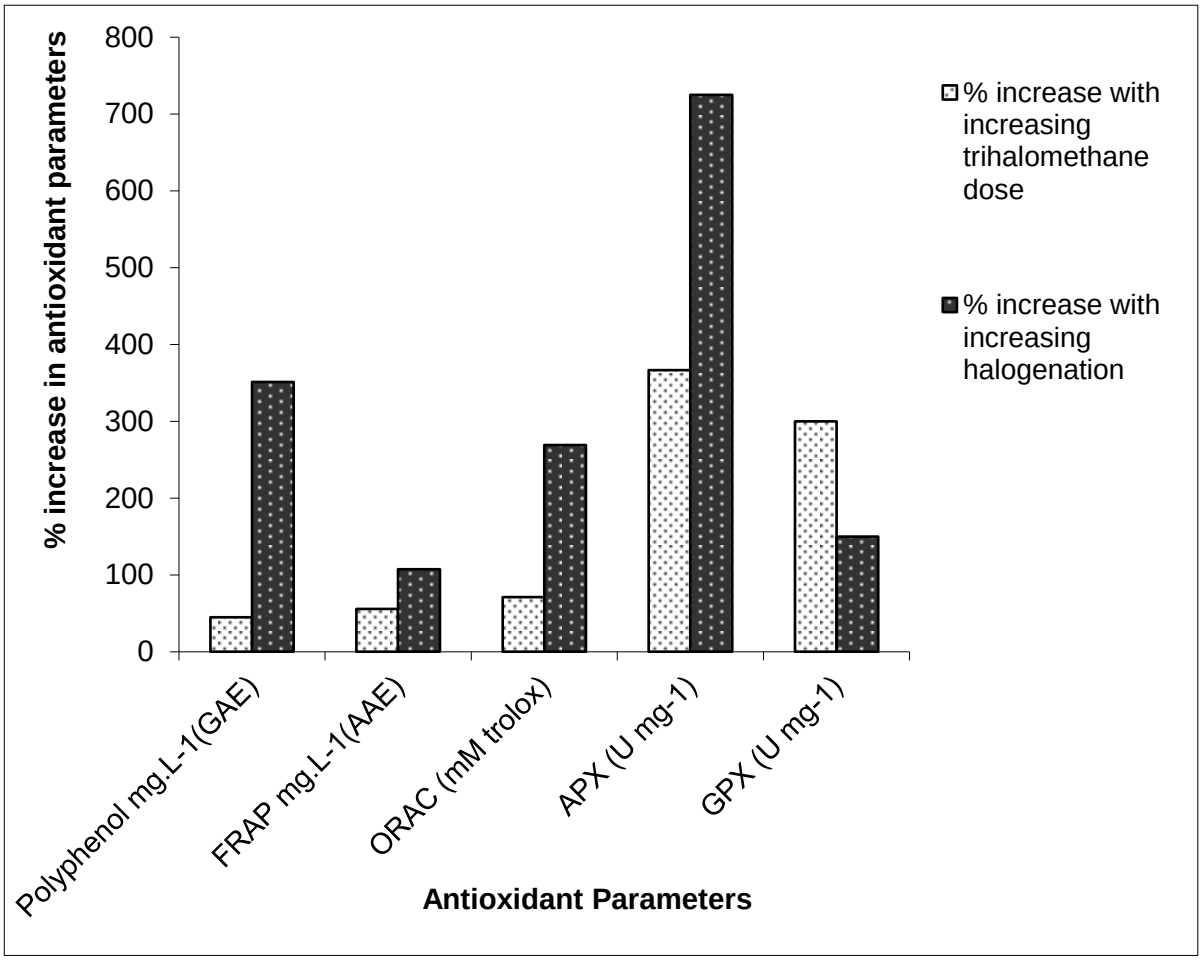


Fig 30: Comparison between the effects of increasing trihalomethane dose and increasing halogenation on oxidative stress parameters of tomato plants after a 30 day exposure

4.2.2. Effects of increasing trihalomethane dose on membrane lipid composition in lyophilized leaf tissues of tomato plants

Quantities of various lipid components extracted were determined by division of the peak area of known amounts of internal standard (IS) with the peak areas of the lipid component of interest to generate GC/MS relative response factor (RRF).

Relative Response Factor (RRF) = $\mathrm{RFA} / \mathrm{RFB}\ (\mathrm{IS})$

Where RFA = Response factor A and RFB = Response factor B

Equation 4.2.2.1

Although the lipidome fraction is known to contain several hundred molecules, we report lipid components with a statistically significant difference at P≤0.05 in relative response factors from the control only or a lipid component expressed in the chemically stressed variety but not detected in the control extracts. The RRF can be used to calculate the unknown concentration of analyte A in the presence of a known concentration of analyte B using the following equation:

Concentration CA = $\mathrm{PAA} / \mathrm{PAB}\ x 1 / \mathrm{RRF}\ x \mathrm{CB}$

Where: CA = Concentration A, PAA = Peak area A; PAB = Peak area B; and RRF = Relative response factor; CB = Concentration B

Equation 4.2.2.2

The percentage increases or decreases in concentration (µg/ml) of the lipid components of interest were also reported.

Table 5
Effects of bromodichloromethane on lipid membrane composition of lyophilized leaf tissues of tomato plants (data at 30 days after commencement of treatment)

	Plant Variety			
Lipid Component	TMM control (a)	TMM exposed to 10 $mg.L^{-1}$ of $CHBrCl_2$ (b)	TS control (c)	TS 10 exposed to 10 $mg.L^{-1}$ of $CHBrCl_2$ (d)
α-linolenic acid methyl ester (mean concentration µg ml^{-1})	0.424	1.140	0.427	1.446
mean peak area	29435668	66170341	18470939	60931353
% increase in concentration	124.80%		229.87%	
mean retention Time (min)	25.687	25.695	25.683	25.690
CV %	6.25	3.84	4.25	5.88
P≤ 0.05	*ab		*cd	
Neophytadiene (mean concentration µg ml^{-1})	5.483	0.193	0.290	0.310
mean peak area	38076209	11169295	12529012	13062927
% increase in concentration	-70.67%		4.26%	
mean retention Time (min)	26.276	27.896	22.787	22.484
CV %	5.84	9.42	7.12	6.62
P≤ 0.05	*ab		NS	
Ethyl linoleolate (mean concentration µg ml^{-1})	5.483	10.507	6.188	7.093
mean peak area	380762099	609909194	267540708	298904073
% increase in concentration	60.18%		11.72%	
mean retention Time (min)	26.275	26.326	26.249	26.256
CV %	4.84	8.19	6.16	4.92
P≤ 0.05	*ab		NS	
Palmitic acid (mean concentration µg ml^{-1})	0.358	0.579	0.302	0.854
mean peak area	24883794	33606499	13072285	36004617
% increase in concentration	35.05%		175.43%	
mean retention Time (min)	23.587	23.587	23.586	23.588
CV %	8.56	7.48	3.18	6.46
P≤ 0.05	NS		*cd	

Table 5 continued

	Plant Variety			
Lipid Component	TMM control (a)	TMM exposed to 10 $mg.L^{-1}$ of $CHBrCl_2$ (b)	TS control (c)	TS exposed to 10 $mg.L^{-1}$ of $CHBrCL_2$ (d)
Vitamin E (mean concentration µg ml^{-1})	0.752	0.570	3.849	0.971
mean peak area	43627849	39592567	50989599	4090952
% increase in concentration	-24.16%		-91.97%	
mean retention Time (min)	35.557	35.557	35.564	35.557
CV %	4.42	6.37	5.93	8.64
P≤ 0.05	NS		*cd	
Nonacosane (mean concentration µg ml^{-1})	0.330	0.732	0.707	0.336
mean peak area	22893955	42472291	30556733	14149792
% increase in concentration	85.52%		-53.70%	
mean retention Time (min)	33.634	31.870	31.868	31.866
CV %	6.19	7.44	5.15	7.68
P≤ 0.05	*bd *ab		*cd *db	

Values presented are means of n = 4 replicates, *ab = significance at P≤0.05 respectively when column a & b are compared; NS = not significant

A significant increase in α-linolenic acid was observed in the membrane lipids of both tomato cultivars subjected to chemical stress (Table 5). Neophytadiene was significantly reduced in TMM cultivar only under $CHBrCl_2$ stress, while ethyl linoleolate increased significantly by 60.18 % (Table 5). The TS cultivar exposed to 10 $mg.L^{-1}$ of $CHBrCl_2$ significantly increased the composition of saturated fatty acids in the lipid membrane as illustrated by a 175.43 % increase in palmitic acid peak (Table 5). Vitamin E (α-tocopherol) was significantly depleted in the membrane lipids of the TS cultivar exposed to 10 $mg.L^{-1}$ of $CHBrCl_2$ as illustrated by a 91.97 % decrease in concentration (Table 5). Significant increase in the plant paraffin (n-nonacosane)

was reported for the TMM cultivar while the TS cultivar exhibited a significant decrease in plant paraffin (Table 5).

Table 6
Effects of bromoform on lipid membrane composition of lyophilized leaf tissues of tomato plants (data at 30 days after commencement of treatment)

	Plant Variety			
Lipid Component	TMM control (a)	TMM exposed to 10 $mg.L^{-1}$ of $CHBr_3$ (b)	TS control (c)	TS 10 exposed to 10 $mg.L^{-1}$ of $CHBr_3$ (d)
2-Hydroxy-4-methylanthraquinone (mean concentration µg ml^{-1})	NE	0.392	NE	0.082
mean peak area	NA	19723317	NA	3973528
% increase in concentration	NA		NA	
mean retention Time (min)	NA	16.179	NA	16.177
CV %	NA	6.33	NA	4.92
P≤ 0.05	*bd		*db	
1-Octadecene (mean concentration µg ml^{-1})	NE	0.365	NE	0.259
mean peak area	NA	18325276	NA	12536216
% increase in concentration	NA		NA	
mean retention Time (min)	NA	26.921	NA	26.920
CV %	NA	9.44	NA	6.23
P≤ 0.05	NS		NS	
2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl phenol (mean concentration µg ml^{-1})	0.273	10.255	0.193	7.411
mean peak area	10167382	515786740	7956125	358667752
% increase in concentration	4972.96%		4408.07%	
mean retention Time (min)	29.080	29.138	29.076	29.126
CV %	6.64	4.92	6.18	7.58
P≤ 0.05	*ab		*cd	

Table 6 continued

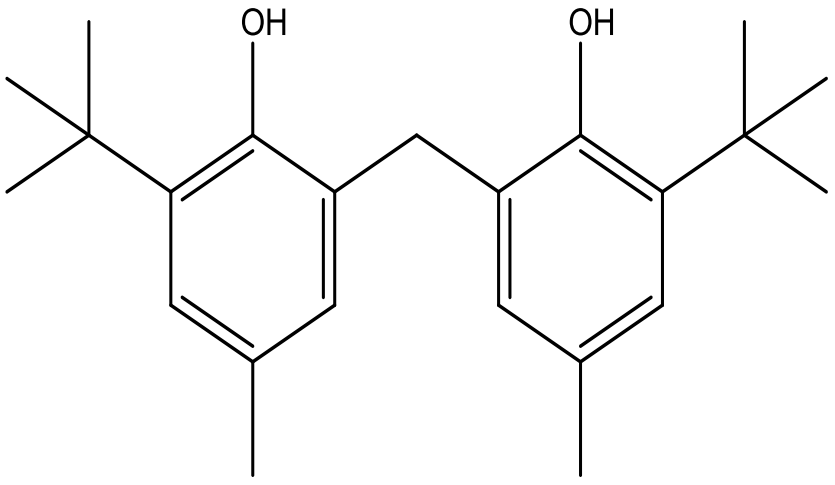
	Plant Variety			
Lipid Component	TMM control (a)	TMM exposed to 10 mg.L^{-1} of $CHBr_3$ (b)	TS control (c)	TS 10 exposed to 10 mg.L^{-1} of $CHBr_3$ (d)
Clionasterol (mean concentration µg ml^{-1})	0.419	1.691	0.229	1.194
mean peak area	15570565	85052915	9442165	57778380
% increase in concentration	446.24%		511.92%	
mean retention Time (min)	37.411	37.436	37.4123	37.426
CV %	6.64	8.42	7.93	3.84
P≤ 0.05	*ab		*cd	
α-linolenic acid methyl ester (mean concentration µg ml^{-1})	0.340	0.265	1.529	6.774
mean peak area	13311694	12636390	63149350	327860317
% increase in concentration	-0.22%		419.18%	
mean retention Time (min)	25.684	25.690	26.228	26.282
CV %	7.19	6.72	4.52	8.66
P≤ 0.05	NSab *bd		*cd *db	
Vitamin E (mean concentration µg ml^{-1})	1.529	1.837	0.386	0.478
mean peak area	56846026	92380040	17945505	23133175
% increase in concentration	62.51%		28.91%	
mean retention Time (min)	35.564	35.573	35.557	35.563
CV %	3.84	4.42	4.89	6.93
P≤ 0.05	*ab *bd		NScd *db	

Values presented are means of n = 4 replicates, *ab = significance at P≤0.05 respectively when column a & b are compared; NS = not significant; NE = not expressed; & NA = not applicable.

The compound 2-Hydroxy-4-methylanthraquinone was not expressed in the lipid membrane of both TMM and TS controls but was expressed in the bromoform stressed cultivars with the TMM cultivar significantly expressing more anthraquinone (Table 6). We report a similar occurrence

with 1-octadecene although there were no significant differences between stressed TMM and TS cultivars exposed to bromoform.

The key phenol 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl increased significantly by over 4000% in concentration of both bromoform stressed tomato cultivars (Table 6). The molecular structure for 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl can be seen in Fig 31. A tentative identification by the Wiley library of the GCMS for the molecular structure of the key phenol produced under bromoform stress based on mass spectral data, gas chromatographic data and retention indices can be seen in figure (11). Significant increase in sterol expression was also detected in the lipid membrane of both the chemically stressed tomato cultivars. A significant increase in α-linolenic acid (ALA) methyl ester was expressed only in the lipid profile of the chemically stressed TS cultivar (Table 6). An opposite occurrence was reported in vitamin E (α-tocopherol) expression where a significant increase was seen in the lipid composition of the TMM cultivar only (Table 6).



2,2'- methylenebis [6-(1,1- dimethylethyl)- 4- methylphenol]

Fig 31: A tentative identification of the molecular structure of the key lipophyllic antioxidant phenol produced by tomato cultivars under trihalomethane stress.

Table 7
Effects of chloroform on lipid membrane composition of lyophilized leaf tissues of tomato plants (data at 30 days after commencement of treatment)

	Plant Variety			
Lipid Component	TMM control (a)	TMM exposed to 10 $mg.L^{-1}$ of $CHCl_3$ (b)	TS control (c)	TS 10 exposed to 10 $mg.L^{-1}$ of $CHCl_3$ (d)
α-linolenic acid methyl ester (mean concentration µg ml^{-1})	0.256	0.919	0.253	0.159
mean peak area	9681386	35371561	7959476	5685085
% increase in concentration	265.36%		-28.57%	
mean retention Time (min)	25.686	25.690	25.6831	25.6144
CV %	6.13	9.74	5.89	4.33
P≤ 0.05	*ab *bd		NScd *db	
Phytol (mean concentration µg ml^{-1})	1.321	2.688	0.803	1.968
mean peak area	50011339	103448627	25271129	70611295
% increase in concentration	106.85%		179.41%	
mean retention Time (min)	25.829	25.832	25.824	25.823
CV %	8.41	6..38	7.92	4.54
P≤ 0.05	*ab		*cd	
Ethyl linoleolate (mean concentration µg ml^{-1})	6.761	9.173	0.044	7.589
mean peak area	255941494	353006725	1382817	272254589
% increase in concentration	37.92%		19588.40%	
mean retention Time (min)	26.270	26.269	26.477	26.247
CV %	5.54	8.82	3.97	9.44
P≤ 0.05	NSab *ac		*cd *ca	

Table 7 continued

	Plant Variety			
Lipid Component	TMM control (a)	TMM exposed to 10 mg.L^{-1} of Chloroform (b)	TS control (c)	TS 10 exposed to 10 mg.L^{-1} of Chloroform (d)
Eicosanoic acid, methyl ester (mean concentration µg ml^{-1})	NE	0.2400	NE	0.1832
mean peak area	NA	9236826	NA	6572310
% increase in concentration	NA		NA	
mean retention Time (min)	NA	28.204	NA	28.200
CV %	NA	4.07	NA	5.93
P≤ 0.05	NS		NS	
Vitamin E (mean concentration µg ml^{-1})	0.660	2.083	0.663	1.061
mean peak area	24993852	80144009	20874276	38053658
% increase in concentration	220.65%		82.30%	
mean retention Time (min)	35.565	35.567	35.562	35.557
CV %	4.28	7.13	6.19	6.03
P≤ 0.05	*ab		*cd	
Palmitic acid (mean concentration µg ml^{-1})	4.195	3.764	2.282	4.299
mean peak area	158803915	144835100	71817558	154238391
% increase in concentration	-8.79%		114.76%	
mean retention Time (min)	24.166	24.146	24.149	24.152
CV %	4.97	4.55	6.86	7.93
P≤ 0.05	NS		*cd	
Triacontane (mean concentration µg ml^{-1})	0.146	0.334	0.708	1.384
mean peak area	5519582	12863383	22269078	49665317
% increase in concentration	133.05%		123.02%	
mean retention time (min)	36.737	36.723	37.109	37.098
CV %	5.32	7.14	4.90	5.56
P≤ 0.05	*ab		*cd	

Values presented are means of n = 4 replicates, *ab = significance at P≤0.05 respectively when column a & b are compared; NS = not significant; NE = not expressed; & NA = not applicable.

There was a significant increase in α-linolenic acid (ALA) in the membrane lipid of leaf tissues of the TMM cultivar under chloroform stress. The TS cultivar did not exhibit a significant response in ALA expression under chloroform stress (Table 7). Phytol expression in the lipid membrane of both tomato cultivars increased significantly under chloroform stress. Ethyl linoleolate increased in concentration by 19588.40% in the TS cultivar under chloroform stress but the response of the TMM cultivar in terms of ethyl linoleolate expression was not significant (Table 7). Long chain fatty acids such as eicosanoic acid methyl esters were expressed in both tomato cultivars under chloroform stress and not expressed in the controls plants. Vitamin E (α-tocopherol) increased significantly in both cultivars under chloroform stress but the TMM cultivar produced more as seen by a 220.65% increase in concentration compared to the 82.30% increase observed in the TS cultivar (Table 7). The TS cultivar significantly increased the palmitic acid content of their lipid composition under chloroform stress. The change in palmitic acid concentration was not significant for (TMM) cultivars. Plant paraffin such as triacontane increased significantly in both tomato cultivars under chloroform stress.

There was a significant decrease of neophytadiene in the membrane lipids of the TMM cultivars under dibromochloromethane stress (Table 8). Dibromochloromethane did not induce a significant decrease in neophytadiene on the TS cultivars. Dibromochloromethane induced a significant increase in the plant paraffin n-eicosane in both tomato cultivars (Table 8). α-linolenic acid (ALA) increased significantly in both tomato cultivars with the TS cultivar exhibiting a 174.34% increase in concentration as compared to a 50.73% increase in TMM cultivars under dibromochloromethane stress. Vitamin E (α-tocopherol) increased significantly in both tomato cultivars under dibromochloromethane stress (Table 8).

Table 8
Effects of dibromochloromethane on lipid membrane composition of lyophilized leaf tissues of tomato plants (data at 30 days after commencement of treatment)

	Plant Variety			
Lipid Component	TMM control (a)	TMM exposed to 10 $mg.L^{-1}$ of $CHBr_2Cl$ (b)	TS control (c)	TS 10 exposed to 10 $mg.L^{-1}$ of $CHBr_2Cl$ (d)
Neophytadiene concentration µg ml^{-1})	1.823	1.056	6.616	6.471
mean peak area	85016448	39896131	222217546	216946168
% increase in concentration	-53.07%		-2.37%	
mean retention Time (min)	23.033	23.029	22.498	22.484
CV %	4.84	7.72	6.43	7.13
P≤ 0.05	*ab		NS	
N-eicosane concentration µg ml^{-1})	0.141	3.577	0.067	6.446
mean peak area	6580461	135166625	2258452	124187573
% increase in concentration	1954.06%		5398.80%	
mean retention Time (min)	35.468	35.311	35.082	35.309
CV %	3.23	8.64	4.32	9.69
P≤ 0.05	*ab		*cd	
α-linolenic acid methyl ester (mean concentration µg ml^{-1})	4.549	8.464	0.271	1.294
mean peak area	212180244	319827785	9090391	24938426
% increase in concentration	50.73%		174.34%	
mean retention Time (min)	26.256	26.267	25.686	25.686
CV %	4.72	4.11	6.24	9.72
P≤ 0.05	*ab		*cd	
Vitamin E (mean concentration µg ml^{-1})	0.458	0.997	0.476	1.536
mean peak area	21345659	37680689	15971032	29592676
% increase in concentration	76.53%		85.29%	
mean retention Time (min)	35.557	35.557	35.557	35.557
CV %	5.65	6.89	4.11	8.67
P≤ 0.05	*ab		*cd	

Values presented are means of n = 4 replicates, *ab = significance at P≤0.05 respectively when column a & b are compared; NS = not significant.

4.3. Discussion

4.3.1 Effects of increasing halogenation on tomato cultivars

The toxicity and physiological response of plants to organo-chlorines has become a recent focus of scientific interest (Faure et al., 2012, Ahammed et al., 2013, Li et al., 2015). Plant exposure to organo-chlorines shows diverse physiological stress responses, of which growth inhibition is a commonly observed phenomena in all photosynthetic species reported whether phytoplankton or crop plants (Gotham and Rhee, 1982, Mitra and Raghu, 1989, San Miguel et al., 2012, Blondel et al., 2014b).

Results from the current study reports for the first time that trihalomethanes may negatively affect the 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 ^oC) than the chlorinated trihalomethanes (chloroform $CHCl_3$ B.P 61.2 ^oC). 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) observed in fig 2, 3, 4, 5 and 6 that correlated with an increasing degree of trihalomethane chlorination (Table 1). Similar correlation relationships on the potential of increasing chlorination inhibiting plant growth have been reported for 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 defense systems that can protect cells from oxidative damage and scavenge harmful ROS that are produced in excess of those normally required for various metabolic reactions (Gill and Tuteja, 2010).

Literature is scanty on the mechanisms of trihalomethanes generating ROS in plants, since no report was found on them stimulating fenton-like reactions in any biological samples. However, exposure of both tomato cultivars to each trihalomethane chemical species generated a different response in both non-enzymatic and enzymatic plant defenses.

The antioxidant activity of plant phenols mainly due to their redox properties has been extensively documented in 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 (fig 7) that correlated with the trihalomethane degree of chlorination (Table 2). The reports of plant exposure to organo-chlorinated compounds generating excessive productions of peroxide (H_2O_2) 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 literature for parameters that measure various non-enzymatic plant responses to stress such as total polyphenol content and the ferric reducing antioxidant power (FRAP) (Reyes-Carmona et al., 2005, Bunea et al., 2011, Ma et al., 2011). 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 7 & 8). 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 total antioxidant capacity of sample extracts, it 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). The general trend of non-enzymatic antioxidant response with respect to increasing chlorination in total polyphenol, FRAP and ORAC were similar (fig 7, 8 and 9). Although the chlorination order of the ORAC plant extracts tested were slightly different. The ORAC assay takes into account free 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 reports the total antioxidant capacity of our plant extracts to peroxyl radicals, and this capacity increases with increasing chlorination of the trihalomethane molecule (fig 9).

The dismutation of the toxic superoxide (O_2^-) radicals into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) is a function of superoxide dismutase (Baranenko, 2005). Dismutation of the superoxide (O_2^-) radicals in this study was random and responded to no particular halogenation order in the tomato cultivars (fig 13). 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 peroxyl 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) that correlated to an increasing order of chlorination in the trihalomethane molecule (Table 2).

Our results were 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 producing the highest amount of H_2O_2 induced peroxidase scavenging enzymes more rapidly and intensively than the least chlorinated compounds.

The total soluble protein content of our plant extracts exposed to the trihalomethanes were random in this study and cannot be associated with any particular chlorination or bromination order for the two tomato cultivars. Furthermore, we observed no particular chlorination or bromination order in the general peroxidation of lipids in plants exposed

to trihalomethanes (fig 10). San Miguel et al. (2012) reported 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 reported. Nevertheless, the di-chlorinated trihalomethane molecule bromodichloromethane ($CHBrCl_2$) induced the highest levels of lipid peroxidation in both tomato cultivars in this study (fig 10). 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 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) into 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 (Gardner, 1995, Blée, 2002). Evidence of the increase in α-linolenic acid that correlated to the degree of chlorination in trihalomethanes could only be seen in the TMM cultivar (fig 14). 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 and Hause, 2013, Wasternack, 2014, Zhao et al., 2014). This indicates that the TMM cultivar may be more tolerant to trihalomethane chemical action as compared to the TS cultivar (fig 14).

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 (fig 15). The accumulation of palmitic acid is a phenomena 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 to any particular halogenation order (fig 16). This was surprising at first, since α-tocopherol is well documented as a lipid soluble peroxyl radical scavenger (Niki, 2014). Since no particular halogenation order was observed in general lipid peroxidation (TBARS), it should be expected that no halogenation order be seen in the lipophyllic antioxidant response with the release of α-tocopherol. This may be due to the efficient scavenging of the peroxyl 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's non-enzymatic and enzymatic antioxidant systems in response to oxidative stress, especially the peroxyl radical scavenging antioxidant defenses which mostly correlated with the trihalomethane degree of chlorination. Antioxidant responses from plants exposed to organo-chlorine compounds have been previously described in literature (Sinha, 2002, Monferran et al., 2007, San Miguel et al., 2013). The ROS production appear to be efficiently scavenged by the peroxidase group of enzymes as no particular chlorination order effects of trihalomethanes were observed in lipid peroxidation (fig 10). In this 30-

day exposure trihalomethanes did not induce sufficient oxidative damage to cause cell or plant death at concentration levels of 10 $mg.L^{-1}$.

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. It would contribute to knowledge to understand which halogenation order plants generally respond to. 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), monoxygenases P450 enzymes become actively involved in the dehalogenation process leading to the loss of Cl- atoms (Siminszky, 2006, Shimazu et al., 2010). 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 (Sinha, 2002, Pennathur et al., 2010).

These ROS and free chlorine radicals generated from exposure to chlorinated compounds in plants may explain the relationships between oxidative stress and the chlorination degree of trihalomethanes. The present study however does demonstrate that tomato plants, if exposed to trihalomethane levels normally found in finished waters is unlikely to induce any significant oxidative damage. Although chlorine produced oxidants' first signs of deleterious effects have been reported to appear at concentrations as low as 0.1 $mg \cdot 1^{-1}$, about two orders of magnitude lower than the actual concentrations in chlorinated effluents (Abarnou and Miossec, 1992).

4.3.2 Effects of increasing trihalomethane concentration on tomato cultivars

Environmental water based organic pollutants such as the organo-halogenated disinfection by-products may induce a variety of physiological or oxidative stress in plants during irrigation, which may compromise plant performance. Since growth inhibition is a universal parameter, it has been confirmed that radical inhibition can be used as a reliable indicator of pollutant toxicity (Di Salvatore et al., 2008, Kummerová et al., 2013).

Cell cycle disruptions and apoptosis have been observed in *Zea mays* roots exposed to organochlorine pesticides (Blondel et al., 2014a). Moreover, monobrominated diphenyl ethers were recently reported as having negative effects on the microscopic structure of the roots of *Populus tomentosa Carr* seedlings (Cai et al., 2015). Thus, this morphological indicator along with physiological indicators of biomass accumulation such as fresh and dry weight was used as suitable parameters to assess tomato (*S. lycopersicum*) seedling tolerance to trihalomethanes.

In this study, the inhibition of radical growth in tomato seedlings increased with trihalomethane concentration in a dose dependent manner (Table 4). The inhibition of radicle growth also increased with an increasing degree of bromine atoms in the trihalomethane molecule. The observed phenomena could be based on the semi-volatile nature of trihalomethanes with focus on the boiling points of each trihalomethane chemical species.

The more brominated trihalomethanes have higher boiling points (bromoform $CHBr_3$ B.P 149.1 ^{o}C), and are less volatile so they stay longer in solutions to cause an effect more

than the chlorinated trihalomethanes (chloroform $CHCl_3$ B.P 61.2 ^oC) as mentioned previously.

However, in growing plants a decrease in eight essential nutrients in plant dry matter was observed with increasing levels of trihalomethane exposure (fig 17 to 22). The decrease in phosphorus and sulphur levels was not significant, while trihalomethane exposure had no effect on calcium and iron levels in plants. The magnitude of the effect of increasing degree of chlorination in the trihalomethane molecules appear to be greater than the effect of increasing trihalomethane dose when comparing the percentage decrease in nutrient concentration with the exception of Cu and Zn (Fig. 29).

There is growing interest in the toxicity and physiological response of plants to organochlorine compounds (Faure et al., 2012, Ahammed et al., 2013, Li et al., 2015). Moreover, mounting evidence is gathering to suggest that organochlorine compounds are able to induce the over-production of reactive oxygen species (ROS) thus damaging plant cell components (Menone et al., 2008, Michałowicz et al., 2009, San Miguel et al., 2012, Ahammed et al., 2013).

Plant cells have developed numerous antioxidant defense systems which are both enzymatic and non-enzymatic in nature. In this study, a significant induction of non-enzymatic antioxidant defenses and peroxyl radical scavenging enzymes (APX and GPX) were observed due to an increase in trihalomethane dose and the number of chlorine atoms in the trihalomethane molecule.

The antioxidant properties of non-structural plant phenols have been reported largely due to their redox properties (Agati et al., 2012, Dangles, 2012, Martín et al., 2015). In

this study, we report a 44.79% increase in total phenolic content from control plants (exposed to no trihalomethanes) to tomato plants exposed to 10 $mg.L^{-1}$ of trihalomethane. The effect of the increasing degree of chlorination in the trihalomethane molecule appear to induce a greater total phenolic response in plants when comparing the percentage increase in phenolic content of mono-chlorinated to tri-chlorinated trihalomethanes (351.25%) (Fig.30). The effect is almost 8 times greater in magnitude than the effect of increasing trihalomethane dose on total phenolic content.

The ferric reducing antioxidant power (FRAP) of plant extracts in quite a number of studies usually correlates or follows a similar trend with the total phenolic content suggesting that phenolic compounds are the major contributors to the non enzymatic antioxidant properties of plants (Dudonne et al., 2009, Bunea et al., 2011, Ma et al., 2011). FRAP values increased by 55.51% in response to increasing trihalomethane dose from control plants to those exposed to 10 $mg.L^{-1}$ of trihalomethane. The percentage increase associated with the effects of increasing chlorination in trihalomethane molecules was again higher (almost double) (107.61%) when compared to the % increase in FRAP linked to the effects of increasing trihalomethane dose (Fig. 30).

The oxygen radical absorbance capacity (ORAC) values (fig 25) and the scavenging activites of the peroxidase enzymes in this study (fig 27 & 28) indicate that trihalomethanes induce the over-production of peroxyl radicals. The measured antioxidant capacity of the ORAC assay depends on which oxidant or free radical is used in the measurement (Cao and Prior, 1998). The azo-compound 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH) was used to generate peroxyl radicals in the measurement of ORAC values in this study so in essence, the ORAC values reports the

total antioxidant capacity of the plant extracts to peroxyl radicals, and this capacity increases with increasing trihalomethane dose.

The interplay between the type of ROS produced and the appropriate scavenging enzymes is important (Mittler, 2002). In this study, the activites of superoxide dismutase (SOD) known to catalyze the dismutation of the superoxide radical (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) displayed no observable trends in response to increasing trihalomethane dose. However, the activites of both peroxidase enzymes (APX & GPX) known for the scavenging of peroxyl radicals in plants increased with increasing trihalomethane dose (Fig. 27 & 28).

The percentage increase associated with increasing trihalomethane dose for both peroxidase enzyme activities were APX (366.67%) and GPX (300%), while the percentage increase from the effect of increasing chlorination of the trihalomethane molecule was almost double for APX activities (725%), but reduced by half for GPX (150%) when compared with the effects of increasing trihalomethane dose (Fig. 30). The study conducted by San Miguel. et al. (2012) on the effects of mono, di and trichloro-benzenes on peroxidase enzyme activities of *Zea mays* reported trends in consonance with our results. This suggests that the most chlorinated compounds induce the greater amount of peroxyl radicals hence activating peroxidase scavenging enzymes more rapidly than the least chlorinated compounds as mentioned previously.

The general peroxidation of lipids measured as malondialdehyde (MDA) content increased with increasing trihalomethane dose (fig 26), although no particular trends were observed in response to increasing chlorination of the trihalomethane molecule (fig 10). This contradicts the trends reported in San Miguel et al. (2012).

The increased efficiency observed in the scavenging activites of ascorbate peroxidase (APX) with increasing chlorination of trihalomethane molecules may have minimised the effects of the peroxyl radicals on lipid peroxidation. In light of this, it is probable that no discernable trend would be observed for (MDA) content with increasing chlorination of the trihalomethane molecule.

Evidence is gathering to suggest that increasing chlorination induces oxidative stress in plants and this can occur through several processes reported in literature. The prevalent hypothesis suggests that during the scavenging of peroxyl radicals by peroxidases induced by chlorine atoms, monoxygenases P450 enzymes and plant dehalogenases become actively involved in the dechlorination of halogenated organic compounds primarily by sequestration (Doty et al., 2000, Nzengung and Jeffers, 2001, Siminszky, 2006, Shimazu et al., 2010).

The metabolism of halogenated organic compounds and their corresponding oxidative or reductive by-products in plants suggests that more than one pathway requiring different enzymes may be involved in the phyto-transformation of halogenated compounds. Four mechanisms have been identified in the removal of halogenated organic compounds by plants, that is the rapid sequestration and partitioning to lipophilic plant cuticles; phyto-reduction to less halogenated metabolites; phyto-oxidation to haloethanols, haloacetic acids, and unidentified metabolites; and assimilation as non-phytotoxic products presumably by covalent binding with the plant tissues (Nzengung and Jeffers, 2001).

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.

The proposed mechanism of action of trihalomethanes suggests that the free chlorine radicals generated during the dehalogenation processes of trihalomethanes may also damage transport protein channels of some nutrients primarily transported by active transport mechanisms in the symplast of plants. Hence, this would result in the reduction of nutrient transport efficiency and consequently nutrient levels in plants. Concentration levels of nutrients such as P, Ca, and Fe, in which plants transport both by active transport through symplast movements and passive transport through the apoplast was not significantly affected by trihalomethanes (Schachtman et al., 1998, Yang and Jie, 2005, Morrissey and Guerinot, 2009). Furthermore, the exposure of plants to trihalomethanes induced the increased production of ROS such as peroxides (H_2O_2) known to displace chloride ions from chlorinated compounds producing secondary radicals of variable reactivity (Sinha, 2002, Pennathur et al., 2010).

Future research at the molecular level may confirm and shed more light on this mechanism. In conclusion, this study highlights that the magnitude of plant responses to organohalogens is more dependent on the halogenation number and less on concentration. Since no plant death was reported even at the highest concentrations of 10 $mg.L^{-1}$ of the trihalomethane, it is unlikely that significant oxidative damage be induced in plants exposed to trihalomethanes at levels found in potable waters.

4.3.3 Effect of increasing trihalomethane dose on membrane lipids in leaves of tomato cultivars

The non-enzymatic antioxidant phenolic compounds are primarily derived from the shikimic acid pathway, phenylpropanoid pathway, and flavonoid pathway, and a full-scale investigation has been carried out on the functional verification and transcriptional regulation of some key enzymes and genes involved in these pathways (Tian et al., 2008, Akagi et al., 2009, Yatusevich et al., 2010). The key lipophyllic phenolic antioxidant produced in both tomato cultivars under trihalomethane stress was tentatively identified as 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl phenol (Table 6). The molecular structure of this phenolic compound is illustrated in (Fig 31).

Lipophyllic phenolic antioxidants have been reported to outperform well known lipophyllic antioxidants such as α-tocopherol (Hernández et al., 2009) and this is largely due to their redox properties (Agati et al., 2012, Dangles, 2012, Martín et al., 2015). Peroxidases and polyphenoloxidases are able to oxidize phenolics, yielding quinones (Lattanzio et al., 2006). The hydroxyl and alkyl substitutions of such quinone derivatives produced in the membrane lipids of both tomato cultivars under bromoform stress (Table 6) have been known to demonstrate antioxidant properties. This is done by scavenging hydroxyl radicals produced, hence inhibiting the peroxidation of α-linoleic acid (Yen et al., 2000).

In general, vitamin E (α-tocopherol) increased significantly in the membrane lipids of tomato cultivars exposed to trihalomethane chemical species, with the exception of $CHBrCl_2$, where a significant decrease was reported (Table 5). The mechanism of action of α-tocopherol is in the scavenging of lipid peroxyl radicals, thereby preventing the propagation of lipid peroxidation, and protects lipids and other membrane components

by physically quenching and reacting chemically with singlet oxygen (Waśkiewicz et al., 2014).

However, given that α-tocopherol increases membrane rigidity, its concentration, together with that of the other membrane components, may be regulated to afford adequate fluidity for membrane function (Yoshida et al., 2003, Munné-Bosch, 2007).

Vitamin E (α-tocopherol) plays a vital role in conferring tolerance to several abiotic stresses (e.g., salinity, drought, metal toxicity, ozone and UV radiation). Several reports indicate that stress-tolerant plants exhibit an enhanced level of α-tocopherol, whereas sensitive ones show a decreased level of α-tocopherol under stressful conditions leading to oxidative damage (Hasanuzzaman et al., 2014). This suggests that plant exposure to $CHBrCl_2$ would have a greater tendency to induce lipid peroxidation and subsequently oxidative damage when compared to other trihalomethane chemical species.

Exposure to bromoform significantly increased the phytosterol (Stigmast-5-en-3-ol) in both tomato cultivars (Table 6). The sterol or mevalonate pathway in plants represents a sequence of more than 30 enzyme-catalyzed reactions, all associated with membranes (Benveniste, 1986, Hartmann, 1998). Phytosterols are synthesized via a pathway that starts with reduction of HMG-CoA (six carbons) to mevalonate (five carbons). Six mevalonate units are then assembled into two farnesyl diphosphate molecules, which are combined to make squalene (30 carbons or ‘‘three terpenes’’). Enzymatic ring closure steps then form cycloartenol (also 30 carbons), and additional enzymatic reactions form common plant triterpenes such as phytosterols, triterpene alcohols and brassinosteroids (Benveniste, 1986).

In general, phytosterols are also thought to stabilize plant membranes, with an increase in the sterol/phospholipid ratio leading to membrane rigidification (Itzhaki et al., 1990). However, individual phytosterols differ in their effect on membranes stability. Stigmasterol has been reported to have a disordering effect on membranes and inefficient in reducing water membrane permeability (Schuler et al., 1991, Ovečka and Lichtscheidl, 2006).

The molar ratio of stigmasterol to other phytosterols in the plasma membrane increases during senescence (Brenac and Sauvaire, 1996). Phytosterols have also been reported to prevent the formation of H_2O_2 radicals (Wang et al., 2002). Critical concentrations of s-avenasterol, fucosterol and citrostadienol have been shown to exhibit antioxidant properties (Gordon, 1990).

Plant exposure to chloroform induced an alternative pathway from bromoform exposure, with the production of phytol, a compound formed from the non-mevalonate pathway or 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP pathway) (Table 7). This alternative pathway of isoprenoid biosynthesis leads to the formation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Rohmer, 1999, Eisenreich et al., 2004, Hunter, 2007). Sterols are formed via the mevalonate (MVA) route, whereas chloroplast isoprenoids, such as phytol are synthesized via the MEP route.

Chlorophyll is known to consist of a porphyrin ring with magnesium and the phytol side chain (Kohlmeier, 2003). Phytol is an essential component of chlorophyll biosynthesis, which suggests that plant exposure to chloroform may enhance chlorophyll degradation, although this was not experimentally confirmed. This in turn stimulates genes involved in

the MEP pathway producing new phytol for chlorophyll regeneration (Vavilin and Vermaas, 2007).

Exposure to trihalomethane chemical species stimulated an increase in wax biosynthesis in plants with the exception of bromoform, although the TS tomato cultivar exposed to $CHBrCl_2$ demonstrated a significant decrease in wax biosynthesis. Wax biosynthesis requires the coordinated activity of a large number of enzymes for the formation of saturated very-long-chain fatty acids and their further transformation in several aliphatic compounds. Epicuticular plant waxes such as triacontane, nonacosane and eicosane are normally expressed as a defense mechanism to drought, even though water stress was not induced in these experiments (Bondada et al., 1996, Sánchez et al., 2001).

Decreased expressions of several other defense bio-molecules were isolated in the lipid membrane. Neophytadiene a diterpene which was significantly reduced in the membrane lipids of the TMM cultivar exposed to $CHBrCl_2$ and $CHBr_2Cl$ (Tables 5 & 8) was reported as one of several bio-molecules plants secrete as an anti-inflammatory and anti-microbial compound (Dar et al., 2012). Neophytadiene can also be hydrated to phytol (Changi et al., 2012), an essential component of chlorophyll biosynthesis, which suggests that plant exposure to $CHBrCl_2$ and $CHBr_2Cl$ may enhance chlorophyll degradation although this requires experimental confirmation (Vavilin and Vermaas, 2007). Neophytadiene has also been reported to be a pre-cursor molecule for conversion to α-tocopherol (Daines et al., 2003, Netscher, 2007). However, reduced levels of neophytadiene in plants exposed to $CHBrCl_2$ and $CHBr_2Cl$ did not translate into a significant increase in phytol or could be directly associated with α-tocopherol response.

The release of volatiles was recorded in the TMM cultivar exposed to $CHBrCl_2$ and the TS cultivars exposed to $CHCl_3$ (Tables 5 & 7). Ethyl linoleolate has been identified as a major volatile compound produced in plant leaves and plays an important role in plant protection as an anti-bacterial agent (Zouari et al., 2011). Abiotic stress factors have been reported to induce the release of volatiles in plants (Gouinguené and Turlings, 2002). Volatile release under biotic and abiotic stress has already been documented on this species of tomato plants (Maes and Debergh, 2003).

The presence of octadecene expressed in the membrane lipids of bromoform stressed plants only (Table 6) can be argued as evidence of lipid peroxidation. The production of octadecene as an antioxidant in plant lipids could be a mechanism of oxidative response as alkenes are well known to react with free radicals (Giese, 1983).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Based on the observations 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. The decrease in nutrient levels was observed to correlate with an increasing degree of chlorination in the trihalomethane molecule.

The trihalomethanes demonstrated the potential to induce oxidative stress as observed in the tomato cultivars. This is based on the significant increase in total phenolic content, FRAP, ORAC, and the induction of the peroxidase antioxidant enzymes (APX and GPX). α-linolenic acid stress signaling in the TMM cultivar correlated with an increasing degree of chlorination in the trihalomethane molecule. However, this trend was not observed in the TS cultivars. This may suggest that this variety could be more tolerant to chemical action.

The increase in trihalomethane dosage was observed to induce a decrease in radicle length and seedling biomass accumulation. Radicle inhibition and loss of seedling biomass corresponded to an increasing number of bromine atoms in the trihalomethane molecule. After repeated experiments, it is suspected that these observed trends in tomato seedlings exposed to trihalomethanes may be based on trihalomethane volatility. It is known that brominated trihalomethanes have higher boiling points (bromoform $CHBr_3$ B.P 149.1 ^oC), and are less volatile than the chlorinated trihalomethanes (chloroform $CHCl_3$ B.P 61.2 ^oC). This suggests that the brominated trihalomethanes stay in solution much longer than the chlorinated trihalomethanes, hence, cause a longer lasting effect on exposed seedlings.

Measures were taken to reduce trihalomethane volatility in the experiments on growing plants which may explain why we observed changes in trends from the effects of increased bromination to chlorination in the physiological parameters determined. This could indicate that in natural systems or warmer climates the effects of the more brominated trihalomethanes are prevalent.

In the experiments on growing plants, a decrease in seven essential nutrients required for plant growth was observed that correlated to an increase in the number of chlorine atoms in the trihalomethane molecule. Also, the increase in trihalomethane dose induced a decrease in eight essential nutrients in growing plants. Oxidative stress was induced in growing tomato plants by increase in trihalomethane dose and degree of chlorination.

Changes in membrane lipids of plants exposed to increasing trihalomethane dose were based on two major factors, which were the adjustments of membrane fluidity with the increase in plant sterols and fatty acids content, and secondly, the increase in lipophyllic antioxidants such as phenols, quinones and α-tocopherol concentrations.

The World Health Organisation (WHO) stated that efficient disinfection must never be compromised in an attempt to meet the guidelines for disinfection by-products and that the microbiological quality of the water must always take precedence. As such disinfected waste-water usually contains disinfection by-products in levels that exceed the limits set for potable water, basically due to the high concentrations of chlorine required to disinfect reclaimed water due to the high amount of organic wastes and fulvic acids present.

The current guideline limit set for total trihalomethanes in treated wastewater considered as class ll or class lll surface water discharge by the Florida department of environmental Protection (FDEP) is 0.4708 mgL^{-1} for chloroform, 0.022 mgL^{-1} for bromodichloromethane, 0.034 mgL^{-1} for dibromochloromethane and 0.36 mgL^{-1} for bromoform. Crop plants exposed to trihalomethane chemicals, even at the highest concentrations of 10 mgL^{-1}, did not cause sufficient oxidative damage to induce plant cell death. Although evidence was provided that supports trihalomethane ability to affect plant performance, the current limits for class ll and class lll treated effluents by the FDEP is unlikely to affect plant performance.

The guidelines for the trihalomethane content in class ll or class lll treated wastewaters have not yet been proposed in South Africa, although guidelines for trihalomethane content in class l potable water exists currently. It is recommended that guidelines and limits for South Africa be proposed on the trihalomethane content of class ll or class lll treated wastewater designated for agricultural use. These guidelines and limits need not be as stringent as those recommended for class l potable water.

Hence, a limit of <0.5 mg/L^{-1} for total trihalomethanes and a bromodichloromethane limit of < 0.1 mg/L^{-1} is recommended for treated wastewater designated for agricultural purposes only. Although deleterious effects have been reported on trihalomethanes at concentrations as low as 0.1 mg/L^{-1}, this limit is set considering other factors at play, such as the volatility of trihalomethanes in warm temperatures during irrigation, poor to medium solubility in water, possible biodegradation by soil microorganisms and plant tolerance at these concentrations.

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Appendix

Table 9
Effects of trihalomethane chemical species each at a concentration of 10 mg.L^{-1} and plant variety on nutrient concentration of the dry matter yields of tomato plants at (data at 30 days after commencement of treatment)

		Chemical species				
Nutrients mg kg^{-1}	Plant Varieties	$CHBr_2Cl$	$CHBrCl_2$	$CHBr_3$	$CHCl_3$	Mean p varieties
N	TMM	2.840	1.727	1.263	0.993	1.706
	TS	2.540	1.754	1.545	1.229	1.767
	Mean C varieties	2.690	1.741	1.404	1.111	
	CV%			1.93%		
	P≤0.05			*		NS
	Interaction			*		
P	TMM	0.836	0.710	0.543	0.461	0.638
	TS	0.853	0.713	0.632	0.534	0.683
	Mean C varieties	0.845	0.712	0.588	0.498	
	CV%			1.88%		
	P≤0.05			*		NS
	Interaction			NS		
K	TMM	9.951	6.617	3.906	2.855	5.832
	TS	7.499	6.335	4.060	3.343	5.309
	Mean C varieties	8.725	6.476	3.983	3.099	
	CV%			2.92%		
	P≤0.05			*		NS
	Interaction			*		
Ca	TMM	1.525	1.546	1.493	1.312	1.469
	TS	1.411	1.439	1.409	1.320	1.395
	Mean C varieties	1.468	1.493	1.451	1.316	
	CV%			2.10%		
	P≤0.05			NS		NS
	Interaction			NS		
Mg	TMM	0.285	0.323	0.287	0.248	0.286
	TS	0.313	0.321	0.287	0.266	0.297
	Mean C varieties	0.299	0.322	0.287	0.257	
	CV%			1.68%		
	P≤0.05			*		NS
	Interaction			NS		
S	TMM	0.656	0.477	0.317	0.282	0.433
	TS	0.566	0.412	0.341	0.315	0.409
	Mean C varieties	0.611	0.445	0.329	0.299	
	CV%			2.71%		
	P≤0.05			*		NS
	Interaction			NS		

Table 9 continued

		Chemical species				
Nutrients mg kg^{-1}	Plant varieties	$CHBr_2Cl$	$CHBrCl_2$	$CHBr_3$	$CHCl_3$	Mean p varieties
Na	TMM	946.2	1248.9	932.6	802.0	982.425
	TS	859.7	921.9	982.8	883.0	911.850
	Mean C varieties	902.95	1085.40	957.70	842.50	
	CV%			2.31%		
	P≤0.05			*		NS
	Interaction			*		
Mn	TMM	56.8	40.8	35.5	38.7	42.950
	TS	38.9	33.5	40.1	42.4	38.725
	Mean C varieties	47.85	37.15	37.80	40.55	
	CV%			2.36%		
	P≤0.05			*		NS
	Interaction			*		
Fe	TMM	1081.4	1192.0	1148.8	1779.3	1300.38
	TS	1109.2	766.4	2549.4	2440.0	1716.25
	Mean C varieties	1095.30	979.20	1849.10	2109.65	
	CV%			10.79%		
	P≤0.05			NS		NS
	Interaction			NS		
Cu	TMM	8.9	7.2	5.8	4.8	6.675
	TS	7.8	6.1	7.0	7.0	6.975
	Mean C varieties	8.35	6.65	6.40	5.90	
	CV%			2.35%		
	P≤0.05			*		NS
	Interaction			*		
Zn	TMM	193.8	160.5	147.3	126.0	156.900
	TS	150.3	135.6	176.1	140.1	150.525
	Mean C varieties	172.05	148.05	161.70	133.05	
	CV%			2.85%		
	P≤0.05			*		NS
	Interaction			*		
B	TMM	37.6	33.6	26.1	22.2	29.875
	TS	31.9	30.9	24.0	22.1	27.225
	Mean C varieties	34.75	32.25	25.05	22.15	
	CV%			1.68%		
	P≤0.05			*		*
	Interaction			NS		

Values presented are means of n = 4 replicates, * = effect of trihalomethanes significant at P≤0.05 respectively; NS = not significant, TMM = tomato moneymaker, TS = tomato star, $CHBrCl_2$ = Bromodichloromethane, $CHBr_3$ = Bromoform, $CHCl_3$ = Chloroform, $CHBr_2Cl$ = Dibromochloromethane

Table 10
Effects of trihalomethane chemical species each at 10 $mg.L^{-1}$ and plant variety on non enzymatic and enzymatic biomarkers of oxidative stress in lyophilized leaf tissues of tomato plants (data at 30 days after commencement of treatment)

		Chemical species				
	Plant Varieties	$CHBr_2Cl$	$CHBrCl_2$	$CHBr_3$	$CHCl_3$	Mean p variety
Polyphenols	TMM	3.832	6.965	12.435	16.755	9.997
$mg.L^{-1}$(GAE)	TS	3.439	7.258	11.747	16.052	9.624
	Mean C varieties	3.635	7.112	12.091	16.403	
	CV%		3.05%			
	P≤0.05		*			NS
	Interaction		NS			
FRAP	TMM	22.172	31.169	40.241	43.801	34.346
$mg.L^{-1}$(AAE)	TS	19.370	29.918	35.465	42.257	31.753
	Mean C varieties	20.771	30.543	37.853	43.029	
	CV%		2.93%			
	P≤0.05		*			NS
	Interaction		NS			
ORAC	TMM	7.175	10.726	7.755	26.020	12.919
mM trolox	TS	6.188	10.576	9.325	23.309	12.350
	Mean C varieties	6.681	10.651	8.540	24.665	
	CV%		3.28%			
	P≤0.05		*			NS
	Interaction		NS			
APX activity	TMM	0.005	0.016	0.002	0.024	0.012
U mg^{-1}	TS	0.004	0.012	0.002	0.042	0.015
	Mean C varieties	0.004	0.014	0.002	0.033	
	CV%		24.54%			
	P≤0.05		*			NS
	Interaction		NS			
GPX activity	TMM	0.010	0.021	0.005	0.026	0.016
U mg^{-1}	TS	0.011	0.013	0.005	0.023	0.013
	Mean C varieties	0.010	0.017	0.005	0.025	
	CV%		16.76%			
	P≤0.05		*			NS
	Interaction		NS			
SOD activity	TMM	0.031	0.012	0.020	0.044	0.027
U mg^{-1}	TS	0.028	0.010	0.013	0.050	0.025
	Mean C varieties	0.029	0.011	0.016	0.047	
	CV%		7.15%			
	P≤0.05		*			NS
	Interaction		NS			
BCA	TMM	76.823	113.899	283.550	70.215	136.122
mg/mL	TS	75.817	81.583	217.654	86.203	115.314
	Mean C varieties	76.320	97.741	250.602	78.209	
	CV%		4.18%			
	P≤0.05		*			*
	Interaction		*			
TBARS	TMM	1.872	3.220	3.005	2.329	2.607
$mM^{-1}cm^{-1}$	TS	1.867	3.156	2.753	2.526	2.576
	Mean C varieties	1.869	3.188	2.879	2.428	
	CV%		3.55%			
	P≤0.05		*			NS
	Interaction		NS			

Values presented are means of n = 4 replicates, * = significance at P≤0.05 respectively; NS = not significant, TMM = tomato moneymaker, TS = tomato star. $CHBrCl_2$ = Bromodichloromethane, $CHBr_3$ = Bromoform, $CHCl_3$ = Chloroform, $CHBr_2Cl$ = Dibromochloromethane FRAP = Ferric reducing ability of plasma, ORAC = Oxygen radical absorbance capacity, APx = Ascorbate peroxidase, GPx = Guaiacol peroxidase, SOD = Superoxide dismutase, BCA = pierce bicinchoninic acid protein assay, TBARS = Thiobarbituric acid reaction for general lipid peroxidation.

Table 11
Effects of trihalomethane concentration and trihalomethane chemical species on nutrient concentrations of the dry matter yields of tomato plants (data at 30 days after commencement of treatment)

		Concentration $mg.L^{-1}$					
Nutrients $mg\ kg^{-1}$	Chemical species	0 control	2.5 $mg.L^{-1}$	5.0 $mg.L^{-1}$	7.5 $mg.L^{-1}$	10 $mg.L^{-1}$	Mean chem. Species
N	$CHBr_2Cl$	3.140	2.993	2.788	2.508	2.023	2.690
	$CHBrCl_2$	1.935	1.925	1.770	1.640	1.433	1.741
	$CHBr_3$	1.985	1.530	1.275	1.143	1.088	1.404
	$CHCl_3$	1.278	1.278	1.085	1.015	0.900	1.111
	Mean Conc.	2.085	1.932	1.730	1.577	1.361	
	CV%			1.93%			
	P≤0.05			*			*
	Interaction			NS			
P	$CHBr_2Cl$	0.940	0.859	0.845	0.790	0.790	0.845
	$CHBrCl_2$	0.888	0.765	0.730	0.613	0.563	0.712
	$CHBr_3$	0.670	0.653	0.603	0.593	0.420	0.588
	$CHCl_3$	0.583	0.568	0.518	0.425	0.395	0.498
	Mean Conc.	0.770	0.711	0.674	0.605	0.542	
	CV%			1.88%			
	P≤0.05			NS			*
	Interaction			*			
K	$CHBR_2Cl$	10.335	9.945	8.045	7.918	7.383	8.725
	$CHBRCl_2$	9.778	7.090	6.603	4.458	4.453	6.476
	$CHBr_3$	5.385	4.285	4.243	3.663	2.340	3.983
	$CHCl_3$	3.745	3.700	2.913	2.650	2.488	3.099
	Mean Conc.	7.311	6.255	5.451	4.672	4.166	
	CV%			2.92%			
	P≤0.05			*			*
	Interaction			*			
Ca	$CHBr_2Cl$	1.553	1.405	1.410	1.508	1.465	1.468
	$CHBrCl_2$	1.355	1.253	1.740	1.760	1.355	1.493
	$CHBr_3$	1.383	1.205	1.795	1.680	1.193	1.451
	$CHCl_3$	1.735	1.220	1.145	1.160	1.320	1.316
	Mean Conc.	1.506	1.271	1.523	1.527	1.333	
	CV%			2.10%			
	P≤0.05			NS			NS
	Interaction			*			
Mg	$CHBr_2Cl$	0.340	0.300	0.285	0.288	0.283	0.299
	$CHBrCl_2$	0.343	0.290	0.333	0.350	0.295	0.322
	$CHBr_3$	0.335	0.270	0.315	0.293	0.223	0.287
	$CHCl_3$	0.323	0.253	0.223	0.260	0.228	0.257
	Mean Conc.	0.335	0.278	0.289	0.298	0.257	
	CV%			1.68%			
	P≤0.05			*			*
	Interaction			NS			

Table 11 continued

		Concentration mg.L^{-1}					
Nutrients mg kg^{-1}	Chemical species	0	2.5 mg.L^{-1}	5.0 mg.L^{-1}	7.5 mg.L^{-1}	10 mg.L^{-1}	Mean chem. Species
S	$CHBr_2Cl$	0.698	0.650	0.615	0.550	0.543	0.611
	$CHBrCl_2$	0.570	0.465	0.418	0.400	0.370	0.445
	$CHBr_3$	0.445	0.335	0.303	0.283	0.280	0.329
	$CHCl_3$	0.395	0.326	0.280	0.253	0.243	0.299
	Mean Conc.	0.527	0.444	0.404	0.372	0.359	
	CV%			2.71%			
	P≤0.05			NS			*
	Interaction			NS			
Na	$CHBr_2Cl$	857.50	804.75	1023.75	1016.50	812.25	902.95
	$CHBrCl_2$	1101.0	900.25	1152.75	1253.50	1019.50	1085.40
	$CHBr_3$	1161.2	841.25	1120.00	886.00	780.00	957.70
	$CHCl_3$	911.75	617.75	1032.00	892.25	758.75	842.50
	Mean Conc.	1007.88	791.00	1082.13	1012.06	842.63	
	CV%			2.31%			
	P≤0.05			*			*
	Interaction			NS			
Mn	$CHBr_2Cl$	61.50	58.50	50.50	42.50	26.25	47.85
	$CHBrCl_2$	44.25	43.00	38.00	31.75	28.75	37.15
	$CHBr_3$	52.50	38.75	36.00	34.75	27.00	37.80
	$CHCl_3$	50.25	42.75	41.00	35.50	33.25	40.55
	Mean Conc.	52.125	45.750	41.375	36.125	28.813	
	CV%			2.36%			
	P≤0.05			*			*
	Interaction			*			
Fe	$CHBr_2Cl$	1224.75	789.50	1251.25	1105.50	1105.50	1095.30
	$CHBrCl_2$	1603.50	847.50	507.25	966.25	971.50	979.20
	$CHBr_3$	3040.25	931.00	2352.75	1429.00	1492.50	1849.10
	$CHCl_3$	988.75	1465.25	3072.75	2634.00	2387.50	2109.65
	Mean Conc.	1714.31	1008.31	1796.00	1533.69	1489.25	
	CV%			10.79%			
	P≤0.05			NS			NS
	Interaction			NS			
Cu	$CHBr_2Cl$	9.25	8.75	8.25	8.00	7.50	8.35
	$CHBrCl_2$	7.75	6.75	6.50	6.25	6.00	6.65
	$CHBr_3$	8.50	7.75	5.75	5.25	4.75	6.40
	$CHCl_3$	7.00	6.75	6.50	5.25	4.00	5.90
	Mean Conc.	8.125	7.500	6.750	6.188	5.563	
	CV%			2.35%			
	P≤0.05			*			*
	Interaction			NS			

Table 11 continued

Nutrient	Chemical		Concentration	mg.L^{-1}			Mean Chem.
mg kg^{-1}	species	0 control	2.5 mg.L^{-1}	5.0 mg.L^{-1}	7.5 mg.L^{-1}	10 mg.L^{-1}	Species
Zn	$CHBr_2Cl$	217.25	176.00	166.75	155.00	145.25	172.05
	$CHBrCl_2$	188.25	182.00	132.25	126.75	111.00	148.05
	$CHBr_3$	197.25	183.25	174.00	145.25	108.75	161.70
	$CHCl_3$	154.25	146.50	140.75	134.25	89.50	133.05
	Mean Conc.	189.25	171.875	153.438	140.313	113.625	
	CV%			2.85%			
	P≤0.05			*			*
	Interaction			NS			
B	$CHBr_2Cl$	40.00	36.00	33.50	32.50	31.75	34.75
	$CHBrCl_2$	39.75	35.75	31.25	27.25	27.25	32.25
	$CHBr_3$	31.25	25.50	25.00	22.75	20.75	25.05
	$CHCl_3$	25.50	23.00	23.00	20.00	19.25	22.15
	Mean Conc.	34.125	30.063	28.188	25.625	24.75	
	CV%			1.68%			
	P≤0.05			*			*
	Interaction			NS			

Values presented are means of n = 4 replicates, * = significance at P≤0.05 respectively; NS = not significant, TMM = tomato moneymaker, TS = tomato star. $CHBrCl_2$ = Bromodichloromethane, $CHBr_3$ = Bromoform, $CHCl_3$ = Chloroform, $CHBr_2Cl$ = Dibromochloromethane

Table 12
Pearson correlation coefficient matrix between nutrients in dry matter yield of tomato plants

	N	P	K	Ca	Mg	S	Na	Mn	Fe	Cu	Zn	B
N	1											
P	0.7417 <.0001	1										
K	0.8086 <.0001	0.8663 <.0001	1									
Ca	0.2891 0.0093	0.3805 <0.005	0.2986 0.0071	1								
Mg	0.2848 <0.015	0.5664 <0.0001	0.3608 0.0010	0.6515 <.0001	1							
S	0.8419 <.0001	0.7354 <.0001	0.7266 <.0001	0.5115 <.0001	0.4836 <.0001	1						
Na	0.1476 0.1913	0.1947 0.0835	0.2044 0.0690	0.3578 0.0011	0.4550 <.0001	0.2206 0.0493	1					
Mn	0.3863 0.0004	0.1083 0.3389	0.1983 0.0778	0.2068 0.0658	0.0420 0.7112	0.2855 0.0103	0.3094 0.0052	1				
Fe	-0.1924 0.0873	-0.3958 0.0003	-0.3545 0.0013	-0.2497 0.0255	-0.2976 0.0073	-0.2784 0.0124	0.1049 0.3564	0.3972 0.0003	1			
Cu	0.5052 <.0001	0.3073 0.0056	0.3553 0.0012	0.2688 0.0159	0.1798 0.1105	0.4504 <.0001	0.3943 0.0003	0.6468 <.0001	0.4440 <.0001	1		
Zn	0.3467 0.0016	0.4021 0.0002	0.3464 0.0016	0.3795 0.0005	0.2566 0.0216	0.3265 0.0031	0.3155 <.0001	0.3317 <.0001	0.1929 0.0115	0.4499 <.0001	1	
B	0.7471 <.0001	0.8032 <.0001	0.8584 <.0001	0.5816 <.0001	0.5479 <.0001	0.7948 <.0001	0.2727 0.0004	0.1385 0.0755	-0.1815 0.0190	0.2877 0.0004	0.2564 0.0009	1

Values presented are Pearson's (r) test for association between paired samples at P≤0.05 significance

Table 13
Effects of trihalomethane concentration and trihalomethane chemical species on non enzymatic and enzymatic biomarkers of oxidative stress in lyophilized leaf tissues of tomato plants (data at 30 days after commencement of treatment)

		Trihalomethane Concentration $mg.L^{-1}$					
	Chemical species	0 control	2.5 $mg.L^{-1}$	5.0 $mg.L^{-1}$	7.5 $mg.L^{-1}$	10 $mg.L^{-1}$	Mean chem. Species
Polyphenols	$CHBr_2Cl$	2.361	3.665	3.907	3.958	4.285	3.635
$mg.L^{-1}$(GAE)	$CHBrCl_2$	5.284	6.805	6.875	7.491	9.103	7.112
	$CHBr_3$	9.590	11.625	11.824	12.216	15.200	12.091
	$CHCl_3$	15.096	15.324	16.363	17.008	18.224	16.403
	Mean Conc.	8.083	9.355	9.742	10.168	11.703	
	CV%		3.05%				
	P≤0.05		*				*
	Interaction		NS				
FRAP	$CHBr_2Cl$	14.707	21.743	21.971	22.086	23.348	20.771
$mg.L^{-1}$(AAE)	$CHBrCl_2$	20.777	26.567	33.505	34.737	37.131	30.543
	$CHBr_3$	31.537	32.181	34.616	42.827	48.107	37.853
	$CHCl_3$	36.824	39.927	40.502	44.988	52.903	43.029
	Mean Conc.	25.961	30.105	32.649	36.160	40.372	
	CV%		2.93%				
	P≤0.05		*				*
	Interaction		*				
ORAC	$CHBr_2Cl$	3.718	6.853	7.189	7.645	8.002	6.681
mM trolox	$CHBrCl_2$	7.565	10.912	11.100	11.275	12.404	10.651
	$CHBr_3$	7.117	7.133	7.932	9.279	11.238	8.540
	$CHCl_3$	18.852	21.680	21.971	28.717	32.102	24.665
	Mean Conc.	9.313	9.931	12.048	14.229	15.937	
	CV%		3.28%				
	P≤0.05		*				*
	Interaction		*				
APX activity	$CHBr_2Cl$	0.001	0.004	0.005	0.006	0.007	0.004
U mg^{-1}	$CHBrCl_2$	0.007	0.008	0.011	0.020	0.026	0.014
	$CHBr_3$	0.002	0.002	0.002	0.003	0.004	0.002
	$CHCl_3$	0.012	0.022	0.023	0.031	0.076	0.033
	Mean Conc.	0.006	0.009	0.010	0.011	0.028	
	CV%		24.54%				
	P≤0.05		*				*
	Interaction		NS				
GPX activity	$CHBr_2Cl$	0.005	0.009	0.011	0.012	0.014	0.010
U mg^{-1}	$CHBrCl_2$	0.006	0.010	0.013	0.019	0.035	0.017
	$CHBr_3$	0.004	0.004	0.005	0.006	0.007	0.005
	$CHCl_3$	0.009	0.019	0.019	0.037	0.039	0.025
	Mean Conc.	0.006	0.011	0.012	0.019	0.024	
	CV%		16.76%				
	P≤0.05		*				*
	Interaction		NS				

Table 13 continued

		Trihalomethane Concentration $mg.L^{-1}$					
	Chemical species	0 control	2.5 $mg.L^{1}$	5.0 $mg.L^{1}$	7.5 $mg.L^{-1}$	10 $mg.L^{-1}$	Mean Chem. Species
SOD activity U mg^{-1}	$CHBr_2Cl$	0.037	0.040	0.026	0.019	0.025	0.029
	$CHBrCl_2$	0.024	0.007	0.002	0.010	0.013	0.011
	$CHBr_3$	0.018	0.016	0.010	0.027	0.011	0.016
	$CHCl_3$	0.056	0.036	0.012	0.051	0.079	0.047
	Mean Conc.	0.034	0.025	0.012	0.027	0.032	
	CV%		7.15%				
	P≤0.05		*				*
	Interaction		*				
BCA mg/mL	$CHBr_2Cl$	101.67	87.771	72.176	67.668	52.380	76.320
	$CHBrCl_2$	126.629	115.589	114.210	80.524	51.752	97.741
	$CHBr_3$	184.608	209.798	243.993	302.665	311.946	250.602
	$CHCl_3$	102.078	86.251	85.448	79.020	38.248	78.209
	Mean Conc.	128.746	124.852	128.957	132.469	113.582	
	CV%		4.18%				
	P≤0.05		NS				*
	Interaction		*				
TBARS $mM^{-1}cm^{-1}$	$CHBr_2Cl$	0.838	0.988	1.547	2.925	3.049	1.869
	$CHBrCl_2$	2.191	2.416	3.403	3.947	3.983	3.188
	$CHBr_3$	1.761	2.100	3.058	3.442	4.034	2.879
	$CHCl_3$	1.393	1.819	2.516	3.132	3.280	2.428
	Mean Conc.	1.546	1.831	2.631	3.362	3.587	
	CV%		3.55%				
	P≤0.05		*				*
	Interaction		*				

Values presented are means of n = 4 replicates, * = significance at P≤0.05 respectively; NS = not significant, TMM = tomato moneymaker, TS = tomato star. $CHBrCl_2$ = Bromodichloromethane, $CHBr_3$ = Bromoform, $CHCl_3$ = Chloroform, $CHBr_2Cl$ = Dibromochloromethane FRAP = Ferric reducing ability of plasma, ORAC = Oxygen radical absorbance capacity, APx = Ascorbate peroxidase, GPx = Guaiacol peroxidase, SOD = Superoxide dismutase, BCA = pierce bicinchoninic acid protein assay, TBARS = Thiobarbituric acid reaction for general lipid peroxidation.

Table 14

Pearson correlation coefficient matrix between all biomarkers of oxidative stress in lyophilized leaf tissues of tomato plants

	polyphenols	FRAP	ORAC	APX	GPX	SOD	BCA	TBARS
polyphenols	1							
FRAP	0.88191 <.0001	1						
ORAC	0.72380 <.0001	0.59330 <.0001	1					
APX	0.21586 0.0061	0.24020 0.0022	0.21141 0.0073	1				
GPX	0.14135 0.0746	0.13222 0.0956	0.15818 0.0457	0.62784 <.0001	1			
SOD	0.24926 0.0015	0.22217 0.0047	0.30760 <.0001	0.46416 <.0001	0.51466 <.0001	1		
BCA	0.25911 0.0009	0.25213 0.0013	-0.13271 0.0943	-0.25958 0.0009	-0.30020 0.0001	-0.27430 0.0004	1	
TBARS	0.19615 0.0129	0.25508 0.0011	0.05512 0.4888	0.04025 0.6133	0.01392 0.8613	-0.09906 0.2127	0.32648 <.0001	1

Values presented are Pearson's (r) test for association between paired samples at P≤0.05 significance