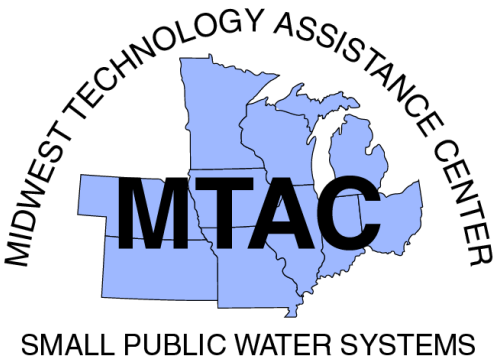

Emerging Health Concerns Related to Water Treatment

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Emerging Health Concerns Related to Water Treatment

Final Report

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June 2008

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Executive Summary

Drinking water utilities provide an exceedingly important public health service through their generation of high quality, safe and palatable tap water. The disinfection of drinking water in public facilities primarily employs chemical disinfectants such as chlorine, chloramines, ozone and chlorine dioxide. These disinfectants are oxidants that convert naturally occurring and synthetic organic material, bromide, and iodide in the raw water into chemical disinfection by-products (DBPs). DBPs are an unintended consequence and were first discovered over 30 years ago. Each disinfection method generates a different spectrum and distribution of DBPs; to date over 600 DBPs have been identified. While reducing the public health risk of acute infection by waterborne pathogens, the unintended generation of DBPs poses a chronic health risk. DBPs represent an important class of environmentally hazardous chemicals that are regulated by the U.S. Environmental Protection Agency (U.S. EPA) and carry long-term human health implications. Epidemiological studies demonstrated that individuals who consume chlorinated drinking water have an elevated risk of cancer. DBPs have been linked to reproductive and developmental effects, including the induction of spontaneous abortions in humans.

Although chlorine has been used for over 100 years in the United States as a water disinfectant, the majority of DBPs present in drinking water have yet to be chemically characterized. With only approximately 30% of the total organic halide identified to specific DBP chemical classes, and a small fraction of these evaluated for their biological and toxicological effects, it is clear that a great deal of work remains in the characterization of DBPs.

A comparative, *in vitro* analysis measured chronic cytotoxicity and acute genomic DNA damage in Chinese hamster ovary (CHO) cells induced by three chemical classes of emerging DBPs. Haloacetonitriles and haloacetamides (nitrogen-containing DBPs) and haloacetaldehydes (carbon-based DBPs) were evaluated such that a rank order of their chronic cytotoxicity to CHO cells was generated. For the haloacetonitriles the cytotoxicity from most toxic to least toxic was dibromoacetonitrile > bromoacetonitrile > ≈ iodoacetonitrile > bromochloroacetonitrile > dichloroacetonitrile > chloroacetonitrile > trichloroacetonitrile. The cytotoxicity order for the haloacetamides was diiodoacetamide > iodoacetamide > bromoacetamide > tribromoacetamide > bromoiodoacetamide > dibromochloroacetamide > bromochloroacetamide > chloroiodoacetamide > bromodichloroacetamide > dibromoacetamide > chloroacetamide > dichloroacetamide > trichloroacetamide. Finally the order of cytotoxicity for the haloacetaldehydes was tribromoacetaldehyde ≈ chloroacetaldehyde > dibromoacetaldehyde > dichloroacetaldehyde >> trichloroacetaldehyde. The induction of genomic DNA damage by these DBPs was quantitatively measured using single cell gel electrophoresis (SCGE) analysis. The rank order for the genotoxicity of the haloacetonitriles was iodoacetonitrile > bromoacetonitrile ≈ dibromoacetonitrile > bromochloroacetonitrile > chloroacetonitrile > trichloroacetonitrile > dichloroacetonitrile. The rank order for the genotoxicity of the haloacetamides was tribromoacetamide > diiodoacetamide ≈ iodoacetamide > bromoacetamide > dibromochloroacetamide > bromoiodoacetamide > bromodichloroacetamide > chloroiodoacetamide > bromochloroacetamide > dibromoacetamide > chloroacetamide > trichloroacetamide with dichloroacetamide not genotoxic. The genotoxic rank order for the haloacetaldehydes was chloroacetaldehyde ≈ dibromoacetaldehyde > tribromoacetaldehyde > dichloroacetaldehyde. Trichloroacetaldehyde was not genotoxic. These data demonstrate the wide range of responses by these emerging and important DBPs. The selection of these three DBP classes was based on information from recent U.S. EPA DBP priority and occurrence

studies. Mammalian cell cytotoxicity and genotoxicity data provided a rank ordering of the relational toxicities of regulated and emerging DBPs and related agents both within an individual chemical class and among classes. The use of alternative disinfectants generates new DBP compounds and alters the distribution of DBP chemical classes. The water supply community will be able to consider these factors when employing alternatives to chlorine disinfection. In addition these data will be available to prioritize DBPs for future *in vivo* toxicological studies and risk assessment.

Introduction

One of the most important and perhaps the most significant public health achievement of the twentieth century was the disinfection of drinking water and its distribution infrastructure [1]. In a recent paper Dr. Mark Shannon, Director of the University of Illinois WaterCAMP*W*S Program stated, “One of the most pervasive problems afflicting people throughout the world is inadequate access to clean water and sanitation. Problems with water are expected to grow worse in the coming decades, with water scarcity occurring globally, even in regions currently considered water-rich. Addressing these problems calls out for a tremendous amount of research to be conducted to identify robust new methods of purifying water at lower cost and with less energy, while at the same time minimizing the use of chemicals and impact on the environment” [2].

The public drinking water community provides an exceedingly important public health service for the nation by its generation of high quality, safe and palatable tap water. Each day approximately 250,000 public water purification facilities in the United States provide over 1.3×10^{10} liters of high quality, drinking water to over 270 million Americans [3]. The disinfection of drinking water in public facilities primarily uses chemical disinfectants such as chlorine, chloramines, ozone and chlorine dioxide [4, 5]. These disinfectants are also oxidants that convert naturally occurring and synthetic organic material, bromide, and iodide in the raw water into chemical disinfection by-products (DBPs). DBPs are an unintended consequence and were first discovered over three decades ago [6, 7]. Each disinfection method generates a different spectrum and distribution of DBPs [5, 8]; to date over 600 DBPs have been identified [9, 10]. While reducing the public health risk of acute infection by waterborne pathogens, the generation of DBPs poses a chronic health risk. DBPs represent an important class of environmentally hazardous chemicals that carry long-term human health implications [3, 10-14]. Epidemiological and laboratory based studies demonstrated that individuals who consume chlorinated drinking water have an elevated risk of cancer of the bladder, stomach, pancreas, kidney, and rectum as well as Hodgkin’s and non-Hodgkin’s lymphoma [15-34]. DBPs have been linked to deleterious reproductive and developmental effects [10, 35-39], including the induction of spontaneous abortions in humans [40-49].

Although chlorine has been used for over 100 years as a water disinfectant, the majority of DBPs present in drinking water have yet to be chemically defined [5, 8, 50-52]. Identified DBPs account for less than 50% of the total organic halide (TOX) produced in chlorinated drinking water. Many drinking water disinfection facilities use multiple chemical disinfection methods. A summary of the percentages of DBP classes in water surveyed in the U.S. EPA Nationwide Occurrence Study is presented in Figure 1 [50]. With only approximately 30% of the total organic halide identified to specific DBP chemical classes, it is clear that a great deal of work remains in the characterization of DBPs.

Evolving U.S. EPA Regulations

In 1979 the U.S. EPA issued a regulation to control total trihalomethanes (chloroform, bromodichloromethane, dibromochloromethane, and bromoform) at an annual average of 100 μg/L (ppb) in drinking water [53]. In 1998 the U. S. EPA issued the Stage 1 Disinfectants (D)/DBP Rule, which lowered permissible levels of total THMs to 80 μg/L and regulated five haloacetic acids (chloro-, dichloro-, trichloro-, bromo-, and dibromoacetic acids) at 60 μg/L, bromate at 10 μg/L, and chlorite at 1000 μg/L [54]. Stage 1 regulations required monitoring

based on running annual averages in a utility's distribution system. Recently the Stage 2 D/DBP Rule was enacted that maintained the Stage 1 Rule maximum contaminant levels (MCLs) for the DBPs plus the additional restriction that the MCLs are based on locational running annual averages [55]. The Stage 2 D/DBP Rule maintains the MCLs for bromate and chlorite; however, the U.S. EPA plans to review the bromate MCL as part of their 6-year review process. Besides the United States, the United Nations World Health Organization has issued guidelines for DBPs [12] as well as the European Union [56].

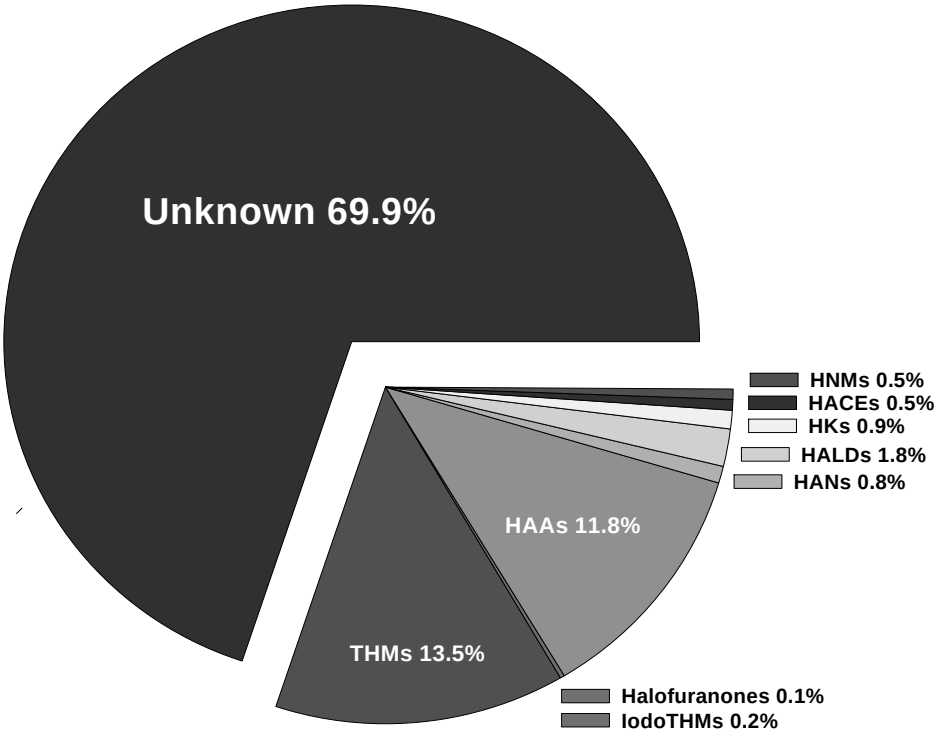

Figure 1. Summary distribution of DBP chemical classes in water analyzed in the U.S. EPA Nationwide Occurrence Study as a component of TOX . The abbreviations are THMs = trihalomethanes, HAAs = haloacetic acids, HNMs = halonitromethanes, HACEs = haloacetamides, HKs = haloketones, HALDs = haloacetaldehydes, HANs = haloacetonitriles. Data summarized from the U.S. EPA Nationwide Occurrence Study [50].

As a result of the more stringent effluent limitations on THMs and HAAs embodied in the U.S. Environmental Protection Agency's Disinfection By-Products Rule, drinking water utilities are in the process of incorporating different disinfectant combinations, particularly the use of chloramination and ozonation, to minimize THM and HAA formation. While these strategies effectively reduce the formation of these regulated DBPs [5, 8], recent findings indicate that they may foster the formation of highly toxic N-DBPs. For example, nitrosamines form primarily during chloramination [57-60]. Ozonation followed by chlorination significantly enhances the formation of chloropicrin, a member of the nitroalkane family [61, 62]. Because utilities are altering their disinfection protocols, there is a current need for research regarding

disinfection methodologies that minimize N-DBP formation. These data indicate that unknown and potentially hazardous novel DBPs may be generated by using alternative disinfection methods especially when heightened levels of bromine or iodine are present in the raw waters.

Emerging Health Concerns Related to Nitrogen-Containing DBPs

Several recent developments point to emerging important, and quantifiable, human health risks. Moreover, these developments promise new, exciting avenues for research that could lay the groundwork for an overhaul in the methods by which DBP regulations are developed. First, alterations in source water quality are resulting in the formation of new DBP families that exhibit far higher toxicities than the carbon-based trihalomethane (THM) and haloacetic acid (HAA) disinfection byproducts (C-DBPs) that are currently regulated. The decreasing availability of pristine waters supplies fostered by population growth is encouraging utilities to exploit waters impaired by agricultural runoff or wastewater effluents [63]. Whether via direct dissolved organic nitrogen (DON) inputs from wastewater effluents or via algal blooms fostered by inorganic nitrogen loadings from agricultural runoff, such waters often feature higher DON concentrations [64] that serve as precursors for nitrogen-containing DBPs (N-DBPs). Of particular concern due to their toxicities are N-DBPs featuring nitro (-NO_2; e.g., halonitromethanes), nitrile (-CN; e.g., haloacetonitriles), nitroso (-NO; e.g., N-nitrosodimethylamine (NDMA), and amine (-CNH_2; e.g. haloacetamides) functional groups. For example the haloacetonitriles constitute 10% of the 50 DBPs predicted to be the most carcinogenic [65]. The U.S. EPA estimates that drinking water concentrations of nitrosamines as low as 10^{-12} M would result in a 10^{-6} lifetime cancer risk [66].

Combining recent advances in analytical biology with our emerging knowledge regarding alterations in source water quality may enable the avoidance of one of the major pitfalls that had befallen prior C-DBP research: the inability to predict the most important C-DBPs likely to form, and to quantify their toxicological importance. While >600 C-DBPs have been identified over the past 30 years, these compounds account for only ~ 40% of the total organic halides (TOX) in chlorinated drinking waters [50]. With ozonation and chloramination, the percentage of unidentified TOX is even higher (i.e., > 80%) [5, 8].

Research Objectives

In this project we applied *in vitro* mammalian cell chronic cytotoxicity, genomic DNA damage and acute cytotoxicity assays to investigate two important emerging classes of nitrogen-containing DBPs (N-DBPs) the haloacetonitriles and the haloacetamides as well as an emerging class of poorly studied C-DBPs, the haloaldehydes. With changing disinfection practices the formation of these emerging DBPs deserves attention by managers of drinking water utilities in Illinois and the nation.

Materials and Methods

Biological and Chemical Reagents

General reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St. Louis, MO). Media and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Logan, UT) or from Fisher Scientific Co. (Itasca, IL).

Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cells are widely used in toxicology. The transgenic CHO cell line AS52 [67, 68] was derived from the parental CHO K1-BH4 line [69, 70]. Clone 11-4-8 was isolated from AS52 by Dr. E. Wagner and it expresses a stable chromosome complement, a consistent cell doubling time as well as functional p53 protein [71-73].

Selection and Source of DBPs and Associated Chemical Agents

A major problem for the toxicological analysis of emerging DBPs is that the large majority of the agents listed by Woo et al. [74] and Krasner et al. [50] are not commercially available. However, through collaboration with Dr. Susan Richardson, U. S. EPA, and with support from the American Water Works Research Foundation, most of the priority DBPs were synthesized as analytical standards and were provided for this study. Reagent grade or higher chemical agents were purchased from commercial vendors when available. The DBPs and related chemicals were shipped to the laboratory, logged into a database, and usually stored in dark conditions at 4°C. Prior to the biological experiments a 1 M or a 2 M solution of the DBP was prepared in dimethylsulfoxide (DMSO). This stock solution was immediately stored under dark conditions at −22°C. For each experiment freshly prepared serial dilutions into Hams F12 medium were made to treat the mammalian cells. A list of the sources and purities of the DBPs and related chemicals analyzed in this study is presented in Table 1.

Table 1. Sources and purities of the DBPs and related chemical agents used in the *in vitro* CHO cell toxicity assays for this project.

DBP or Chemical Agent	Chemical Class	MW	Purity %	Source
Bromoacetonitrile	Halonitrile	119.95	97	Chem. Service
Bromochloroacetonitrile	Halonitrile	154.39	>95	Chem. Service
Chloroacetonitrile	Halonitrile	75.50	>99	Chem. Service
Dibromoacetonitrile	Halonitrile	198.84	97	Chem. Service
Dichloroacetonitrile	Halonitrile	109.94	>99	Chem. Service
Iodoacetonitrile	Halonitrile	166.95	98	CanSyn Chem. Co.
Trichloroacetonitrile	Halonitrile	144.39	98	Aldrich Chem. Co.
Bromoacetamide	Haloamide	137.96	98	U.S. EPA
Bromochloroacetamide	Haloamide	172.41	>99	CanSyn Chem. Co.
Bromodichloroacetamide	Haloamide	206.85	>95	CanSyn Chem. Co.
Bromoiodoacetamide	Haloamide	263.86	85	CanSyn Chem. Co.
Chloroacetamide	Haloamide	93.51	>95	U.S. EPA
Chloroiodoacetamide	Haloamide	219.41	>95	CanSyn Chem. Co.
Dibromoacetamide	Haloamide	216.86	>95	CanSyn Chem. Co.
Dibromochloroacetamide	Haloamide	251.31	>95	CanSyn Chem. Co.
Dichloroacetamide	Haloamide	127.96	98	U.S. EPA

Table 1. Sources and purities of the DBPs and related chemical agents used in the *in vitro* CHO cell toxicity assays for this project.

DBP or Chemical Agent	Chemical Class	MW	Purity %	Source
Diiodoacetamide	Haloamide	310.85	>99	CanSyn Chem. Co.
Iodoacetamide	Haloamide	184.96	>97	Sigma Chem. Co.
Tribromoacetamide	Haloamide	295.75	>95	CanSyn Chem. Co.
Trichloroacetamide	Haloamide	162.40	99	U.S. EPA
Chloroacetaldehyde	Haloaldehyde	78.50	>95	CanSyn Chem. Co.
Dibromoacetaldehyde	Haloaldehyde	201.85	>95	CanSyn Chem. Co.
Dichloroacetaldehyde	Haloaldehyde	112.94	>95	CanSyn Chem. Co.
Tribromoacetaldehyde	Haloaldehyde	280.74	97	CanSyn Chem. Co.
Trichloroacetaldehyde	Haloaldehyde	165.40	>95	CanSyn Chem. Co.

Maintenance of CHO Cells

Stock cultures of the CHO cells were frozen in a solution of 90% fetal bovine serum (FBS):10% dimethylsulfoxide (DMSO) (v/v) and stored at −80°C. Cells were grown on glass culture plates in Hams F12 medium plus 5% FBS at 37°C in a humidified atmosphere of 5% CO_2. The cells exhibit normal morphology, express cell contact inhibition and grow as a monolayer without expression of neoplastic foci. CHO cells were transferred when the culture became confluent.

CHO Cell Chronic Cytotoxicity Assay

The CHO cell microplate chronic cytotoxicity assay measures the reduction in cell density as a function of the concentration of the test agent over a 72 h period [75, 76]. A 96-well flat-bottomed microplate was used to evaluate a series of chemical concentrations. One column served as the blank control consisting of 200 μL of F12 +FBS medium only. The concurrent negative control column consisted of 3×10^3 CHO cells plus F12 +FBS medium. The wells of the remaining columns contained 3×10^3 CHO cells, F12 +FBS and a known DBP concentration in a total of 200 μL (Figure 2). The wells were covered with a sheet of sterile AlumnaSeal™ and the cells were incubated for 72 h at 37°C at 5% CO_2. After the treatment time, the medium from each well was aspirated, the cells fixed in methanol for 10 min and stained for 10 min with a 1% crystal violet solution in 50% methanol. The microplate was washed, 50 μL of DMSO/methanol (3:1 v/v) were added to each well, and the plate was incubated at room temperature for 10 min. The microplate was analyzed at 595 nm with a BioRad microplate reader; the absorbancy of each well was recorded and stored on a spreadsheet file. This assay was calibrated and there is a direct relationship between the absorbancy of the crystal violet dye associated with the cell and the number of viable cells (Figure 3) [75].

The averaged absorbancy of the blank wells was subtracted from the absorbancy data from each well. The mean blank-corrected absorbancy value of the negative control was set at 100%. The absorbancy for each treatment group well was converted into a percentage of the negative control. This procedure normalized the data, maintained the variance and allowed the combination of data from multiple microplates. For each DBP concentration, 8 replicate wells were analyzed per experiment, and the experiments were repeated 2-4×. These data were used to generate a concentration-response curve for each DBP (Figure 4). Regression analysis was applied to each DBP concentration-response curve, which was used to calculate the %C½ value,

which is analogous to a LC_{50} value. The %C½ value is the calculated DBP concentration that induced a cell density that was 50% of the negative control (Figure 4).

The data from the cytotoxicity experiments were transferred to Excel spreadsheets and analyzed using the statistical and graphical functions of SigmaPlot 8.02, SigmaStat 3.1 and Table Curve 4.03 (Systat Software Inc., San Jose, CA). The crystal violet absorbancy data collected by the Bio-Rad microplate reader was saved as a text file (.txt) with the experiment number and transferred to an Excel spreadsheet. The original absorbancy data, the blank-corrected and the conversion to the percent of the negative control values were saved on the spreadsheet for each DBP analyzed. For each DBP, a summary page was prepared and all of the statistical data was conducted on the percent of the negative control values. A concentration-response cytotoxicity curve for each DBP was generated from the summary page and a one-way analysis of variance (ANOVA) test was conducted to determine if the DBP induced a significant level of cell killing at a specific concentration. If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted. The power of the test statistic $(1-\beta)$ was maintained as ≥ 0.8 at $\alpha = 0.05$.

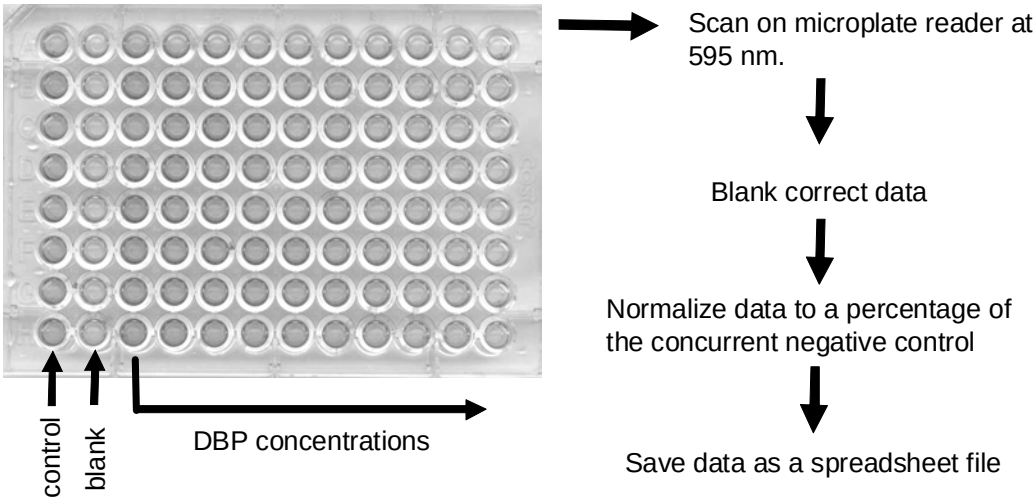

Figure 2. A stained microplate illustrating the CHO cell chronic cytotoxicity assay. The control (column 1) contained cells not exposed to the test compound. The blank column without cells was used to determine the absorbancy of the crystal violet histological dye that was not associated with cells and to normalize the absorbancy data. The DBP or test chemical was assayed from low concentration (column 3) to high concentration (column 12) with 8 replicate cultures per concentration.

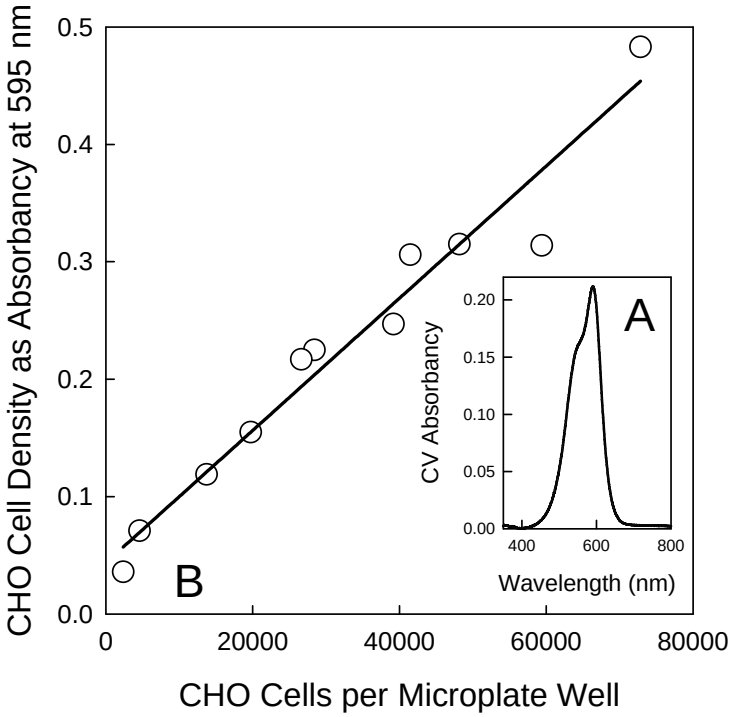

Figure 3. Calibration of the CHO cell cytotoxicity assay. (A) Absorption spectrum of crystal violet in the range from 340–800 nm. The maximum absorbancy of crystal violet was between 585–595 nm. (B) A comparison of the number of cells per microplate well determined by Coulter counting or by the absorbancy after crystal violet stain.

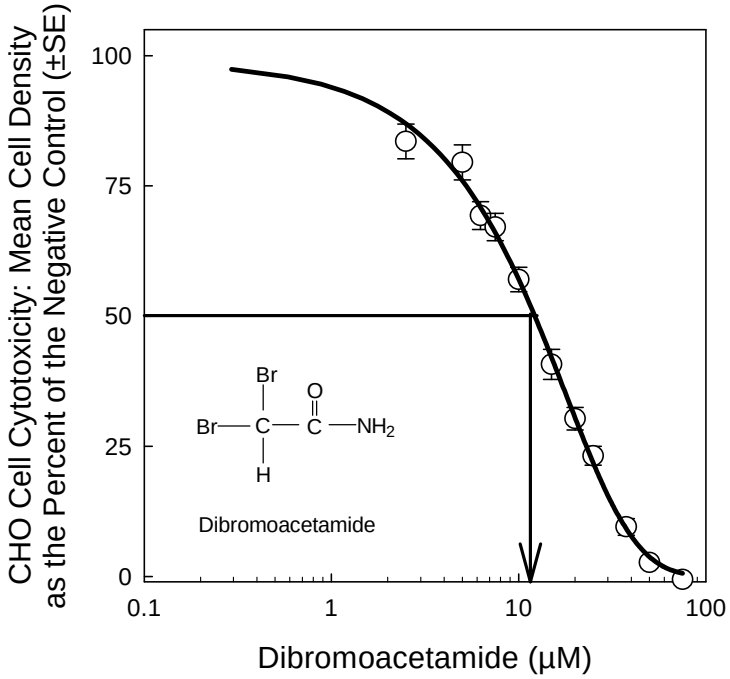

Figure 4. CHO cell chronic cytotoxicity concentration-response curve illustrating the determination of the %C½ value. This value is the calculated DBP concentration (based upon regression of the concentration-response data) that induced a cell density that was 50% of the negative control and is analogous to the LC_{50} value. The R^2 from the regression analysis was 0.99 and the %C½ value = 12.2 μM.

CHO Single Cell Gel Electrophoresis (SCGE) Assay

SCGE is a molecular genetic assay that can quantitatively measure the level of genomic DNA damage induced in individual nuclei of cells [77-79]. The day before treatment, 4×10^4 CHO cells were added to each microplate well in 200 μL of F12 + 5% FBS and incubated. The next day the cells were washed with Hank's balanced salt solution (HBSS) and treated with a series of concentrations of a specific DBP in F12 medium without FBS in a total volume of 25 μL for 4 h at 37°C, 5% CO_2. The wells were covered with sterile AlumnaSeal™. With each experiment a negative control, a positive control (3.8 mM ethylmethanesulfonate, EMS) and 9 concentrations of a specific DBP were conducted concurrently. After incubation the cells were washed 2× with HBSS and harvested with 50 μL of 0.01% trypsin + 53 μM EDTA. The trypsin was inactivated with 70 μL of F12 + FBS. To measure acute cytotoxicity a 10 μL aliquot of cell suspension was mixed with 10 μL of 0.05% trypan blue vital dye in phosphate-buffered saline (PBS) [80]. SCGE data were not used if the acute cytotoxicity exceeded 30%. Prior to the experiment clear microscope slides were coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. After cell treatment, the cell suspension from each well was embedded in a layer of low melting point agarose prepared with PBS and placed upon the slides. After the microgels solidified on a tray placed over ice, a final layer of 0.5% low

melting point agarose was placed upon the previous layers. The cellular membranes were removed by an overnight immersion in lysing solution at 4°C. The slides were placed in an alkaline buffer (pH 13.5) in an electrophoresis tank and the DNA was denatured for 20 min. The microgels were electrophoresed at 25 V, 300 mA (0.72 V/cm) for 40 min at 4°C. The microgels were removed from the tank, neutralized with Tris buffer, pH 7.5, rinsed in cold water, dehydrated in cold methanol, dried at 50°C and stored at room temperature in a covered slide box.

For microscopic analysis the microgels were hydrated in cold water for 20-30 min and stained with 65 μL of ethidium bromide (20 μg/mL) for 3 min. The microgels were rinsed in cold water and were analyzed with a Zeiss fluorescence microscope with an excitation filter of 546/10 nm and a barrier filter of 590 nm. For each experiment 2 microgels were prepared per treatment group. Twenty-five randomly chosen nuclei were analyzed in each microgel using a charged coupled device camera. A computerized image analysis system (Komet version 3.1, Kinetic Imaging Ltd., Liverpool, UK) was employed to determine the tail moment (integrated value of migrated DNA density multiplied by the migration distance) of the nuclei as a measure of DNA damage. The digitalized data (Figure 5) were automatically transferred to a computer based spreadsheet for subsequent statistical analysis. The experiments were repeated 3 times for each DBP.

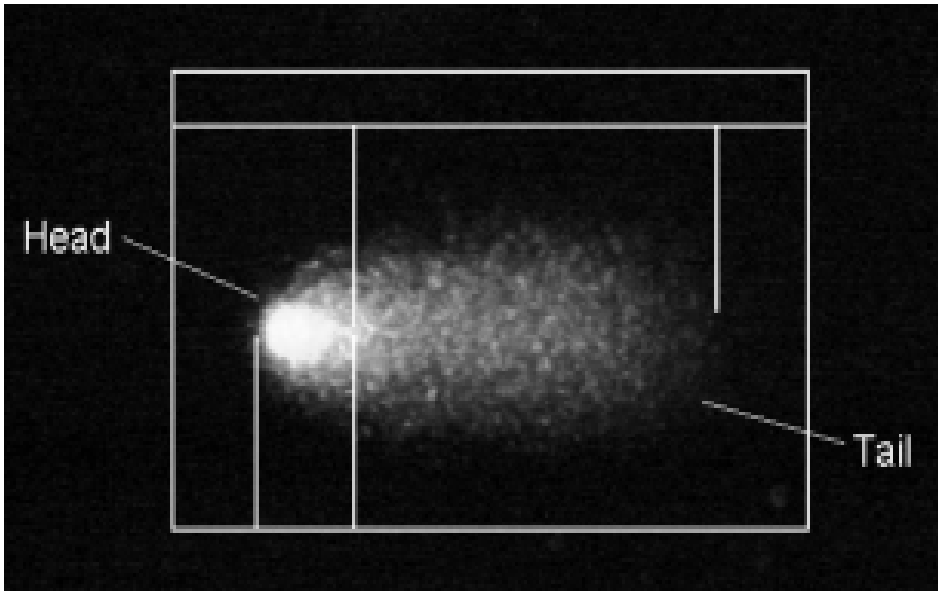

Figure 5. SCGE image illustrating genomic DNA damage in a nucleus after being electrophoresed in an agarose microgel. The level of DNA damage is directly related to the amount of DNA that migrates in the microgel (tail). The calipers are from a computer program for the assay that measures the amount and distance of DNA migration.

The SCGE tail moment data for each nucleus for each microgel was generated using the Komet 3.1 software. These data were stored on a spreadsheet and the median tail moment value for that microgel was calculated and transferred to a data spreadsheet. In addition the acute

cytotoxicity of the treated cells was entered into the same data spreadsheet. Within the DBP concentration range that allowed for 70% or greater viable cells, a concentration-response curve was generated. The data were plotted and a regression analysis was used to fit the curve (Figure 6). The SCGE genotoxic potency value was calculated for each DBP. The SCGE genotoxic potency value was determined from the concentration-response curve. It represents the midpoint of the curve within the concentration range that expressed above 70% cell viability.

The data were transferred to the SigmaStat 3.1 program spreadsheet for an ANOVA statistical test. The tail moment values in the SCGE assay are not normally distributed and violate the requirements for analysis by parametric statistics. The median tail moment value for each microgel was determined as described above and the data were averaged amongst all of the microgels for each DBP concentration. Averaged median or mean values express a normal distribution according to the central limit theorem [81]. The averaged median tail moment values obtained from repeated experiments were analyzed with a one-way ANOVA test [82]. If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted. The power of the test statistic $(1-\beta)$ was maintained as ≥ 0.8 at $\alpha=0.05$.

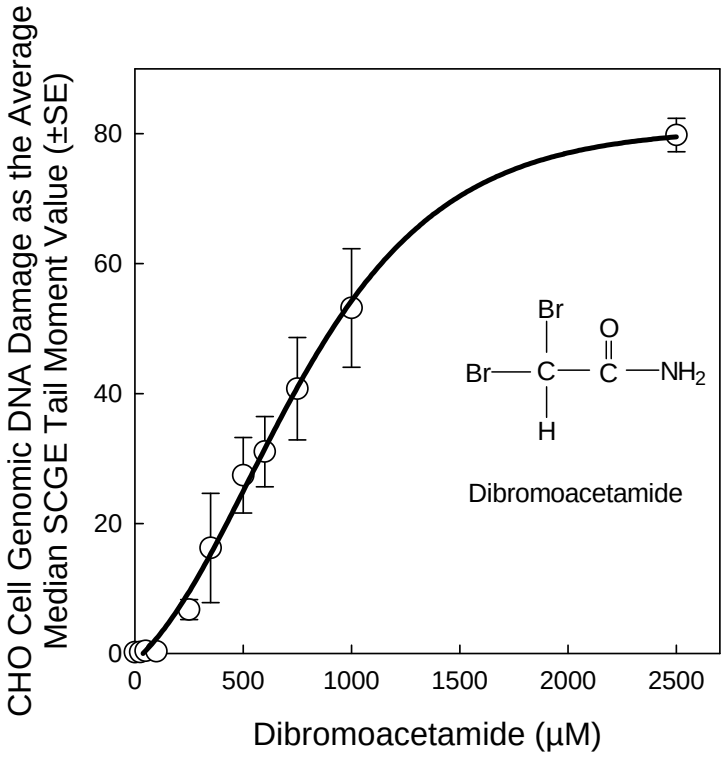

Figure 6. CHO cell SCGE genotoxicity concentration-response curve for dibromoacetamide.

Safety

Safety is a principal concern in the laboratory. Manipulations of toxic, genotoxic and/or carcinogenic chemicals were conducted using disposable papers and gloves in a certified stage two containment biological/chemical safety hood. The disposal of hazardous material was in compliance with the University of Illinois regulations. All staff involved on this project received safety training under the auspices of the University of Illinois Institutional Biological Safety Committee. The regulations of the Division of Research Safety of the University of Illinois were implemented throughout this project. Experimental designs for each DBP analyzed were prepared in hard-copy data books. The information for each experiment, identified by a unique experiment number, was placed in a file within an Excel spreadsheet.

Results and Discussion

Haloacetonitriles

The haloacetonitriles were measured in several occurrence studies [50, 51, 83-85] with bromochloroacetonitrile, chloroacetonitrile, dibromoacetonitrile, and trichloroacetonitrile (HAN4) the most commonly measured species. In the U.S. EPA's Information Collection Rule (ICR), the haloacetonitriles (HAN4) ranged from <0.5 to 41.0 µg/L, and were generally 12% of the levels of the four regulated trihalomethanes. These haloacetonitriles were formed using chlorine and/or chloramine disinfection; plants using chloramines (with and without chlorine) had the highest levels. Higher haloacetonitrile levels were from distribution system waters treated with post-chloramination versus free chlorine. Seven halonitriles were evaluated in this study, bromoacetonitrile, bromochloroacetonitrile, chloroacetonitrile, dibromoacetonitrile, dichloroacetonitrile, iodoacetonitrile, and trichloroacetonitrile (Table 1).

CHO Cell Cytotoxicity Analysis of the Haloacetonitriles

The CHO cell chronic cytotoxicity of the seven halonitriles analyzed in this study are presented in Table 2. In the table, the lowest concentration of a specific haloacetonitrile was identified by the ANOVA test statistic that induced a significant toxic response as compared to their concurrent negative controls. The %C½ value was the concentration of the haloacetonitrile that induced a 50% reduction of the cell density as compared to the negative controls. Finally, the R^2 refers to the fit of the regression analysis from which the %C½ value was calculated. All concentrations are presented in molar (M) units of measure.

Table 2. CHO cell chronic cytotoxicity of the haloacetonitrile DBPs and related chemicals.

Compound	Lowest Tox. Conc. (M)	R^2	%C½ (M)	ANOVA Test Statistic
Bromoacetonitrile	1.0×10^{-6}	0.98	3.21×10^{-6}	$F_{11,228} = 98.3$; $P \leq 0.001$
Bromochloroacetonitrile	7.0×10^{-6}	0.96	8.46×10^{-6}	$F_{11,171} = 36.2$; $P \leq 0.001$
Chloroacetonitrile	5.0×10^{-5}	0.99	6.83×10^{-5}	$F_{13,188} = 65.9$; $P \leq 0.001$
Dibromoacetonitrile	1.0×10^{-6}	0.99	2.85×10^{-6}	$F_{11,179} = 271.5$; $P \leq 0.001$
Dichloroacetonitrile	1.0×10^{-5}	0.99	5.73×10^{-5}	$F_{10,171} = 63.4$; $P \leq 0.001$
Iodoacetonitrile	1.0×10^{-7}	0.98	3.30×10^{-6}	$F_{12,163} = 148.4$; $P \leq 0.001$
Trichloroacetonitrile	2.5×10^{-5}	0.93	1.60×10^{-4}	$F_{17,282} = 36.8$; $P \leq 0.001$

A comparison of the relative cytotoxicity of the halonitriles analyzed in this study is presented in Figure 7. The lowest concentration that induced a significant cytotoxic response ranged from 100 nM (iodoacetonitrile) to 50 µM (chloroacetonitrile) (Table 2). The %C½ values ranged from 2.85 µM (dibromoacetonitrile) to 160 µM (trichloroacetonitrile). The rank order for cytotoxicity (highest to lowest) based on the %C½ values was dibromoacetonitrile > bromoacetonitrile > ≈ iodoacetonitrile > bromochloroacetonitrile > dichloroacetonitrile > chloroacetonitrile > trichloroacetonitrile.

CHO Cell Genotoxicity Analysis of the Haloacetonitriles

In this study seven halonitriles were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 3. In the table, the lowest concentration of a specific haloacetonitrile was identified by the ANOVA test statistic that induced significant

genomic DNA strand breakage (as measured by SCGE median tail moment values) as compared to their concurrent negative controls. The SCGE genotoxic potency value was calculated for each chemical from the concentration-response curve. It represents the midpoint of the curve within the concentration range that expressed above 70% cell viability of the treated cells. Finally, the R^2 refers to the fit of the regression analysis from which the SCGE genotoxic potency value was calculated. All concentrations are presented in molar (M) units of measure.

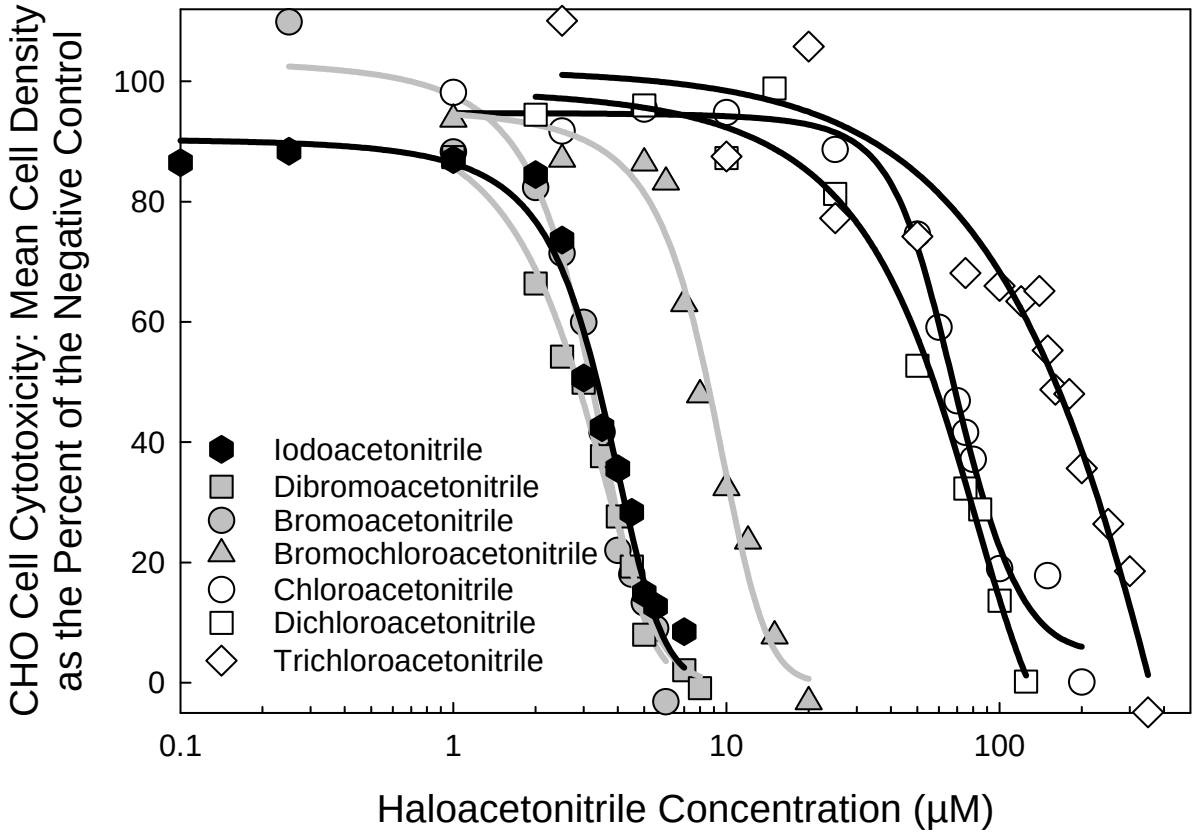

Figure 7. Comparison of the concentration-response curves for the CHO cell chronic cytotoxicity of seven haloacetonitriles.

Table 3. CHO cell genotoxicity of the haloacetonitrile DBPs and related chemicals.

Compound	Lowest Genotox. Conc. (M)	R^2	SCGE Gen. Potency (M)	ANOVA Test Statistic
Bromoacetonitrile	4.00×10^{-5}	0.99	3.85×10^{-5}	$F_{6, 36} = 32.7$; $P \leq 0.001$
Bromochloroacetonitrile	2.50×10^{-4}	0.98	3.24×10^{-4}	$F_{10, 41} = 19.1$; $P \leq 0.001$
Chloroacetonitrile	2.50×10^{-4}	0.99	6.01×10^{-4}	$F_{11,42} = 28.9$; $P \leq 0.001$
Dibromoacetonitrile	3.00×10^{-5}	0.95	2.97×10^{-5}	$F_{9, 46} = 46.1$; $P \leq 0.001$

Dichloroacetonitrile	2.40×10^{-3}	0.98	2.75×10^{-3}	$F_{17,62} = 14.2$; $P \leq 0.001$
Iodoacetonitrile	3.00×10^{-5}	0.98	3.71×10^{-5}	$F_{10,53} = 46.6$; $P \leq 0.001$
Trichloroacetonitrile	1.00×10^{-3}	0.98	1.01×10^{-3}	$F_{7,32} = 30.5$; $P \leq 0.001$

The lowest concentration that induced a significant SCGE genotoxic response ranged from 30 µM (iodoacetonitrile or dibromoacetonitrile) to 2.4 mM (dichloroacetonitrile). The SCGE genotoxic potency value ranged from 29.7 µM (dibromoacetonitrile) to 2.75 mM (dichloroacetonitrile) (Table 3). The rank order of genotoxic potency from most potent to least was dibromoacetonitrile > iodoacetonitrile ≈ bromoacetonitrile > bromochloroacetonitrile > chloroacetonitrile > trichloroacetonitrile > dichloroacetonitrile. A comparison of the genotoxicity for these halonitriles is presented in Figure 8.

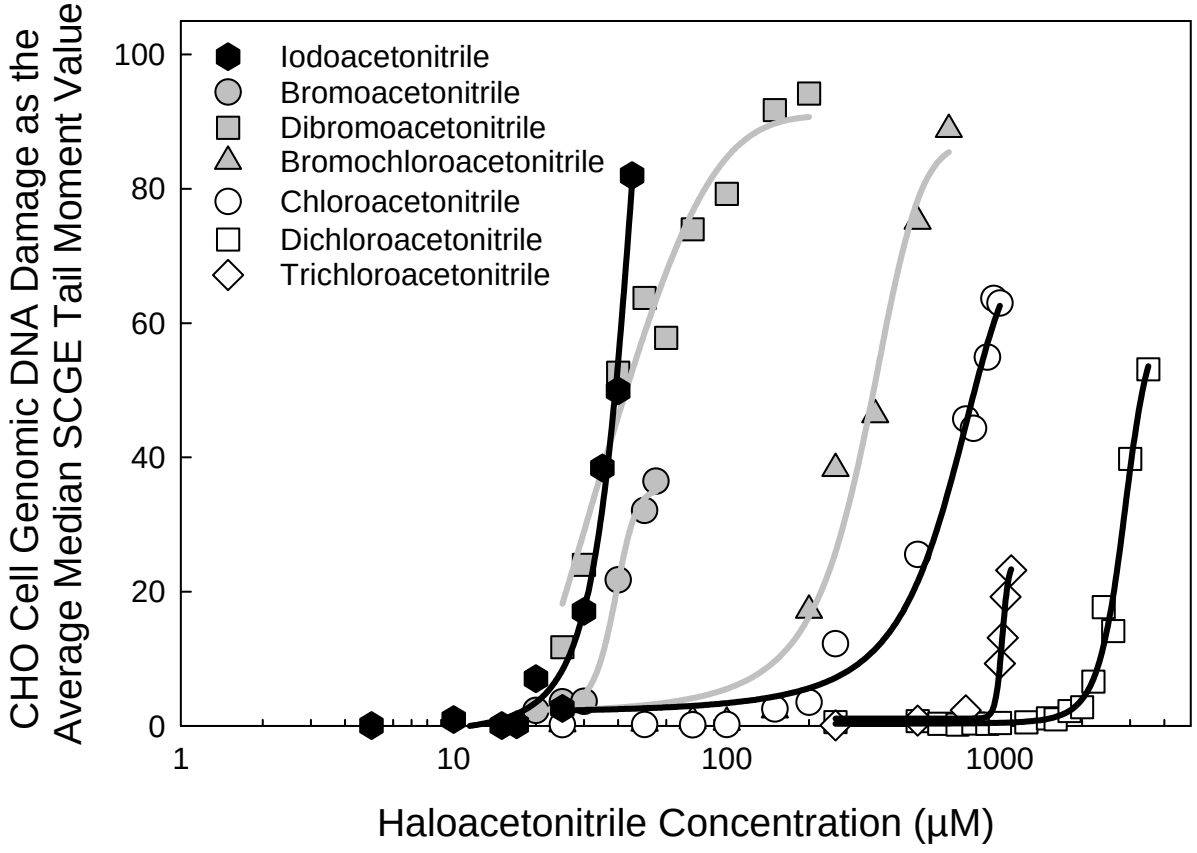

Figure 8. Comparison of the SCGE genotoxicity concentration-response curves of seven haloacetonitriles.

Haloacetamides

Haloacetamides have been identified as DBPs from drinking water treatment plants and from laboratory studies [61, 62, 86]. Thirteen haloamides were evaluated in this study, bromoacetamide, bromochloroacetamide, bromodichloroacetamide, bromoiodoacetamide,

chloroacetamide, chloroiodoacetamide, dibromoacetamide, dibromochloroacetamide, dichloroacetamide, diiodoacetamide, iodoacetamide, tribromoacetamide, and trichloroacetamide (Table 1).

CHO Cell Cytotoxicity Analysis of the Haloacetamides

The CHO cell chronic cytotoxicity of the 13 haloacetamides analyzed in this study are presented in Table 4. In the table, the lowest concentration of a specific haloacetamide was identified by the ANOVA test statistic that induced a significant toxic response as compared to their concurrent negative controls. The %C½ value was the concentration of the haloacetamide that induced a 50% reduction of the cell density as compared to the negative controls. Finally, the R^2 refers to the fit of the regression analysis from which the %C½ value was calculated. All concentrations are presented in molar (M) units of measure.A comparison of the relative cytotoxicity of the haloacetamides analyzed in this study is presented in Figure 9.

The lowest concentration that induced a significant cytotoxic response ranged from 25 nM (diiodoacetamide) to 800 μM (dichloroacetamide) (Table 4). The %C½ values ranged from 678 nM (diiodoacetamide) to 2.05 mM (trichloroacetamide). The rank order for cytotoxicity (highest to lowest) of the 13 haloacetamides based on their %C½ values was diiodoacetamide > iodoacetamide > bromoacetamide > tribromoacetamide > bromoiodoacetamide > dibromochloroacetamide > chloroiodoacetamide > bromodichloroacetamide > dibromoacetamide > bromochloroacetamide > chloroacetamide > dichloroacetamide > trichloroacetamide.

Table 4. CHO cell chronic cytotoxicity of the haloacetamide DBPs and related chemicals.

Compound	Lowest Tox. Conc. (M)	R^2	%C½ (M)	ANOVA Test Statistic
Bromoacetamide	5.00×10^{-7}	0.99	1.89×10^{-6}	$F_{12, 282} = 57.15$; $P \leq 0.001$
Bromochloroacetamide	1.00×10^{-6}	0.96	1.71×10^{-5}	$F_{16, 183} = 111.05$; $P \leq 0.001$
Bromodichloroacetamide	2.00×10^{-6}	0.98	8.68×10^{-6}	$F_{10, 197} = 173.96$; $P \leq 0.001$
Bromoiodoacetamide	2.00×10^{-6}	0.98	3.81×10^{-6} [a]	$F_{10, 164} = 85.99$; $P \leq 0.001$
Chloroacetamide	7.50×10^{-5}	0.98	1.48×10^{-4}	$F_{13, 176} = 99.20$; $P \leq 0.001$
Chloroiodoacetamide	2.00×10^{-6}	0.96	5.97×10^{-6}	$F_{14, 193} = 111.78$; $P \leq 0.001$
Dibromoacetamide	2.50×10^{-6}	0.99	1.22×10^{-5}	$F_{11, 283} = 174.56$; $P \leq 0.001$
Dibromochloroacetamide	1.00×10^{-6}	0.96	4.75×10^{-6}	$F_{9, 174} = 40.56$; $P \leq 0.001$
Dichloroacetamide	8.00×10^{-4}	0.95	1.92×10^{-3}	$F_{12, 271} = 79.20$; $P \leq 0.001$
Diiodoacetamide	2.50×10^{-8}	0.98	6.78×10^{-7}	$F_{10, 149} = 144.35$; $P \leq 0.001$
Iodoacetamide	5.00×10^{-7}	0.98	1.42×10^{-6}	$F_{17, 332} = 133.23$; $P \leq 0.001$
Tribromoacetamide	2.00×10^{-6}	0.97	3.14×10^{-6}	$F_{10, 275} = 122.62$; $P \leq 0.001$
Trichloroacetamide	5.00×10^{-4}	0.96	2.05×10^{-3}	$F_{11, 251} = 77.05$; $P \leq 0.001$

[a] The calculated %C½ value for bromoiodoacetamide alone assuming an additive model for the diiodoacetamide and dibromoacetamide contaminants was 3.35×10^{-6} M.

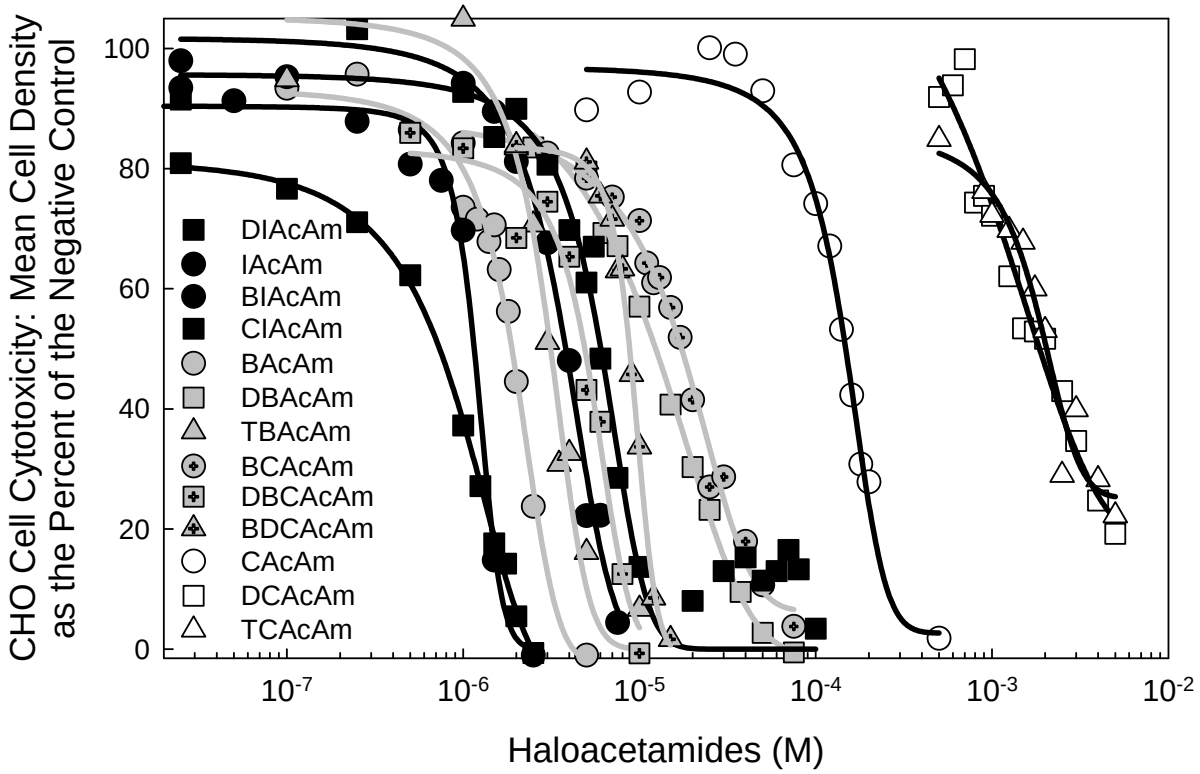

Figure 9. Comparison of the concentration-response curves for the CHO cell chronic cytotoxicity of the haloacetamides. The abbreviations are DIAcAm = diiodoacetamide, IAcAm = iodoacetamide, BIAcAm = bromoiodoacetamide, ClAcAm = chloroiodoacetamide, BAcAm = bromoacetamide, DBAcAm = dibromoacetamide, TBAcAm = tribromoacetamide, BCAcAm = bromochloroacetamide, DBCAcAm = dibromochloroacetamide, BDCAcAm = bromodichloroacetamide, CAcAm = chloroacetamide, DCAcAm = dichloroacetamide, and TCAcAm = trichloroacetamide.

CHO Cell Genotoxicity Analysis of the Haloacetamides

In this study13 haloacetamides were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 5. In the table, the lowest concentration of a specific haloacetamide was identified by the ANOVA test statistic that induced significant genomic DNA strand breakage (as measured by SCGE median tail moment values) as compared to their concurrent negative controls. The SCGE genotoxic potency value was calculated for each chemical from the concentration-response curve. It represents the midpoint of the curve within the concentration range that expressed above 70% cell viability of the treated cells. Finally, the R^2 refers to the fit of the regression analysis from which the SCGE genotoxic potency value was calculated. All concentrations are presented in molar (M) units of measure.

Table 5. CHO cell genotoxicity of the haloacetamide DBPs and related chemicals.

Compound	Lowest Genotox. Conc. (M)	R^2	SCGE Gen. Potency (M)	ANOVA Test Statistic
Bromoacetamide	2.50×10^{-5}	0.99	3.68×10^{-5}	$F_{9, 38} = 29.77$; $P \leq 0.001$
Bromochloroacetamide	4.00×10^{-4}	0.99	5.83×10^{-4}	$F_{9, 48} = 53.86$; $P \leq 0.001$
Bromodichloroacetamide	7.50×10^{-5}	0.99	1.46×10^{-4}	$F_{9, 39} = 58.41$; $P \leq 0.001$
Bromoiodoacetamide	2.50×10^{-5}	0.99	7.21×10^{-5} [a]	$F_{10, 54} = 29.38$; $P \leq 0.001$
Chloroacetamide	7.50×10^{-4}	0.99	1.38×10^{-3}	$F_{11, 46} = 25.02$; $P \leq 0.001$
Chloroiodoacetamide	2.00×10^{-4}	0.99	3.02×10^{-4}	$F_{17, 62} = 35.19$; $P \leq 0.001$
Dibromoacetamide	5.00×10^{-4}	0.99	7.44×10^{-4}	$F_{10, 47} = 21.09$; $P \leq 0.001$
Dibromochloroacetamide	2.50×10^{-5}	0.98	6.94×10^{-5}	$F_{8, 37} = 185.59$; $P \leq 0.001$
Dichloroacetamide	NA	NA	NS $>1\times10^{-2}$	$F_{11, 34} = 1.026$; $P = 0.417$
Diiodoacetamide	2.50×10^{-5}	0.98	3.39×10^{-5}	$F_{11, 60} = 29.12$; $P \leq 0.001$
Iodoacetamide	3.00×10^{-5}	0.99	3.41×10^{-5}	$F_{15, 43} = 13.11$; $P \leq 0.001$
Tribromoacetamide	3.00×10^{-5}	0.97	3.25×10^{-5}	$F_{17, 62} = 35.19$; $P \leq 0.001$
Trichloroacetamide	5.00×10^{-3}	0.98	6.54×10^{-3}	$F_{9, 50} = 5.75$; $P \leq 0.001$

NS = not statistically different from the negative control, NA = non applicable. [a] The calculated SCGE genotoxic potency value for bromoiodoacetamide alone assuming an additive model for the diiodoacetamide and dibromoacetamide contaminants was 1.62×10^{-5} M.

A comparison of the CHO cell genotoxicity for these haloacetamides is presented in Figure 10. The lowest concentration that induced a significant SCGE genotoxic response ranged from 25 μM for diiodoacetamide, bromoiodoacetamide, bromoacetamide, and dibromochloroacetamide to 5 mM for trichloroacetamide. The SCGE genotoxic potency value ranged from 32.5 μM for tribromoacetamide to 6.5 mM for trichloroacetamide (Table 5). The rank order of genotoxic potency from most potent to least was tribromoacetamide > diiodoacetamide ≈ iodoacetamide > bromoacetamide > dibromochloroacetamide > bromoiodoacetamide > bromodichloroacetamide > chloroiodoacetamide > bromochloroacetamide > dibromoacetamide > chloroacetamide > trichloroacetamide. Dichloroacetamide was not genotoxic (Table 5).

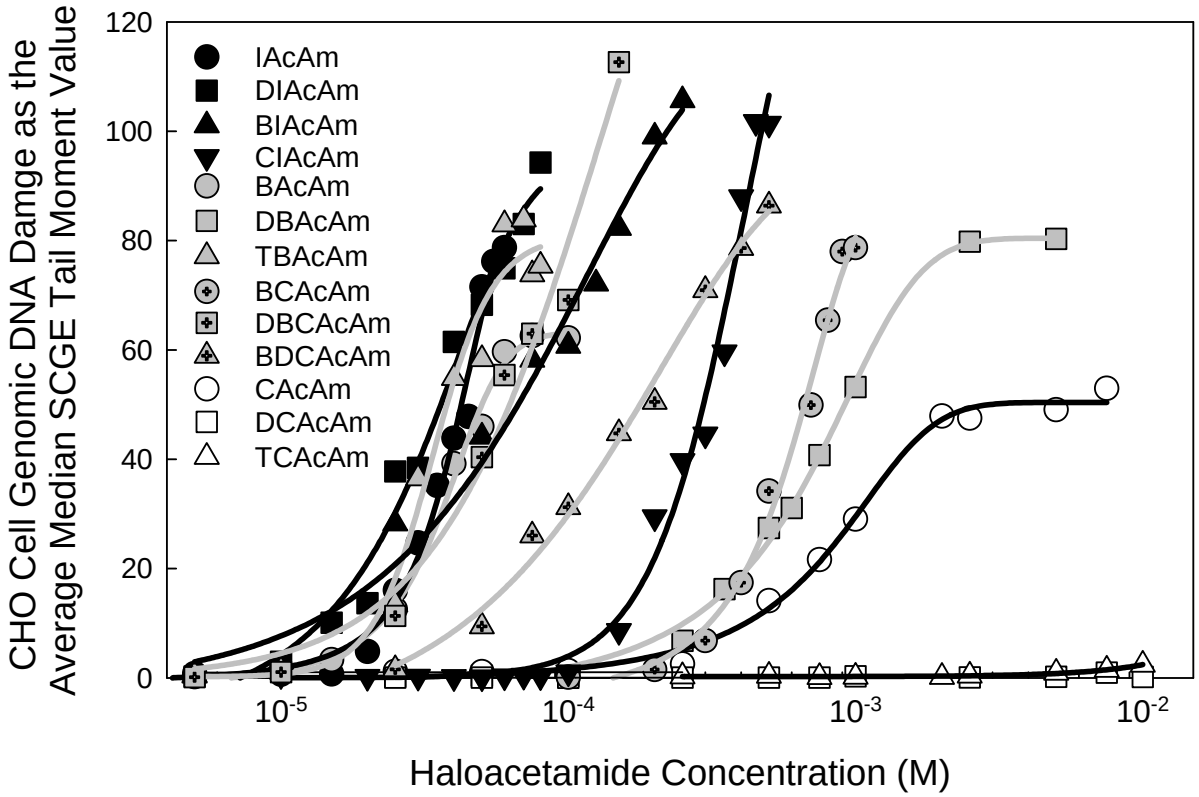

Figure 10. Comparison of the SCGE genotoxicity concentration-response curves of 13 haloacetamides. The abbreviations are IAcAm = iodoacetamide, DIAcAm = diiodoacetamide, BIAcAm = bromoiodoacetamide, CIAcAm = chloroiodoacetamide, BAcAm = bromoacetamide, DBAcAm = dibromoacetamide, TBAcAm = tribromoacetamide, BCAcAm = bromochloroacetamide, DBCAcAm = dibromochloroacetamide, BDCAcAm = bromodichloroacetamide, CAcAm = chloroacetamide, DCAcAm = dichloroacetamide, and TCAcAm = trichloroacetamide.

Haloacetaldehydes

The aldehydes are toxic DBPs and several were measured in the ICR report, including formaldehyde, acetaldehyde, glyoxal, methylglyoxal, and trichloroacetaldehyde (chloral hydrate). Although the non-halogenated aldehydes are DBPs produced primarily by ozone treatment [9, 87], chlorine and chlorine dioxide treatment can form formaldehyde [9, 65, 85]. The haloaldehydes evaluated in this study were chloroacetaldehyde, dibromoacetaldehyde, dichloroacetaldehyde, tribromoacetaldehyde, and trichloroacetaldehyde (Table 1).

CHO Cell Cytotoxicity Analysis of the Haloacetaldehydes

The CHO cell chronic cytotoxicity of the five haloacetaldehydes analyzed in this study are presented in Table 6. In the table, the lowest concentration of a specific haloacetaldehyde was identified by the ANOVA test statistic that induced a significant toxic response as compared to their concurrent negative controls. The %C½ value was the concentration of the haloacetaldehyde that induced a 50% reduction of the cell density as compared to the negative

controls. Finally, the R^2 refers to the fit of the regression analysis from which the %C½ value was calculated. All concentrations are presented in molar (M) units of measure. A comparison of the relative cytotoxicity of the haloacetaldehydes analyzed in this study is presented in Figure 11.

Table 6. CHO cell chronic cytotoxicity of the haloacetaldehyde DBPs and related chemicals.

Compound	Lowest Tox. Conc. (M)	R^2	%C½ (M)	ANOVA Test Statistic
Chloroacetaldehyde	5.00×10^{-7}	0.99	3.60×10^{-6}	$F_{11, 176} = 240.7$; $P \leq 0.001$
Dibromoacetaldehyde	2.00×10^{-6}	0.99	4.70×10^{-6}	$F_{10, 177} = 164.8$; $P \leq 0.001$
Dichloroacetaldehyde	8.00×10^{-6}	0.91	2.93×10^{-5}	$F_{19, 328} = 36.1$; $P \leq 0.001$
Tribromoacetaldehyde	2.00×10^{-6}	0.99	3.58×10^{-6}	$F_{18, 102} = 42.8$; $P \leq 0.001$
Trichloroacetaldehyde	3.75×10^{-4}	0.94	1.16×10^{-3}	$F_{24, 333} = 33.9$; $P \leq 0.001$

The lowest concentration that induced a significant cytotoxic response ranged from 0.5 μM (chloroacetaldehyde) to 375 μM (trichloroacetaldehyde) (Table 6). The %C½ values ranged from 3.58 μM (tribromoacetaldehyde) to 1.16 mM (trichloroacetaldehyde). Based on the %C½ values the rank order for cytotoxicity was tribromoacetaldehyde ≈ chloroacetaldehyde > dibromoacetaldehyde > dichloroacetaldehyde >> trichloroacetaldehyde.

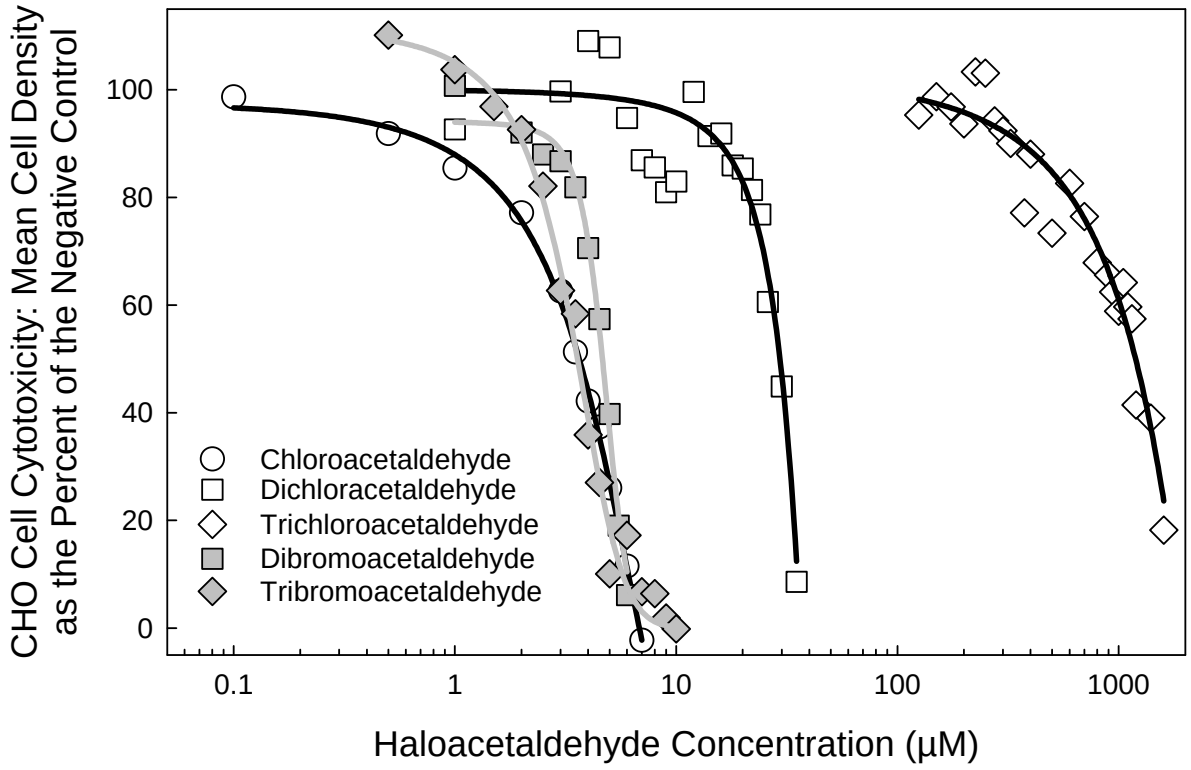

Figure 11. Comparison of the concentration-response curves for the CHO cell chronic cytotoxicity of the haloacetaldehydes.

CHO Cell Genotoxicity Analysis of the Haloacetaldehydes

In this study five haloacetaldehydes were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 7. In the table, the lowest concentration of a specific haloacetaldehyde was identified by the ANOVA test statistic that induced significant genomic DNA strand breakage (as measured by SCGE median tail moment values) as compared to their concurrent negative controls. The SCGE genotoxic potency value was calculated for each chemical from the concentration-response curve. It represents the midpoint of the curve within the concentration range that expressed above 70% cell viability of the treated cells. Finally, the R^2 refers to the fit of the regression analysis from which the SCGE genotoxic potency value was calculated. All concentrations are presented in molar (M) units of measure.

Table 7. CHO cell genotoxicity of the haloacetaldehyde DBPs and related chemicals.

Compound	Lowest Genotox. Conc. (M)	R^2	SCGE Gen. Potency (M)	ANOVA Test Statistic
Chloroacetaldehyde	1.25×10^{-4}	0.98	1.59×10^{-4}	$F_{9, 58} = 56.7$; $P \leq 0.001$
Dibromoacetaldehyde	1.00×10^{-4}	0.98	1.64×10^{-4}	$F_{8, 43} = 55.7$; $P \leq 0.001$
Dichloroacetaldehyde	7.00×10^{-4}	0.96	8.83×10^{-4}	$F_{21, 88} = 31.9$; $P \leq 0.001$
Tribromoacetaldehyde	2.50×10^{-4}	0.97	3.55×10^{-4}	$F_{11, 64} = 70.99$; $P \leq 0.001$
Trichloroacetaldehyde	NS	–	NS	$F_{20, 37} = 1.2$; $P = 0.29$

NS = not statistically different from the negative control.

A comparison of the CHO cell genotoxicity for these agents is presented in Figure 12. The lowest concentration that induced a significant SCGE genotoxic response ranged from 100 µM for dibromoacetaldehyde to 700 µM for dichloroacetaldehyde. The SCGE genotoxic potency value ranged from 159 µM for chloroacetaldehyde to 883 µM for dichloroacetaldehyde (Table 7). The rank order of genotoxic potency from most to least potent was chloroacetaldehyde ≈ dibromoacetaldehyde > tribromoacetaldehyde > dichloroacetaldehyde. Trichloroacetaldehyde was not genotoxic (Figure 12).

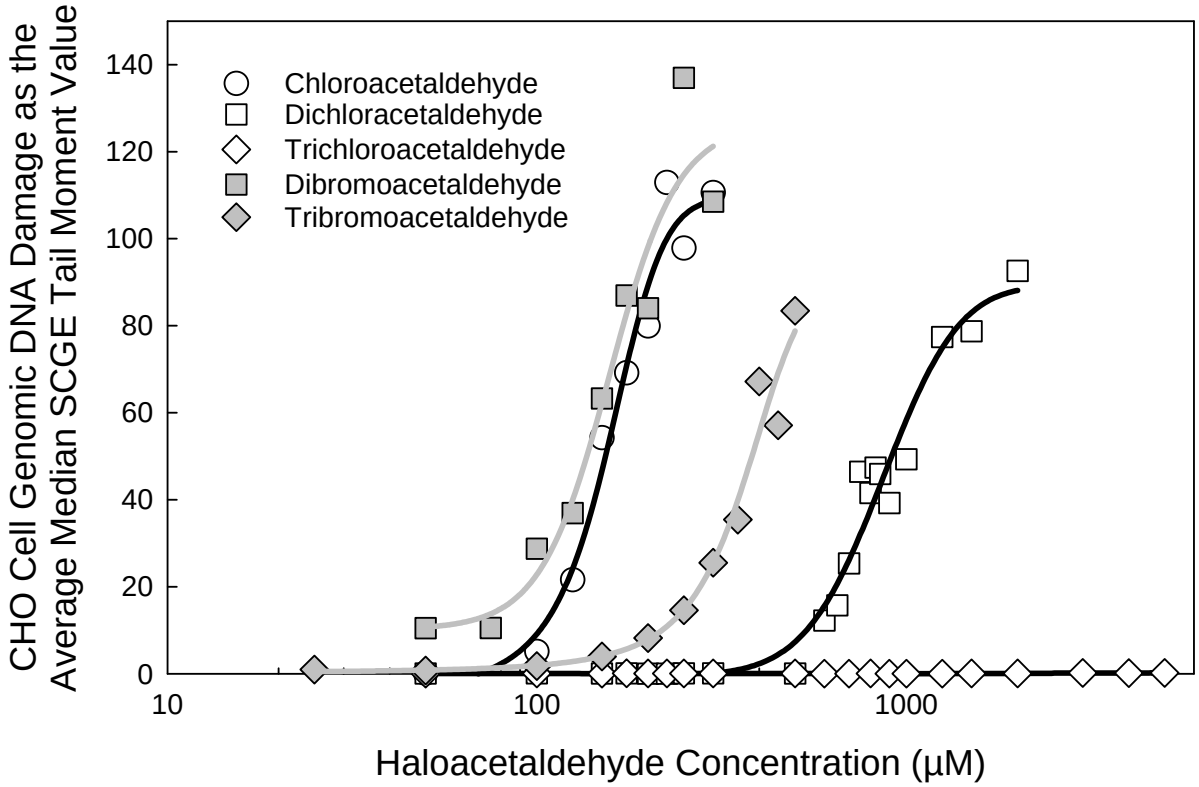

Figure 12. Comparison of the SCGE genotoxicity concentration-response curves of the haloacetaldehydes.

Conclusion

A comparative, *in vitro* analysis that measured chronic cytotoxicity and acute genomic DNA damage in CHO cells of three chemical classes of emerging DBPs was conducted. The N-DBPs (haloacetonitriles and haloacetamides) and a C-DBP class (haloacetaldehydes) were evaluated such that a rank order of their chronic cytotoxicity to CHO cells was generated. For the haloacetonitriles the cytotoxicity from most toxic to least toxic was dibromoacetonitrile > bromoacetonitrile > ≈ iodoacetonitrile > bromochloroacetonitrile > dichloroacetonitrile > chloroacetonitrile > trichloroacetonitrile. The cytotoxicity order for the haloacetamides was diiodoacetamide > iodoacetamide > bromoacetamide > tribromoacetamide > bromoiodoacetamide > dibromochloroacetamide > bromochloroacetamide > chloroiodoacetamide > bromodichloroacetamide > dibromoacetamide > chloroacetamide > dichloroacetamide > trichloroacetamide. Finally the order of cytotoxicity for the haloacetaldehydes was tribromoacetaldehyde ≈ chloroacetaldehyde > dibromoacetaldehyde > dichloroacetaldehyde >> trichloroacetaldehyde. The induction of genomic DNA damage by these DBPs was quantitatively measured using SCGE analysis. The rank order for the genotoxicity of the haloacetonitriles was iodoacetonitrile > bromoacetonitrile ≈ dibromoacetonitrile > bromochloroacetonitrile > chloroacetonitrile > trichloroacetonitrile > dichloroacetonitrile. The rank order for the genotoxicity of the haloacetamides was tribromoacetamide > diiodoacetamide

≈ iodoacetamide > bromoacetamide > dibromochloroacetamide > bromoiodoacetamide > bromodichloroacetamide > chloroiodoacetamide > bromochloroacetamide > dibromoacetamide > chloroacetamide > trichloroacetamide with dichloroacetamide not genotoxic. The genotoxic rank order for the haloacetaldehydes was chloroacetaldehyde ≈ dibromoacetaldehyde > tribromoacetaldehyde > dichloroacetaldehyde. Trichloroacetaldehyde was not genotoxic. These data demonstrate the wide range of responses by these emerging and important DBPs. The selection of these three DBP classes was based on information from the U.S. EPA DBP priority study [74] and the U.S. EPA Nationwide Occurrence Study [50]. Mammalian cell cytotoxicity and genotoxicity data provided a rank ordering of the relational toxicities of regulated and emerging DBPs and related agents both within an individual chemical class and among classes. The use of alternative disinfectants generates new DBP compounds and alters the distribution of DBP chemical classes. The water supply community will be able to consider these factors when employing alternatives to chlorine disinfection. In addition these data will be available to prioritize DBPs for future *in vivo* toxicological studies and risk assessment.

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