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The Occurrence and Toxicity of Disinfection Byproducts in European Drinking Waters in Relation with the HIWATE Epidemiology Study

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Abstract

The HIWATE (**H**ealth **I**mpacts of long-term exposure to disinfection byproducts in drinking **WATE**r) project was a systematic analysis that combined the epidemiology on adverse pregnancy outcomes and other health effects with long term exposure to low levels of drinking water disinfection byproducts (DBPs) in the European Union. The present study focused on the relationship of the occurrence and concentration of DBPs with *in vitro* mammalian cell toxicity. Eleven drinking water samples were collected from 5 European countries. Each sampling location

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SUPPORTING INFORMATION

Additional information is available in the Supporting Information on experimental methods, including additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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corresponded with an epidemiological study for the HIWATE program. Over 90 DBPs were identified; the range in the number of DBPs and their levels reflected the diverse collection sites, different disinfection processes, and the different characteristics of the source waters. For each sampling site, chronic mammalian cell cytotoxicity correlated highly with the numbers of DBPs identified and the levels of DBP chemical classes. Although there was a clear difference in the genotoxic responses among the drinking waters, these data did not correlate as well with the chemical analyses. Thus, 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. This study represents the first to integrate quantitative *in vitro* toxicological data with analytical chemistry and human epidemiologic outcomes for drinking water DBPs.

Keywords

DBPs; disinfection byproducts; occurrence; cytotoxicity; genotoxicity; drinking water; HIWATE; epidemiology

INTRODUCTION

The introduction of water disinfection greatly reduced the incidence of waterborne infectious diseases [1]. Although chlorine is the most common disinfectant, alternatives include ozone, chloramines, chlorine dioxide, and UV radiation [2–4]. An unintended consequence of disinfection is the formation of drinking water disinfection byproducts (DBPs) from the reaction between organic and inorganic materials in the water and disinfectants. Chemical classes of DBPs include halomethanes, haloacetic acids (HAAs) and nitrogen-containing DBPs (N-DBPs); to date, more than 600 DBPs have been identified in drinking water [5, 6]. The spectrum of DBP generation depends on the source water, pH, temperature, disinfection type and processes [5, 7–9]. Less than 20 DBPs are currently regulated in the United States and in other countries [6, 10].

Previous epidemiological studies reported associations between DBPs in chlorinated water and increased cancer risk [11–15] as well as DBPs and adverse pregnancy outcomes including spontaneous abortion, low birth weight (LBW), small-for-gestational-age (SGA), still birth, and preterm delivery [16–19]. HAAs were teratogenic in mice embryos [20]; mixtures of trihalomethanes (THMs) and HAAs were teratogenic in rats [21].

In 2006, the European Union (EU) established the project HIWATE (**H**ealth **I**mpacts of long-term exposure to disinfection byproducts in drinking **WATE**r) to investigate potential human health risks associated with long-term exposure to DBPs [22]. Pregnancy cohorts (N ~23,000) were included from France, Lithuania, Spain, Italy, and the United Kingdom (Table 1). These locations encompassed a variety of disinfectants and treatments including chlorine, ozone, chlorine dioxide, and desalination with reverse osmosis. Metrics for adverse pregnancy outcomes were LBW, SGA, preterm delivery, fetal growth restriction (FGR), and parameters derived from ultrasound medical diagnosis.

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This project represents the first systematic analysis combining DBP analytical chemistry and *in vitro* mammalian cell toxicology with adverse pregnancy outcomes. Our objectives were to (i) obtain disinfected drinking water from HIWATE cities, extract and concentrate the organic fraction and chemically analyze for DBPs, (ii) determine the relative chronic cytotoxicity and acute genotoxicity in mammalian cells for each HIWATE sample, and (iii) analyze for correlations between the toxicity data and the occurrence and concentrations of DBPs.

EXPERIMENTAL SECTION

Chemicals and Reagents

General reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) and Fisher Scientific Co. (Itasca, IL). Media and fetal bovine serum (FBS) were purchased from Fisher Scientific Co. (Itasca, IL). Chemical standards were purchased from Sigma-Aldrich, ChemService (West Chester, PA), Orchid Cellmark (Westminster, BC, Canada), and TCI America (Waltham, MA) at the highest level of purity.

Sample Preparation

Drinking water samples (20 L) were collected from 11 different distribution systems from 7 cities within 5 European countries, where an epidemiologic study of reproductive outcomes was being conducted. Samples were collected from March–June 2010 using 2L Teflon bottles (headspace-free) and were commercially shipped in coolers with icepacks to the U.S. Environmental Protection Agency (U.S. EPA) laboratory in Athens, GA. Water samples were extracted immediately upon arrival using XAD resins [23]. The final extract (2 mL in ethyl acetate) was equally divided for GC/MS analysis and genotoxicity/cytotoxicity analysis. For toxicity analyses the solvent ethyl acetate was evaporated with a stream of dry N_2 and exchanged to dimethylsulfoxide (DMSO) resulting in a $10^5\times$ concentration. These samples were stored in glass Supelco 1-mL Micro Reaction Vessels (No. 27036) at −20°C.

Broad-Screen GC/MS Analyses

Half of the extract was derivatized with diazomethane [24] to identify halo-acids (through their corresponding methyl esters) while the other half was analyzed directly for other DBPs. Comprehensive gas chromatography/mass spectrometer (GC/MS) analyses were performed on a high-resolution magnetic sector mass spectrometer (Autospec, Waters, Inc.) in electron ionization mode, equipped with an Agilent model 6890 gas chromatograph and operated at an accelerating voltage of 8 kV and source temperature of 200°C, in both low-resolution (1000) and high-resolution (10,000) modes. Injections of 1 μL of the extracts were introduced via a split/splitless injector (in splitless mode) onto a GC column (ZB-5, 30-m × 0.25-mm ID, 0.25-μm film thickness, Phenomenex (Torrance, CA). The GC temperature program consisted of an initial temperature of 35°C (4 min) followed by an increase at 9°C/min to 285°C (held for 30 min). Transfer lines were held at 280°C and the injection port at 250°C. To prevent decomposition of THMs, separate analyses were made with an injection port temperature of 180°C [25]. For analysis of data by the Massworks expert system [26], extracts were analyzed in the continuum mode at 1000 resolution.

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Mass spectra of unknown compounds in the drinking water extracts were subjected to library database searching (National Institute of Standards and Technology and Wiley databases). For DBPs not present in either database, high-resolution-MS and Massworks software (Cerno Bioscience, Norwalk, CT) were used to provide empirical formulas for molecular ions and fragments. Mass spectra were also interpreted extensively to provide tentative structural identifications. When possible, pure standards were obtained to confirm identifications through a match of GC retention times and mass spectra.

GC×GCTOFMS Measurements

GC×GC-time-of-flight (TOF)-MS measurements were conducted using a Leco Pegasus 4D GC×GC-TOF mass spectrometer (Leco Corp., St. Joseph, Michigan). 1 μL of the extracts was introduced via a split/splitless injector (in splitless mode). A DB-VRX (45 m, 0.25 mm i.d., 1.4 um film thickness, Agilent, Santa Clara CA) served as the primary column and a Stabilwax® (1.5 m, 0.25 mm i.d., 0.25 um film thickness, Restek, Bellefonte, PA) as the secondary column. The primary GC oven program consisted of an initial temperature of 45°C (3 min), an increase at 10°C/min to 145°C (3 min), an increase at 5°C/min to 240°C, and final hold of 20 min. The secondary GC oven was 13°C above the primary GC oven. The modulator offset was 20°C above to the primary GC oven. The modulation period was 7 s with 1.5 s hot pulse. The transfer line and source temperature were maintained at 248°C and 200°C, respectively. The MS data were acquired from *m/z* 35 to 500 at rate of 150 spectra/s in electron ionization mode.

Quantitative Chemical Analyses

THMs (chloroform, bromodichloromethane, dibromochloromethane, and bromoform), haloacetonitriles (dichloroacetonitrile, bromochloroacetonitrile, dibromoacetonitrile, and trichloroacetonitrile), haloketones (1,1-dichloro- and 1,1,1-trichloropropanone), trichloroacetaldehyde (chloral hydrate), and trichloronitromethane (chloropicrin) were extracted using a modified form of U.S. EPA Method 551.1 [27]. HAAs (chloro-, bromo-, dichloro-, trichloro-, bromochloro-, dibromo-, bromodichloro-, dibromochloro-, and tribromoacetic acid) were analyzed using a modified form of U.S. EPA Method 552.3 [28]. The limit of detection for each DBP was 1 μg/L, with the exception of chloroacetic acid (detection limit was 2 μg/L).

Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cell line AS52, clone 11-4-8 was used for the biological assays [29–31]. CHO cells were maintained on glass culture plates in Ham's F12 medium containing 5% fetal bovine serum (FBS), 1% antibiotics (100 U/mL sodium penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B in 0.85% saline), and 1% glutamine at 37°C in a humidified atmosphere of 5% CO_2.

CHO Cell Chronic Cytotoxicity Assay

This assay measures the reduction in cell density on flat-bottom 96-well microplates as a function of the concentration of the test sample over a period of approximately 72 h (~3 cell cycles) [32, 33]. Microliters of the sample in DMSO were diluted with F12 +FBS medium

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to analyze a range of concentration factors. This assay was calibrated; the detailed procedure was published [32, 33] and is presented in the Supporting Information (SI). For each HIWATE sample concentration factor, 4 replicate wells were analyzed. The experiments were repeated 2–3 times. A concentration-response curve was generated for each sample. A regression analysis was conducted with each curve. The LC_{50} (%C½) values were calculated from the regression analysis and represents the sample concentration factor that induced a 50% reduction in cell density as compared to the concurrent negative controls.

CHO Cell Single Cell Gel Electrophoresis (SCGE) Assay

Single cell gel electrophoresis (SCGE, or Comet) assay quantitatively measures genomic DNA damage in individual nuclei induced by a test agent [34–36]. We employed microplate methodology [35]; the detailed procedure is presented in the SI. The SCGE metric for genomic DNA damage induced by the HIWATE samples was the %Tail DNA value which is the amount of DNA that migrated from the nucleus into the microgel [37]. Within each concentration factor range with >70% cell viability, a concentration-response curve was generated and regression analysis was used to fit the curve. The concentration factor inducing a 50%Tail DNA value was calculated from each concentration-response curve.

Statistical Analyses

For the cytotoxicity assay, a one-way analysis of variance (ANOVA) test was conducted to determine if the HIWATE sample induced a statistically significant level of cell death at a specific concentration factor. If a significant F value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was performed to identify the lowest cytotoxic concentration factor. The power of the test statistic ($1-\beta$) was maintained as ≥ 0.8 at $\alpha = 0.05$.

For the SCGE assay, the %Tail DNA values are not normally distributed which limits the use of parametric statistics [38]. The mean %Tail DNA value for each microgel was calculated and these values were averaged among all of the microgels for each HIWATE sample concentration factor. Averaged mean values express a normal distribution according to the central limit theorem [38]. A one-way ANOVA test was conducted on these averaged %Tail DNA values [39]. If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted with the power ≥ 0.8 at $\alpha = 0.05$.

The mammalian cell cytotoxicity and genotoxicity analyses were compared with the following analytical chemical metrics: (i) the numbers of DBPs identified in each HIWATE sample, (ii) the chromatographic peak area for the entire sample, (iii) peak areas for specific classes of DBPs, (iv) the total concentration of 21 selected DBPs and, (v) concentrations of specific DBP classes within the group of 21 DBPs. A Pearson's Product Moment correlation test was conducted.

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RESULTS AND DISCUSSION

Chemical Analyses

Over 90 DBPs were identified in the samples, including several haloacids (including 3- and 4-carbon acids and di-acids), halophenols, haloamides, halonitromethanes, haloketones, haloaldehydes, and haloalkenes (Table S1, SI). Approximately 300 chromatographic peaks were observed in the original GC/MS chromatograms (Figure S1, SI;including DBPs and other compounds present in the raw waters prior to disinfection). With GC×GC-TOF-MS analyses, these peaks were resolved into >1000 peaks (Figure S2, SI). Several DBPs identified were not in mass spectral library databases and these identifications were made through the methods outlined previously utilizing Massworks software. Several new DBPs were presumptively identified, including *cis*- and *trans*-2,3-dibromo-3-chloropropenoic acid, 3,3-dibromo-2-chloropropenoic acid, and several halophenols and haloalkenes. Twenty-one target DBPs, including 4 U.S-regulated THMs, 9 HAAs, 4 haloacetonitriles (HANs), 2 haloketones (HKs), trichloroacetaldehyde (chloral hydrate), and trichloronitromethane (chloropicrin) were quantified (Table 2).

Substantial differences were observed in the DBPs from the different locations. As expected, drinking waters from coastal Spain (Barcelona and Valencia) had relatively high DBP levels with many brominated (and some iodinated) species due to higher levels of total organic carbon (TOC), bromide and iodide in their source waters (surface water), as well as the use of chlorine as a disinfectant. Drinking waters from coastal Spain averaged 90 and 33 μg/L for THM4 and HAA9, respectively (Table 2). In contrast, drinking water from Modena, Italy had fewer DBPs at much lower levels; these were primarily chlorine-containing species. The source water for Modena is a low-TOC groundwater that is treated with low chlorine dioxide doses (0.1 mg/L), which forms fewer DBPs as compared to other disinfectants [3, 40–42]. None of the 21 target DBPs were detected in the drinking water from Modena, but a few were detected in the broad screen analyses due to lower detection limits. Drinking water from other locations (samples 4, 6, 8–11, Table 1) expressed intermediate DBP levels with a mix of chloro-bromo species probably due to lower levels of bromide and TOC in their source waters as compared to waters from costal Spain (Table S1, SI and Table 2).

Of the N-DBPs [43], haloacetonitriles and haloamides were prevalent in drinking waters from coastal cities in Spain (samples 1–3, 7), which involved treatment with chlorine, alone or in combination with ozone or chlorine dioxide. Previous research demonstrated that ozonation increased the formation of halonitromethanes when used prior to chlorination or chloramination [44–46]. While chloramination increases the formation of some N-DBPs [5], none of the cities in this study employed chloramines.

CHO Cell Chronic Cytotoxicity

CHO cell chronic cytotoxicity analyses of each HIWATE sample are summarized in Table 3. The concentration factor is the fold concentration of the isolated organic material as compared to the original water. The lowest concentration factor of each sample which induced a statistically significant reduction in cell density as compared to its concurrent negative control was determined by an ANOVA test statistic. The data from replicated

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experiments were averaged and plotted (Figure 1A, Figures S3–S13, SI); regression analyses were used to calculate the LC_{50} (%C½) value for each sample. Based on the LC_{50} values, the descending rank order of chronic cytotoxicity was, sample 3 > sample 1 > sample 2 ≈ sample 4 > sample 7 > sample 10 > sample 9 > sample 8 ≈ sample 11 > sample 6 > sample 5. Samples from Barcelona, Spain were ranked as the 3 most cytotoxic. We calculated the cytotoxicity index value (LC_{50}^{-1} ×1000) for each HIWATE sample (Figure 1B, Table S2, SI).

CHO Cell Acute Genotoxicity

CHO cell acute genotoxicity analyses of each HIWATE sample are summarized in Table 4. The lowest genotoxic concentration factor was that which induced a statistically significant amount of genomic DNA damage as compared to the concurrent negative control. Figure 2A (Figures S14–S24, SI) illustrates the concentration-response curves for the HIWATE samples. Based on 50% Tail DNA values, the descending rank order of genotoxicity was, sample 10 > sample 4 > sample 7 > sample 1 ≈ sample 2 > sample 3 > sample 9 > sample 11 > sample 6 > sample 8 >> sample 5. We calculated the genotoxic index value as 50% Tail DNA^{-1} ×10^4 for each sample (Figure 2B; Table S2, SI).

Correlation of Toxicology, Chemistry and Epidemiology

To investigate correlations between DBP occurrence and DBP classes with mammalian cell toxicity, we applied a Pearson's Product Moment statistical test [38]. The cytotoxic potency index values statistically significantly correlated with the number of identified DBPs (r = 0.78; $P \leq 0.005$, Table 5) and the level of 21 target DBPs (r = 0.77; $P \leq 0.006$, Table 2). The genotoxic potency index values were not correlated with either of these metrics or with any DBP chemical class (Table 2 and Table 5). Interestingly, the cytotoxicity and genotoxicity indices indicated a good correlation (r = 0.74; $P \leq 0.009$). The cytotoxic potency index showed a good correlation with the U.S-regulated DBPs (r = 0.78; $P \leq 0.006$) and unregulated DBPs (r = 0.60; $P \leq 0.05$; Table 2).

Cytotoxicity was significantly correlated with the relative concentrations of the following DBP classes: THMs (r = 0.74; $P \leq 0.01$), haloacids (r = 0.75; $P \leq 0.008$), other monoacids (r = 0.68; $P \leq 0.021$), halodiacids (r = 0.80; $P \leq 0.003$), haloamides (r = 0.68; $P \leq 0.021$), haloaromatics (r = 0.64; $P \leq 0.035$), brominated (r = 0.68; $P \leq 0.022$), chlorinated (r = 0.78; $P \leq 0.005$), and iodinated (r = 0.82; $P \leq 0.002$) DBPs (Table 6). There were no statistically significant correlations with genotoxicity and the above DBP classes, although there were associations or trends in relationships between genotoxicity and the relative concentrations of haloacids (r = 0.54; $P \leq 0.088$), haloaromatics (r = 0.52; $P \leq 0.103$), chlorinated (r = 0.56; $P \leq 0.073$) and iodinated (r = 0.53; $P \leq 0.093$) DBPs (Table 6). It should be noted that some highly polar components might have been missed by GC/MS and this may explain, in part, the reduced correlation seen with the genotoxicity data and analytical chemistry of the water samples. Recently several papers have been published on novel methods to detect polar iodinated/brominated DBPs [47–49].

Epidemiology results on water DBPs and birth outcomes from Lithuania, Spain and France were recently published [50–52] and the present analysis included water samples from the

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geographic areas covered in those studies. An expanded discussion of the associations among the epidemiology studies and this work is presented in the Supporting Information (Table S3 SI). It should be noted, however, that the drinking water samples for the epidemiologic analyses and the current analytical chemical and toxicological evaluations were not collected at the same time. Existing epidemiological studies on birth outcomes including those in the HIWATE project, have evaluated a limited number of DBPs (usually only THMs) through environmental analyses of drinking water or, in the case of the French study [52] through an evaluation of biomarkers of haloacetic acid metabolites in urine. The analyses of water toxicity presented in this paper were limited in number due to their complexity, but they provide an overall evaluation of differences of toxicity in different geographic areas. It is the first time that this evaluation was done to specifically correspond with areas examined in epidemiological studies. Expanding the chemical and toxicological characterization of water samples may enhance the resolving power of epidemiological investigations and the evaluation of dose-response relationships. In addition, the relationship between the analytical chemistry, quantitative *in vitro* toxicology, and the epidemiology may provide additional mechanistic evidence on potential health effects of water DBPs.

This paper focused on the relationship of the occurrence and concentration of DBPs with mammalian cell toxicity. The range of the number of DBPs identified and their levels reflect the diverse collection sites, different disinfection processes, and the different characteristics of the source waters. CHO cytotoxicity was well correlated with the numbers of DBPs identified and the levels of DBP chemical classes. Although there was a clear difference in genotoxic responses, these data did not correlate well with chemical analyses of the HIWATE samples. Thus, 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.

We are continuing to compare the epidemiology with the *in vitro* toxicity and analytical chemistry analyses. Future study will investigate the possible association between chronic cytotoxicity, acute genotoxicity, multivariate comparisons of identified DBPs and epidemiology across the entire HIWATE program. We plan to compare other *in vitro* and molecular toxicity metrics and rates of adverse pregnancy measurements. Finally, we propose to determine the contribution of source water, and disinfection chemistry to the observed toxicity and epidemiology results and develop solutions to protect the public health and the environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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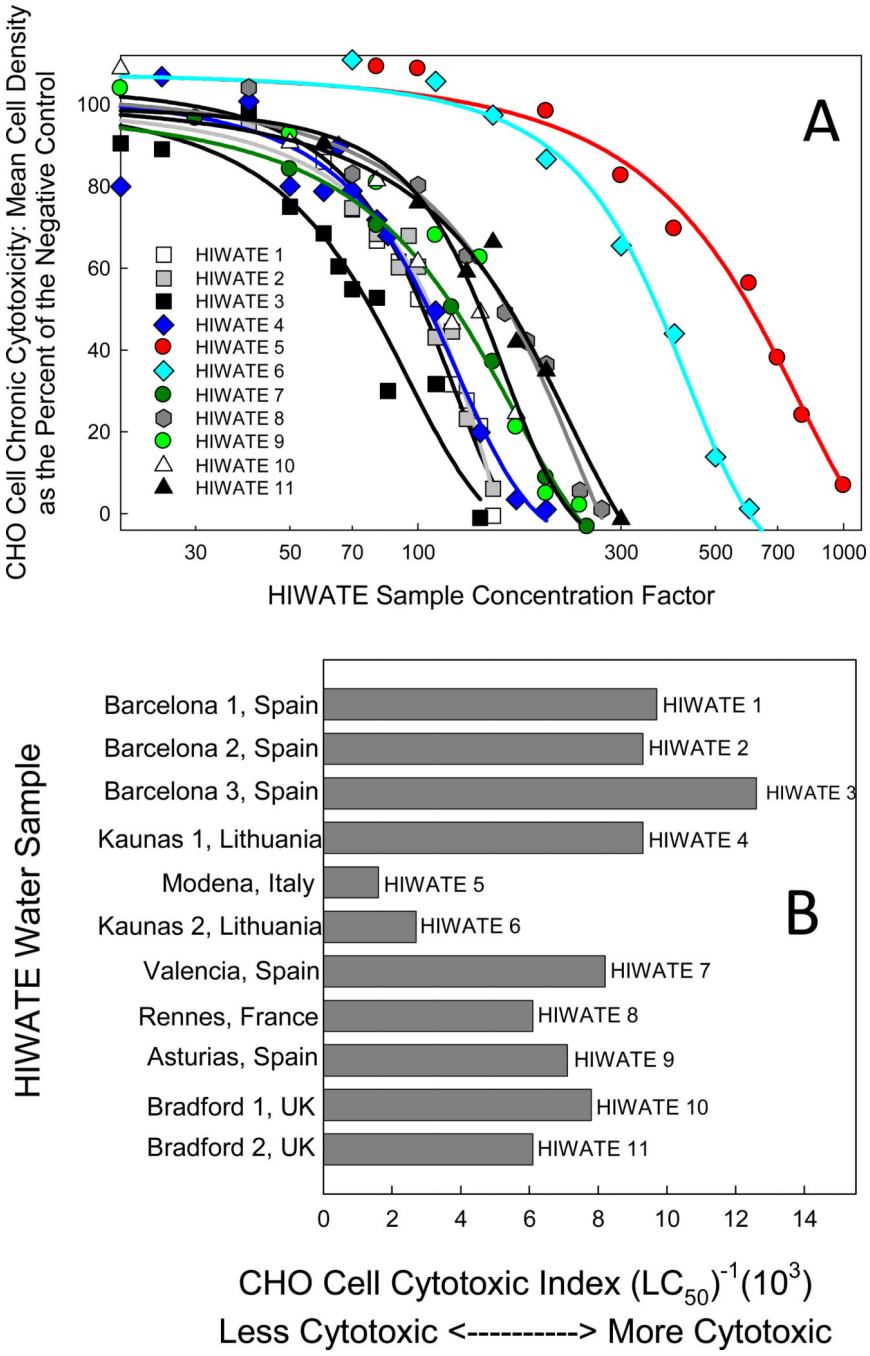

Figure 1.

(A) Log-linear plot of the concentration-response curves of 11 HIWATE samples illustrating CHO cell chronic (72-h) cytotoxicity. (B) The distributions of the CHO cell cytotoxic index values for each HIWATE sample.

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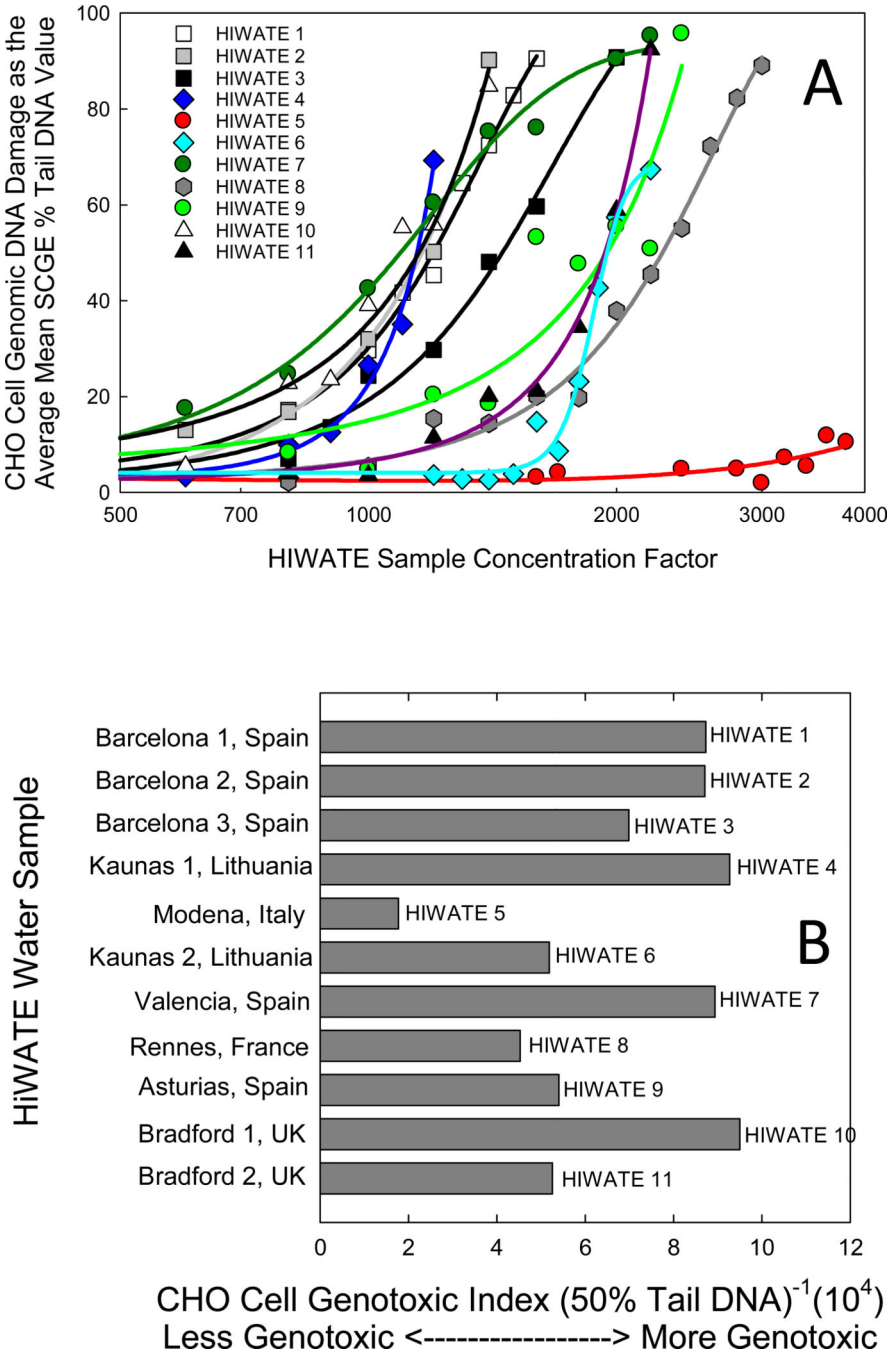

Figure 2.

(A) Log-linear plot of the concentration-response curves of 11 HIWATE samples illustrating CHO cell acute (4-h) genotoxicity. (B) The distributions of the CHO cell genotoxic index values for each HIWATE sample.

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Table 1

HIWATE water sampling locations and applied disinfection methods

Sample Number	Sampling Location (Site)	Disinfection Method
HIWATE 1	Barcelona, Spain (Badalona)	Cl_2-Cl_2
HIWATE 2	Barcelona, Spain (Hospitalet del Llobregat)	Blend of Cl_2-Cl_2, Cl_2-O_3-Cl_2, Desal-RO-ClO_2
HIWATE 3	Barcelona, Spain (Sabadell)	Blend of (ClO_2/Cl_2)-Cl_2, Cl_2-Cl_2
HIWATE 4	Kaunas, Lithuania (Petruniusai)	Cl_2
HIWATE 5	Modena, Italy	ClO_2
HIWATE 6	Kaunas, Lithuania (Viciunai)	Cl_2
HIWATE 7	Valencia, Spain	Cl_2-Cl_2
HIWATE 8	Rennes, France	O_3-Cl_2
HIWATE 9	Asturias, Spain	Cl_2
HIWATE 10	Bradford, U.K. (Shipley)	Cl_2
HIWATE 11	Bradford, U.K. (Airedale)	Cl_2

Cl_2 = chlorination, O_3 = ozonation, ClO_2 = chlorine dioxide, Desal-RO = desalination with reverse osmosis.

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Table 2

Levels of DBPs by chemical classes and correlation with CHO cell cytotoxic potency index and genotoxic potency index.

HIWATE Sample Number	21 DBPs[c] (μg/L)	4 THMs (μg/L)	9 HAAs (μg/L)	4 HANs (μg/L)	2 HKs (μg/L)	CH (μg/L)	CP (μg/L)	U.S.-Regulated DBPs (μg/L)	Unregulated DBPs (μg/L)
1	115	70.9	36.0	6.47	0.21	1.27	<LOD [d]	94.7	20.1
2	91.1	66.8	19.5	4.70	<LOD	<LOD	<LOD	77.1	13.9
3	202	139	51.5	8.88	1.11	1.80	<LOD	168	33.8
4	3.24	3.24	<LOD	<LOD	<LOD	<LOD	<LOD	3.24	<LOD
5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6	1.11	1.11	<LOD	<LOD	<LOD	<LOD	<LOD	1.11	<LOD
7	118	83.9	26.7	4.80	<LOD	2.46	<LOD	103	14.6
8	27.8	14.7	13.3	<LOD	<LOD	<LOD	<LOD	26.5	1.25
9	92.9	55.0	<LOD	4.02	6.86	23.2	3.78	55.0	37.9
10	40.6	22.6	13.3	<LOD	3.28	1.43	<LOD	29.0	11.6
11	45.2	29.3	11.6	<LOD	3.08	1.23	<LOD	36.8	8.37
Cytotoxic Potency Index[a]	$r = 0.77$ $P \leq 0.006$	$r = 0.77$ $P \leq 0.006$	$r = 0.75$ $P \leq 0.009$	$r = 0.73$ $P = 0.011$	$r = 0.04$ $P = 0.913$	$r = 0.04$ $P = 0.905$	$r = -0.02$ $P = 0.947$	$r = 0.76$ $P \leq 0.006$	$r = 0.60$ $P = 0.051$
Genotoxic Potency Index[b]	$r = 0.36$ $P = 0.273$	$r = 0.37$ $P = 0.260$	$r = 0.40$ $P = 0.221$	$r = 0.36$ $P = 0.281$	$r = -0.08$ $P = 0.827$	$r = -0.12$ $P = 0.720$	$r = -0.18$ $P = 0.600$	$r = 0.38$ $P = 0.248$	$r = 0.22$ $P = 0.521$

[a] The CHO cell cytotoxic potency index value corresponds to ($LC_{50}^{-1} \times 10^3$) for each HIWATE sample.

[b] The CHO cell genotoxic potency index value is the reciprocal HIWATE sample concentration factor that was calculated to induce a 50% SCGE tail DNA value $\times 10^4$.

[c] These 21 quantitatively measured DBPs are listed in the text.

[d] LOD = limit of detection.

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Table 3

CHO cell chronic cytotoxicity analyses of the HIWATE samples

Sample Number	Concentration Factor Range	Lowest Cytotoxic Concentration Factor [a]	LC_{50} Value [b] (Conc. Factor ±SE)	r^2 [c]	ANOVA Test Statistic [d]
HIWATE 1	0 – 150	60	102.7 ± 4.2	0.95	$F_{10, 37} = 58.4$; $P \leq 0.001$
HIWATE 2	0 – 150	70	107.8 ± 3.8	0.97	$F_{10, 37} = 59.2$; $P \leq 0.001$
HIWATE 3	0 – 300	50	79.1 ± 4.1	0.96	$F_{19, 76} = 130$; $P \leq 0.001$
HIWATE 4	0 – 300	22.5	107.5 ± 3.7	0.97	$F_{19, 76} = 134$; $P \leq 0.001$
HIWATE 5	0 – 1000	300	605.8 ± 4.3	0.98	$F_{9, 33} = 69.2$; $P \leq 0.001$
HIWATE 6	0 – 800	300	366.9 ± 4.1	0.99	$F_{10, 37} = 113$; $P \leq 0.001$
HIWATE 7	0 – 350	50	122.1 ± 3.4	0.99	$F_{11, 44} = 212$; $P \leq 0.001$
HIWATE 8	0 – 300	70	162.5 ± 4.8	0.98	$F_{10, 37} = 71.7$; $P \leq 0.001$
HIWATE 9	0 – 300	80	140.0 ± 4.9	0.98	$F_{10, 37} = 90.8$; $P \leq 0.001$
HIWATE 10	0 – 300	80	128.9 ± 4.6	0.97	$F_{11, 40} = 77.9$; $P \leq 0.001$
HIWATE 11	0 – 600	100	164.4 ± 5.2	0.98	$F_{10, 37} = 78.0$; $P \leq 0.001$

[a] Lowest cytotoxic concentration was the lowest concentration factor of the HIWATE sample in the concentration-response curve that induced a statistically significant reduction in cell density as compared to the concurrent negative controls.

[b] The LC_{50} value is the fold concentration factor of the HIWATE sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The LC_{50} error term was calculated as $\Sigma X \overline{SE}$.

[c] r^2 is the coefficient of determination for the regression analysis upon which the LC_{50} value was calculated.

[d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

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Table 4

CHO cell SCGE genotoxicity analyses of the HIWATE samples

Sample Number	Concentration Factor Range	Lowest Genotoxic Concentration Factor [a]	50% tail DNA Value [b] (Conc. Factor ±SE)	r^2 [c]	ANOVA Test Statistic [d]
HIWATE 1	0 – 1700	1000	1146 ± 9.1	0.99	$F_{9, 33} = 10.5$; $P \leq 0.001$
HIWATE 2	0 – 2000	1000	1148 ± 3.8	0.99	$F_{10, 37} = 43.6$; $P \leq 0.001$
HIWATE 3	0 – 2000	900	1430 ± 2.0	0.99	$F_{8, 37} = 133$; $P \leq 0.001$
HIWATE 4	0 – 1600	1000	1079 ± 3.3	0.99	$F_{10, 37} = 78.0$; $P \leq 0.001$
HIWATE 5	0 – 3800	3600	5659 ± 1.4	0.74	$F_{10, 38} = 3.14$; $P \leq 0.005$
HIWATE 6	0 – 2200	1600	1925 ± 2.0	0.90	$F_{11, 39} = 20.0$; $P \leq 0.001$
HIWATE 7	0 – 2200	600	1119 ± 2.9	0.95	$F_{9, 26} = 76.3$; $P \leq 0.001$
HIWATE 8	0 – 3000	2000	2206 ± 5.6	0.98	$F_{13, 50} = 9.29$; $P \leq 0.001$
HIWATE 9	0 – 2400	1600	1847 ± 6.9	0.89	$F_{10, 42} = 6.87$; $P \leq 0.001$
HIWATE 10	0 – 1600	400	1052 ± 6.2	0.98	$F_{8, 50} = 7.18$; $P \leq 0.001$
HIWATE 11	0 – 2400	1600	1901 ± 3.7	0.99	$F_{10, 33} = 15.9$; $P \leq 0.001$

[a] The lowest genotoxic concentration was the lowest concentration of the HIWATE sample in the concentration-response curve that induced a statistically significant amount of genomic DNA damage as compared to the negative control.

[b] The SCGE 50% Tail DNA value is the HIWATE sample concentration factor determined from a regression analyses of the data that was calculated to induce a 50% SCGE Tail DNA value. The 50% SCGE Tail DNA value error term was calculated as as $\Sigma\bar{X}_{SE}$.

[c] r^2 is the coefficient of determination for the regression analysis upon which the SCGE % Tail DNA value was calculated.

[d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

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Table 5

Description of each HIWATE sample, DBPs identified and gross correlation with the rank order of CHO cell cytotoxicity and genotoxicity

Sample Number	Number of Identified DBPs	Rank order of Number of Identified DBPs	Rank order of Cytotoxic Potency Index [a]	Rank order of Genotoxic Potency Index [b]
HIWATE 1	86	1	2	4
HIWATE 2	76	5	3	5
HIWATE 3	85	2	1	6
HIWATE 4	41	7	3	2
HIWATE 5	13	11	11	11
HIWATE 6	18	10	10	9
HIWATE 7	83	3	5	3
HIWATE 8	77	4	8	10
HIWATE 9	45	6	7	7
HIWATE 10	41	7	6	1
HIWATE 11	40	9	8	8

Correlation with the rank order of CHO cell cytotoxicity: $r = 0.78$ ($P \leq 0.005$). Correlation with the rank order of CHO cell genotoxicity: $r = 0.52$ ($P = 0.105$). Rank order where 1 is the highest response and 11 is the lowest response.

[a] The CHO cell cytotoxic potency index value is in arbitrary units and the value corresponds to ($LC_{50}^{-1} \times 10^3$) for each HIWATE sample.

[b] The CHO cell genotoxic potency index value is the reciprocal HIWATE sample concentration factor that was calculated to induce a 50% SCGE tail DNA value $\times 10^4$ and is presented in arbitrary units.

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Table 6

Pearson Product Moment correlation analyses of the relative concentrations of each DBP group versus CHO cell chronic cytotoxicity or acute genotoxicity

Relative Concentration of DBP Class [a]	**Cytotoxic Potency Index Value** [b] **($LC_{50}^{-1} \times 10^3$)**	**Genotoxic Potency Index Value** [c] **(50% tail $DNA^{-1} \times 10^4$)**
THMs	$r = 0.74$ $P \leq 0.010$	$r = 0.45$ $P = 0.164$
Haloacids	$r = 0.75$ $P \leq 0.008$	$r = 0.54$ $P = 0.088$
Other monoacids	$r = 0.68$ $P \leq 0.021$	$r = 0.42$ $P = 0.201$
Halodiacids	$r = 0.80$ $P \leq 0.003$	$r = 0.40$ $P = 0.221$
Haloamides	$r = 0.68$ $P \leq 0.021$	$r = 0.45$ $P = 0.170$
Haloaromatics	$r = 0.64$ $P \leq 0.035$	$r = 0.52$ $P = 0.103$
Brominated DBPs	$r = 0.68$ $P \leq 0.022$	$r = 0.46$ $P = 0.154$
Chlorinated DBPs	$r = 0.78$ $P \leq 0.005$	$r = 0.56$ $P = 0.073$
Iodinated DBPs	$r = 0.82$ $P \leq 0.002$	$r = 0.53$ $P = 0.093$

[a] Relative concentration is defined as the integrated area for each chromatographic peak summed for each DBP chemical class.

[b] The CHO cell cytotoxic potency index value corresponds to ($LC_{50}^{-1} \times 10^3$) for each HIWATE sample.

[c] The CHO cell genotoxic potency index value is the reciprocal HIWATE sample concentration factor that was calculated to induce a 50% SCGE tail DNA value $\times 10^4$.