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Analytical and Toxicity Characterization of Halo-hydroxyl-benzoquinones as Stable Halobenzoquinone Disinfection Byproducts in Treated Water

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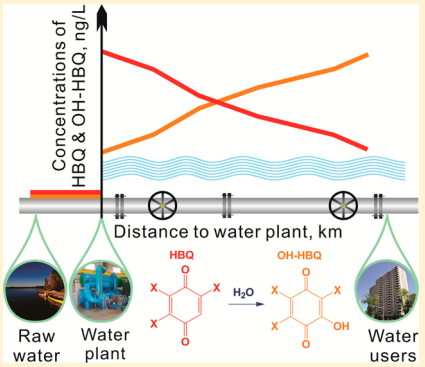
Supporting Information

ABSTRACT: Exposure to chlorination disinfection byproducts (DBPs) is potentially associated with an increased risk of bladder cancer. Four halobenzoquinones (HBQs) have been detected in treated drinking water and have shown potency in producing reactive oxygen species and inducing damage to cellular DNA and proteins. These HBQs are unstable in drinking water. The fate and behavior of these HBQs in drinking water distribution systems is unclear. Here we report the high-resolution mass spectrometry identification of the transformation products of HBQs as halo-hydroxyl-benzoquinones (OH-HBQs) in water under realistic conditions. To further examine the kinetics of transformation, we developed a solid-phase extraction with ultrahigh-performance liquid chromatography tandem mass spectrometry (SPE−UHPLC−MS/MS) method to determine both the HBQs and OH-HBQs. The method provides reproducible retention times (SD < 0.05 min), limits of detection (LODs) at subnanogram per liter levels, and recoveries of 68%−96%. Using this method, we confirmed that decrease of HBQs correlated with increase of OH-HBQs in both the laboratory experiments and several distribution systems, supporting that OH-HBQs were more stable forms of HBQ DBPs. To understand the toxicological relevance of the OH-HBQs, we studied the in vitro toxicity with CHO-K1 cells and determined the IC_{50} of HBQs and OH-HBQs ranging from 15.9 to 72.9 μM. While HBQs are 2-fold more toxic than OH-HBQs, both HBQs and OH-HBQs are substantially more toxic than the regulated DBPs.



Disinfection of drinking water is essential to prevent waterborne disease.[1] However, disinfection byproducts (DBPs) are unintentionally produced from the reactions of disinfectants (e.g., chlorine) with natural organic matter (NOM) in water.[2] Epidemiological studies have found a potential association between exposure to chlorination DBPs and adverse health effects, primarily as an increased risk of bladder cancer.[3] Animal toxicological studies indicate that the current regulated DBPs may not account for the observed risk of bladder cancer.[4] It is likely that more toxicologically relevant DBPs may exist in drinking water but have not yet been identified.[5]

Halobenzoquinones (HBQs) are a class of DBPs that are of toxicological relevance and likely to be carcinogenic.[5] These compounds are predicted to be 10 000 times more toxic than the regulated DBPs such as chloroform based on their lowest observed adverse effect levels (LOAEL).[5] Our research group discovered some HBQs as DBPs in drinking water and swimming pool water.[6−8] Cytotoxicity studies have shown that the four commonly occurring HBQs are highly cytotoxic to T24 bladder cancer cells and can damage DNA and proteins.[9,10]

When DBPs are formed, some will undergo spontaneous transformation reactions in the drinking water treatment plant (WTP) and distribution system (WDS).[11,12] The DBP species and concentrations in the water arriving at customers' taps may be different from when the water originally left the WTP.[13,14] The transformation process can largely affect human exposure and health risk of these DBPs.[15,16] Our previous studies have shown that HBQs are not stable at neutral pH or after exposure to UV irradiation.[7,17] However, the transformation processes of HBQs and their transformation products have not been identified or shown to exist in WTPs and WDSs.

Benzoquinone derivatives (BQs) can undergo spontaneous reactions both in the environment and within living organisms.[18−22] BQs in water can undergo redox, photochemical, and nucleophilic reactions to produce products such as semiquinones, benzene-1,2,4-triols, hydroquinones, and hydroxyl-quinones.[23−28] On the basis of the chemical properties of BQ, we hypothesize that HBQs can undergo oxidation reactions to form halo-hydroxyl-benzoquinones (OH-HBQs) in water and that these OH-HBQs are a stable form of HBQ DBPs in drinking water. To confirm this hypothesis, we

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conducted studies (1) to identify the transformation products of HBQs to elucidate the transformation pathways, (2) to confirm whether OH-HBQs exist as DBPs in drinking water samples, and (3) to demonstrate the toxicological relevance of OH-HBQs.

To identify transformation products, we used high-resolution triple quadrupole time-of-flight (QTOF) mass spectrometry to obtain the mass spectra, accurate mass measurements, and tandem mass spectra of the products. To quantitatively examine the transformation kinetics and determine the products in laboratory reactions, we developed a solid-phase extraction with ultrahigh-performance liquid chromatography tandem mass spectrometry (SPE−UHPLC−MS/MS) method using triple quadrupole ion-trap (QTRAP) mass spectrometry. We further confirmed these products in the field samples. Finally, we evaluated the in vitro toxicity of both the HBQs and the OH-HBQs to elaborate the toxicological relevance of the transformation products.

MATERIALS AND METHODS

Chemicals and Solvents. 2,6-Dibromo-(1,4)-benzoquinone (DBBQ) was purchased from Indofine Chemical Company (Hillsborough, NJ). 3,5-Dichloro-2-methyl-(1,4)-benzoquinone (DCMBQ) and 2,3,6-trichloro-(1,4)-benzoquinone (TriCBQ) were synthesized by Shanghai Acana Pharmtech (Shanghai, China); 2,6-dichloro-(1,4)-benzoquinone (DCBQ) was purchased from Sigma-Aldrich (St. Louis, MO). Chemical structures and molecular weights of these HBQs are listed in Supporting Information Table S1. 3-Hydroxyl-2,6-dichloro-(1,4)-benzoquinone (OH-DCBQ), 5-hydroxyl-2,3,6-trichloro-(1,4)-benzoquinone (OH-DCMBQ), 5-hydroxyl-2,3,6-trichloro-(1,4)-benzoquinone (OH-TriCBQ), and 3-hydroxyl-2,6-dibromo-(1,4)-benzoquinone (OH-DBBQ) were synthesized in our laboratory by dissolving solid DCBQ, DCMBQ, TriCBQ, and DBBQ in Optima water for 12 h at 4 °C, respectively. The purity and identity of the synthesized compounds were assessed using UHPLC−MS analysis. Only one peak was observed in each chromatogram, and isotope patterns confirmed the peak as OH-HBQ. Water (Optima LC/MS grade; the grade means that the solvent goes through 0.03 μm filtration, and the purity is confirmed by UHPLC-UV and HPLC−MS detection), methanol (Optima LC/MS grade), and hydrochloric acid (ACS grade) were purchased from Fisher Scientific (Nepean, ON). Formic acid (HPLC grade, 50% in water) was purchased from Fluka.

Liquid Chromatography−Mass Spectrometry Analysis. A liquid chromatography system (UHPLC, Agilent 1290 Infinity Quaternary LC series) was applied with a Luna C18(2) column (100 mm × 2.0 mm i.d., 3 μm; Phenomenex, Torrance, CA) at room temperature to separate the HBQs and their transformation products. The mobile phase consisted of solvent A (0.1% FA in water) and solvent B (0.1% FA in methanol) with a flow rate of 0.17 mL/min. A gradient program was performed as follows: linearly increased B from 20% to 90% in 20 min; kept B at 90% for 5 min; changed B to 20% for column equilibration at 25.1−30 min. The sample injection volume was 20 μL.

Accurate mass measurements and isotopic patterns were obtained with a quadrupole time-of-flight mass spectrometer (AB SCIEX TripleTOF 5600, AB SCIEX, Concord, ON, Canada) to identify transformation products of the four HBQs. The conditions of the TripleTOF mass spectrometry experiments were as follows: negative ionization mode; ion source voltage, −4500 V; gas I, 60 arbitrary units; gas II, 60 arbitrary units; curtain gas, 25 arbitrary units; source temperature, 450 °C; declustering potential (DP), −90 V; collision energy (CE), −40 V; accumulation time, 0.25 s; scan range, m/z 100−1000. The information dependent acquisition (IDA) was utilized to obtain MS/MS spectra. The MS scan range of IDA was m/z 100−700, and the collision energy spread (CES) was 10 V. The accurate masses of HBQs were set in the inclusion list to track the peaks of HBQs at all times.

Multiple-reaction monitoring (MRM) methods were performed using a triple quadrupole ion-trap tandem mass spectrometer (AB SCIEX QTRAP 5500, Concord, ON, Canada) for the quantification of the four HBQs and their transformation products. The optimized MS instrumental parameters were as follows: ion-spray voltage, −4500 V; source temperature, 450 °C; gas I, 50 arbitrary units; gas II, 60 arbitrary units; curtain gas, 30 arbitrary units; entrance potential, −10 V; accumulation time for each ion pair, 100 ms. The MRM ion pairs and the optimized values of DP, CE, and cell exit potential (CXP) are listed in Supporting Information Table S3. Analyst and PeakView (AB SCIEX) software were used for data analysis. The method confirmed the identity of the peak by matching the relative ratio of two specific parent−product ion pairs and quantified it by the peak area of one ion pair of higher intensity.

Sample Collection and Solid-Phase Extraction. Water samples were collected from defined locations of five WTPs and WDSs, including source water, water plant effluent, and tap water in the distribution systems of different water ages (halfway, maximum distance). Some samples were also collected from locations that showed high concentrations of regulated DBPs. Water samples were stored in amber bottles which were precleaned three times by water and methanol that are HBQ-free. Formic acid (0.25%, v/v) was added to the samples immediately after collection to quench chlorine residual and stabilize HBQs.[8,29] The samples were transported back to our laboratory in coolers with ice packs and analyzed immediately on arrival. The time between collection and analysis was within 2 days.

The water samples were extracted for the HBQs and OH-HBQs using Waters Oasis HLB cartridges (6 mL, 200 mg per cartridge; Milford, MA). The solid-phase extraction (SPE) method was improved upon the previous one for the four HBQs.[7] The details of the SPE procedures for HBQs and OH-HBQs are described in the Supporting Information.

Quality Control and Quality Assurance. A travel-blank sample (500 mL of Optima water, 0.25% FA) was included in each sampling trip. Two SPE-blank samples (500 mL of Optima water, 0.25% FA) were extracted along with other water samples in each batch of SPE. Analysis-blank samples (500 μL, 20% methanol, 80% water, 0.25% FA) were analyzed between every five samples. These blank samples were analyzed to examine whether contamination occurred during sampling, pretreatment, or analysis. Triplicate extractions and triplicate runs of each extract were performed for each water sample to determine the average concentration and standard error. Recoveries and matrix effects of individual analytes were determined from the spiked water samples.

Cell Culture and Cytotoxicity Testing. The CHO-K1 (Chinese hamster ovary, CCL-61, ATCC, Manassas, VA) cell line was chosen to evaluate the toxicity of HBQs and OH-HBQs. This cell line is widely used in DBP toxicity studies, so comparisons can be readily made. The cells were cultured in

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(1:1) DMEM/F12 media (Gibco), 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON, Canada), and 1% penicillin−streptomycin (Invitrogen, Carlsbad, CA) and maintained at 37 °C, 5% CO_2, 90% humidity. The cytotoxicity of HBQs and OH-HBQs was examined using the real-time cell electronic sensing (RT-CES) system (ACEA Biosciences, San Diego, CA). The RT-CES experimental details are described in the Supporting Information.

RESULTS AND DISCUSSION

Figure 1 shows the typical total ion chromatograms (TIC) of four HBQs in freshly prepared solution (red), and solutions stored for 3 h (blue) and 12 h (purple), and the blank (black) at 4 °C. The samples were solid standards dissolved in pure water (Optima LC/MS grade). Before analysis, methanol was added to adjust the ratio of methanol in the sample to 20%. A new peak was clearly detected in the 3 h old HBQ solution, suggesting a product of HBQ degradation in water. After 12 h, HBQs were completely undetectable and only one peak (new) corresponding to individual HBQs was detected. We carefully examined the accurate mass (Supporting Information Table S2) and isotope ratios of the new peaks obtained from IDA analysis (Figure 2). A search using PeakView matched the new peak in DCBQ solution with OH-DCBQ. We then used the same procedures to examine DCMBQ, TriCBQ, and DBBQ. As shown in Figure 2, the accurate mass and isotope patterns of the new peaks in the individual solutions of DCMBQ, TriCBQ, and DBBQ in water correspond to OH-DCMBQ, OH-TriCBQ, and OH-DBBQ, respectively. The mass accuracy for the most abundant isotope of the four OH-HBQs was 0.4, 0.8, 0, and 0.2 ppm for the four OH-HBQs, respectively (Figure 2). Supporting Information Figure S1 shows the extracted ion chromatograms (XIC) of OH-DCBQ, OH-DCMBQ, OH-TriCBQ, and OH-DBBQ produced in the DCBQ, DCMBQ, TriCBQ, and DBBQ solutions, respectively, over 12 h storage time. The formation of OH-DCBQ, OH-DCMBQ, OH-TriCBQ, and OH-DBBQ (Supporting Information Figure S1A−D) increased as the solutions of DCBQ, DCMBQ, TriCBQ, and DBBQ aged, respectively.

Figure 2 shows that the OH-HBQ compounds form $[M - H]^{-\bullet}$, $[M]^{-\bullet}$, and $[M + H]^{-\bullet}$ as the major ions with negative electrospray ionization (ESI). $[M - H]^{-\bullet}$ ion was the most abundant for OH-DCBQ and OH-DBBQ, while $[M + H]^{-\bullet}$ was the most abundant for OH-DCMBQ and OH-TriCBQ under the optimized conditions. The possible ionization pathways are described in Supporting Information Figure S2. The formation of $[M + H]^{-\bullet}$ of OH-HBQs is similar to the ESI pathways of the HBQs that was previously reported.[29] The $[M + H]^{-\bullet}$ ions can be explained by two possible processes: one is direct addition of two electrons and one proton; the other is via two steps: OH-HBQ first undergoes transformation to hydroxyl-halodihydroquinone (OH-HDHQ), which then loses one proton. OH-HBQs also form $[M - H]^{-\bullet}$ ions, which are rarely observed from the ionization of HBQs. This finding could be explained by the ionization of the hydroxyl groups. There were also minor $[M]^{-\bullet}$ ions observed in the mass spectra, which may be produced by direct ionization via addition of an electron.

Having synthesized and confirmed the OH-HBQs, we aimed to confirm the existence of these compounds in the field samples. To achieve this, we developed a UHPLC−MS/MS method to determine both the OH-HBQs and the parent HBQs. Two pairs of transition ions were used in the MRM

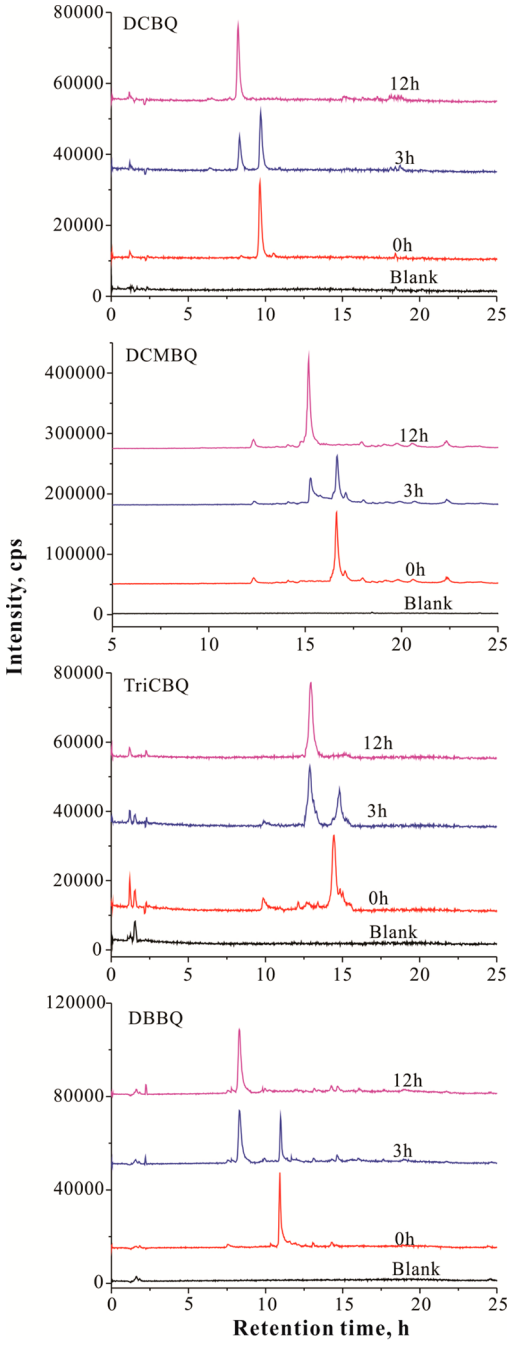

Figure 1. Total ion chromatograms of blank solution (only solvent, black) and (A) DCBQ, (B) DCMBQ, (C) TriCBQ, and (D) DBBQ in freshly prepared (red), 3 h old (blue), and 12 h old (purple) solution. The mass scan range was m/z 100−1000.

measurements, and the MS instrumental parameters were optimized. Supporting Information Table S3 describes the optimized MRM conditions. The baseline separation of the eight OH-HBQs and HBQs was achieved using a C18 reversed-phase LC.

We also optimized the UHPLC and ionization conditions. Formic acid in the mobile phase can stabilize HBQs, while the addition of weak acid in the mobile phase may suppress the signal of negative electrospray ionization.[30] On the basis of the signals of HBQs and OH-HBQs (Supporting Information

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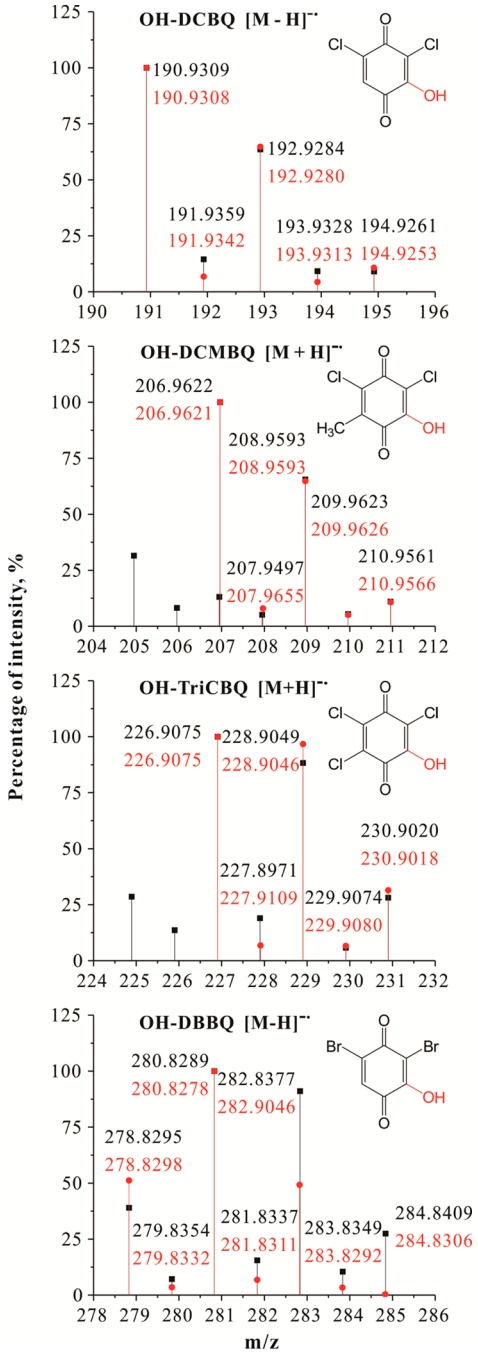

Figure 2. Isotope patterns of four OH-HBQs produced in the solutions of HBQs. The red trace is the theoretical value and the black trace is the measured value.

Figure S3), we used the formic acid concentration in the mobile phase at 0.1% in the MRM quantification methods.

Supporting Information Figure S4 shows typical UHPLC−MS/MS (MRM) chromatograms obtained from analysis of the four HBQs and four OH-HBQs. The identification of the target compounds was based on the following criteria: retention times are identical for the two ion transitions of the specific compound and relative intensity (ratio) of these two ion transitions detected in the samples is consistent with that in the standard solutions. When an HBQ was identified, the ion transition with higher abundance (the first ion pair listed in Supporting Information Table S3 for each compound) was used for quantification. The UHPLC−MS/MS method was validated for analysis of the four HBQs and four OH-HBQs in tap water. The detection limit of the UHPLC−MS/MS method is 0.01−0.7 ng/mL (Supporting Information Table S4, LOD1).

The concentrations of HBQs in water have been previously reported to be around several nanograms per liter to several hundred nanograms per liter.[7,8] Therefore, it is necessary to concentrate the compounds from water samples prior to the UPHLC−MS/MS analysis. No SPE method for OH-HBQs was available; thus, we developed one to concentrate these compounds as well as HBQs. The SPE procedures were optimized, including conditioning of the cartridge, loading of the sample, washing/cleaning, and elution of the analytes. The retention of analytes is dependent on the washing solvent and elution solvent.[31,32] On the basis of our LC separation (Supporting Information Figure S4), methanol is suitable to elute the HBQs and OH-HBQs. The optimized elution condition was 10 mL of methanol (0.25% FA, v/v). The washing step was optimized to remove the interference matrixes and retain the desired analytes. The optimized SPE procedures and recoveries are presented in Supporting Information Figure S5.

To validate the SPE−UHPLC−MS/MS conditions, we examined the retention time, limit of detection (LOD), limit of quantitation (LOQ), recovery, and matrix effect. Supporting Information Table S4 presents the performance of the method: repeatable retention time (SD < 0.05 min), subnanogram per liter LOD (LOD2, 0.02—0.8 ng/L) and LOQ (0.07—2.8 ng/L), and recovery (68—96%). Even after SPE, the matrix effect (79—98%) persisted; therefore, the standard addition method was used for quantification of these compounds in authentic water samples.

Having established a SPE−UHPLC−MS/MS method for both HBQs and OH-HBQs, we were able to quantitatively study the transformation of HBQs to OH-HBQs. Figure 3 presents the time course of HBQs converting to OH-HBQs over 24 h after fresh preparation of an HBQ solution. As the concentrations of HBQs decrease, the concentrations of OH-HBQs increase accordingly. After 12 h, the reaction reached equilibrium. The mass balance (sum) of HBQs and OH-HBQs was maintained around 80%−120%. OH-HBQs were stable for 60 h at the initial pH 7 and initial concentration 50 μg/mL (Supporting Information Figure S6), indicating that OH-HBQs are much more stable than HBQs in water.

We further investigated the presence of OH-HBQs in the field water systems where the HBQs were determined. Using the method for the four HBQs and four OH-HBQs, we analyzed water samples from five WTPs and WDSs. The water treatment processes of the five WTPs are summarized in Supporting Information Table S5. Supporting Information Figure S7 demonstrates that OH-HBQs are present in the treated tap water but not in the source water. To further confirm the presence of OH-HBQs, we investigated the occurrence frequency and concentrations of OH-HBQs in these samples as summarized in Table 1. OH-DCBQ was the most commonly identified OH-HBQs of the four OH-HBQs tested, which was consistent with DCBQ being the most frequently detected HBQ. The concentrations of HBQs and OH-HBQs in each water sample are summarized in Supporting Information Table S6. The samples containing HBQs were confirmed to also contain OH-HBQs, suggesting that the transformation of HBQs to OH-HBQs may occur in the WDSs. To further examine this, we investigated the distribution system

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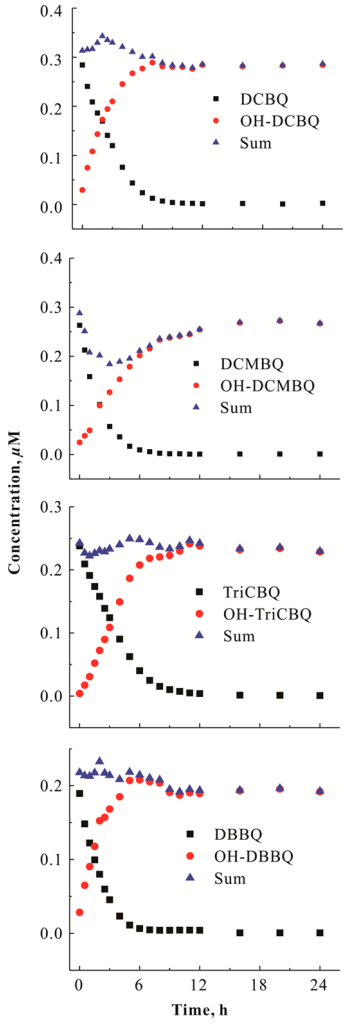

Figure 3. Time course of HBQs to OH-HBQs. Initial HBQ solution was 50 μg/mL at pH 4.5 and was maintained at 4 °C.

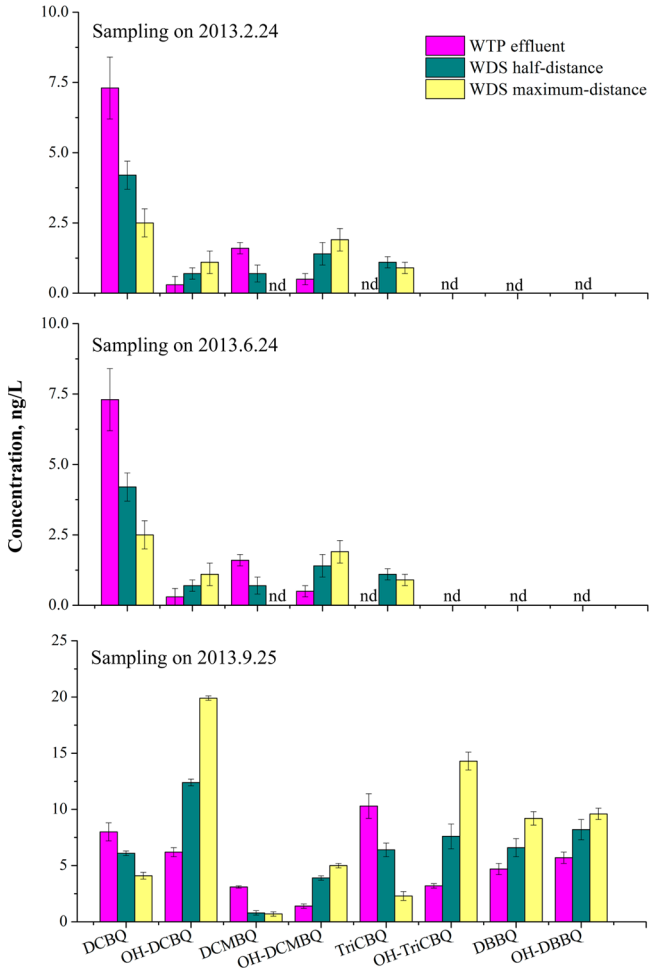

Figure 4. Concentrations of HBQs and OH-HBQs in Plant 1 (sampled on 2013.2.24, 6.24 and 9.25). nd: not detected; the concentration is lower than the detection limit.

of Plant 1. A set of samples were collected from different locations and analyzed for both HBQs and OH-HBQs. The results (Figure 4) show that the concentrations of HBQs in water samples decreased while OH-HBQs increased with the increasing distance from the WTP. Repeated samplings show the same trend in Plant 1 (Figure 4). Similar results were found in Plant 2 WDS where DCBQ was detected (Supporting Information Figure S8). Both laboratory experiments and the field study supported that the decrease of HBQs were correlated with the increase of OH-HBQs.

To assess the health relevance of OH-HBQs as DBPs, we evaluated the toxicity of OH-HBQs compared with that of HBQs. We used the CHO-K1 cells with the RT-CES method (details in the Supporting Information) that has been established in our laboratory.[33,34] Figure 5 illustrates the temporal response and IC_{50} of CHO-K1 cells exposed to DCBQ (as a representation of the tested eight compounds). Each well was dosed with the tested compounds when the cell index (CI) was 1 (after about 20 h of growth). The cells displayed concentration-dependent response curves. At any given time point, as the concentration of DCBQ increased, the normalized CI decreased, demonstrating a concentration-dependent cytotoxic effect on CHO-K1 cells. Cytotoxic responses of OH-DCBQ, DCMBQ, OH-DCMBQ, TriCBQ, OH-TriCBQ, DBBQ, and OH-DBBQ are similar to those in Figure 5.

On the basis of the temporal cytotoxicity profile, we calculated the IC_{50} values for each compound on CHO-K1 cells, as represented by the IC_{50} histogram for DCBQ in Figure

Table 1. Occurrence Frequency and Concentrations of the Four HBQs and OH-HBQs in Treated Water Collected from Five Water Treatment Plants[a]

compd	frequency	concn (ng/L)	compd	frequency	concn (ng/L)
DCBQ	34/37	nd–20	OH-DCBQ	34/37	nd–20
DCMBQ	11/37	nd–4	OH-DCMBQ	12/37	nd–7
TriCBQ	10/37	nd–20	OH-TriCBQ	6/37	nd–20
DBBQ	6/37	nd–10	OH-DBBQ	6/37	nd–10

[a]Note: nd, not detected. The concentration is lower than the detection limit.

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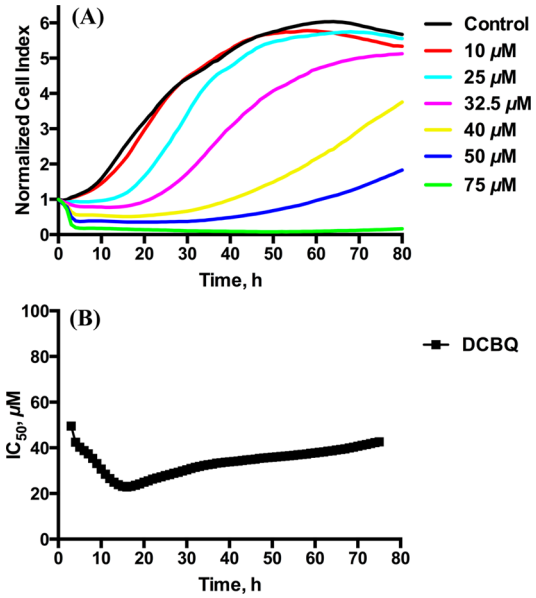

Figure 5. RT-CES profile (A) and IC_{50} histogram (B) of DCBQ on CHO-K1 cells.

5(B). The 24, 48, and 72 h IC_{50} values for all eight compounds are summarized in Table 2. Generally, the IC_{50}'s HBQs and OH-HBQs are at micromole per liter level. The IC_{50}'s of trihalomethanes (THMs) on CHO cells (72 h) were reported at the range of 3.96−11.5 mM,[35] and IC_{50}'s of haloacetic acids (HAAs) were at the range of 8.90 μM to 17.52 mM.[36] (The data are summarized in Supporting Information Table S7.) The IC_{50}'s of HBQs and OH-HBQs (16−91 μM) are much lower (more toxic) than most of the regulated DBPs, indicating that chronic cytotoxicity of HBQs and OH-HBQs are significantly higher than the regulated DBPs. The IC_{50}'s of the eight compounds are in the order DCMBQ < DBBQ < OH-DCMBQ < DCBQ < OH-DBBQ < TriCBQ < OH-DCBQ < OH-TriCBQ. Comparing HBQ and OH-HBQ in pairs, the IC_{50}'s are DCBQ < OH-DCBQ, DCMBQ < OH-DCMBQ, TriCBQ < OH-TriCBQ, and DBBQ < OH-DBBQ, indicating that the addition of the hydroxyl group to HBQ decreases the cytotoxicity and that the transformation process is partially a detoxifying process. The IC_{50} values of the four HBQs are DCMBQ < DBBQ < DCBQ < DBBQ, and those of the four OH-HBQs are OH-DCMBQ < OH-DBBQ < OH-DCBQ < OH-DBBQ, i.e., the same order. This indicated that the basic HBQ structure likely plays a key role in determining the toxicity of the corresponding OH-HBQ.

Table 2. IC_{50} Values of the Four HBQs and Four OH-HBQs on CHO-K1 Cells[a]

	IC_{50} (μM)		
compd	24 h	48 h	72 h
DCBQ	27.3 ± 1.0	35.5 ± 1.0	41.5 ± 1.3
OH-DCBQ	61.0 ± 3.0	69.5 ± 5.6	90.6 ± 33.6
DCMBQ	11.4 ± 0.5	13.7 ± 0.5	15.9 ± 0.9
OH-DCMBQ	20.4 ± 0.6	21.5 ± 0.8	24.0 ± 1.1
TriCBQ	45.5 ± 2.5	63.7 ± 2.1	72.9 ± 3.6
OH-TriCBQ	64.4 ± 3.7	67.1 ± 4.7	69.8 ± 6.8
DBBQ	19.8 ± 1.5	29.2 ± 1.8	35.5 ± 0.7
OH-DBBQ	42.8 ± 6.2	51.8 ± 3.4	50.4 ± 7.6

[a]Note: IC_{50} is the concentration of the compound determined from a regression analysis of the data (Figure 5), at which cell index is reduced to 50% of the negative control.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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