STRUCTURE CONFIRMATION, REACTIVITY, BACTERIAL MUTAGENICITY AND QUANTIFICATION OF 2,2,4-TRIBROMO-5-HYDROXYCYCLOPENT-4-ENE-1,3-DIONE IN DRINKING WATER

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Abstract

Non-target chemical analysis of drinking water revealed the presence of two new disinfectant by-product (DBP) groups in the UK, halogenated-hydroxycyclopentenediones and halogenated-methanesulfonic acids. We unequivocally identified 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione (TBHCD), and quantified it together with dibromomethanesulfonic acid at 122±34 and 326±157 ng L^{-1} in London drinking water, respectively (n=21). We found TBHCD to be photolabile and unstable in tap water and at alkaline pH. Furthermore, spectral and computational data for TBHCD and three other halogenated-hydroxycyclopentenediones indicated they could act as a source of radicals in water and in the body. Importantly, TBHCD was calculated to have a 14.5 kcal mol^{-1} lower C-Br bond dissociation enthalpy than the N-Br bond of *N*-bromosuccinimide, a common radical substitution reagent used in organic synthesis. TBHCD was mutagenic in Salmonella/microsome assays using strains TA98, TA100 and TA102. This work reveals the unique features, activity and toxicity of trihalogenated hydroxycyclopent-4-ene-1,3-diones, prompting a need to more comprehensively assess their risks.

Introduction

A steadily increasing amount of anthropogenic chemical substances are produced worldwide,[1,2] generating an increasing potential for drinking water contamination. Environmental factors are assumed to play a major role in chronic diseases, and yet comprehensive evaluation of environmental exposures and relationship to disease is far from being achieved.[3] In fact, the number of substances analysed by target analysis is limited to a very small fraction of the substances potentially present.[4] The investigation of previously unknown potentially toxic compounds in drinking water is a long and resource-intensive

process. Non-target analysis (NTA) can generate tentative identifications, but these are rarely followed-up by structure confirmation or *in silico* toxicity prediction. For most substances, reference materials are not commercially available and synthesis is required for unequivocal structural confirmation, toxicological testing, and quantification in drinking water before risk assessment can be performed.

The health risks associated with exposure to halogenated disinfection by products (DBPs) in drinking water have been previously documented.[5] Some halogenated disinfection by-products (DBPs) are now considered probably carcinogenic to humans.[6,7] Genetic damage is a distinctive feature of human cancers,[8] and many chlorinated and brominated DBPs cause mutations in pro- and eukaryotic cells.[5,9] However, chromatographic techniques commonly used for DBP analysis, i.e., mainly gas chromatography and reversed-phase liquid chromatography (RPLC), are not necessarily suitable for very polar and ionic species that could have led to substantial underdetection in the past.[10] Here we have used mixed-mode anion-exchange RPLC to conduct an in-depth investigation of acidic contaminants present in London drinking water using non-targeted and suspect screening analysis. This resulted in the discovery of emerging polar DBPs which *in silico* analysis flagged as potentially harmful to humans.[11] In particular, halogenated-methanesulfonic acids (HMSAs), which were previously reported and classified as DBPs in drinking water,[12,13] were subsequently confirmed to be present in London's drinking water. These compounds were found at concentrations close to the μg L^{-1} range, and predicted as mutagenic (specifically, dibromomethanesulfonic acid).[11] All HMSAs were also predicted as developmental toxicants by USEPA Toxicity Estimation Software Tool.[11] While predictions are useful to help with prioritization, their toxicity has to be verified. In addition to these substances, halogenated-hydroxycyclopentenediones (HHCDs) were also tentatively identified. In particular, tribromo-HCD (TBHCD) was previously reported in Swedish

and Chinese drinking water, but with uncertainty about its exact structure.[14-16] Recently, it has been shown that HHCDs are present when chlorination, chloramination, ozonation/chlorination and ozonation/chloramination are employed for potabilization.[17] An in-depth analysis of the distinctive fragmentation pattern found in the product ion scan spectrum (PIS), combined with the definition of a complete formation mechanism, allowed us to assign an unequivocal structure (2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione), which was predicted as mutagenic. However, nuclear magnetic resonance (NMR) analysis and/or X-ray crystallography are critical for unequivocal structure confirmation. Three additional tri-halogenated HCDs were also tentatively identified frequently in London drinking water and predicted as mutagenic: trichloro-HCD (TCHCD), bromodichloro-HCD (BDCHCD), and chlorodibromo-HCD (CDBHCD).[11] Further research into HMSAs and HHCDs is required to understand their prevalence and potential health risks.

Our attempts to isolate TBHCD through bromination of 2,4,6-trihydroxybenzaldehyde were previously unsuccessful due to losses during evaporation.[11] Previously, TBHCD was isolated by freeze-drying 1 L of combined LC fractions; the resulting 10 mg substance were characterised by infrared spectroscopy and mass spectrometry (MS).[14] Unfortunately, no additional spectroscopic evidence was provided, and the exact structure remains to be confirmed. Exhaustive bromination of 4-hydroxycyclopent-4-ene-1,3-dione (HCD) has been documented, but stoichiometry, temperature, reaction concentrations and characterisation data are unavailable.[18] HCD has been previously synthesized by Claisen condensation of diethyl oxalate with diethyl-1,3-acetonedicarboxylate, followed by decarboxylation of the crude intermediate diester under acidic conditions.[19] An alternative procedure using ethyl acetoacetate with a decarboxylation-acetylation-decarboxylation protocol to avoid the need for sublimation has been reported, but insufficient detail exists to replicate it.[20] Overall, a

reliable procedure for laboratory scale preparation of TBHCD is required to enable exposure and toxicological analysis.

The principal aim of this work was to investigate the occurrence, reactivity, stability and toxicity of TBHCD. The objectives included: (a) synthesis and purification of TBHCD via exhaustive bromination of 4-hydroxycyclopent-4-ene-1,3-dione; (b) TBHCD and DBMSA quantification in municipal drinking water samples to provide initial estimate of exposure; (c) *In vitro* mutagenicity testing of both substances using Salmonella/microsome assay with strains TA100, TA98 and TA102; (d) semi-quantification of other HHCDs and HMSAs to evaluate their relative concentration levels in municipal water; (e) investigation of the reactivity of TBHCD.

Results and discussion

Synthesis and characterisation of 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione

As represented schematically in Figure 1A, HCD was obtained via Claisen condensation of diethyl oxalate with diethyl-1,3-acetonedicarboxylate, followed by decarboxylation of the crude intermediate diethyl 2,4,5-trioxocyclopentane-1,3-dicarboxylate under acidic conditions. $^{13}C\{^{1}H\}$ NMR inverse-gated spectroscopy of the sublimed HCD indicated contamination with oxalic acid (δ_C = 161.0 ppm, 11%, see Figure S2 and S3), which presumably arose from the aqueous hydrolysis of unreacted diethyl oxalate, and co-sublimes with HCD. Nonetheless, the crude HCD could be smoothly tribrominated using an excess of bromine in chloroform at 60°C, providing pure TBHCD after concentration and recrystallisation from chloroform. The ^{13}C NMR spectrum of the obtained colourless needles supported our pre-existing tentative structural assignment of TBHCD[11] and was unambiguously confirmed by X-Ray crystallography (see Figures 1B, 1C and S1).[11] The enolic form was confirmed by the O4

longer C–O bond length, the planarity of the C5 ring, and the shortness of the C3–C4 bond compared to the rest of the C–C bonds. The mass spectrum was also consistent with previous reports.[11,14] Characterization data were consistent with a high-purity substance.

Analytical method development, validation and sample analysis

Considering its volatility, a large-volume direct-injection method was developed to avoid TBHCD losses during evaporation (Table S1). A mixed-mode weak-anion-exchange/RPLC separation enabled retention of strongly polar analytes with a pH gradient to deactivate the ion exchange functionality later in the run. A quadrupole time of flight mass spectrometer was operated with narrow-scan windows (0.1 Da) to maximise detector sensitivity. Method performance was evaluated according to Eurachem guidelines.[21] Excellent limits of quantification were determined at 4 and 10 ng L^{-1} for TBHCD and DBMSA with linearity verified up to 2,637 and 10,560 ng L^{-1}, respectively (R^2 >0.995, N≥10). In comparison, the only previously reported limit of detection for TBHCD was 530 ng L^{-1}.[14] Repeatability at high and low spiking level returned relative standard deviations (%RSD) of the area ≤3% for both substances (n=10). Matrix effect from tap water suppressed the signal on average 1% for TBHCD and 8% for DBMSA (n=10, %RSD=2% for areas of both compounds).

As no analyte-free tap water could be retrieved, standard addition was preferentially adopted to account for matrix effects. Calibration with a minimum of five standards returned coefficients of determination of R^2>0.995 (satisfying requirements set by all available guidance).[22-25] Municipal water from seven homes in London were sampled every two weeks for a total of six weeks (sampling coordinates and dates in Table S2, results in Table S3). Mean concentrations were 122 and 326 ng L^{-1} for TBHCD and DBMSA, respectively (see Figure 2 for details of individual sampling points).

Chlorinated HMSAs and HHCDs were semi-quantified employing DBMSA and TBHCD as reference materials, respectively, which shared structural and chromatographic retention time similarity, and in line with other works.[26] The mean semi-quantitative concentrations of DCMSA and BCMSA were 3164 and 1164 ng L^{-1}, in line with concentrations found in tap water samples from Europe, Asia and America.[13] BDCHCD, CDBHCD and TCHCD were semi-quantified respectively at 150, 286 and 138 ng L^{-1} on average. Although monitoring only seven homes, these were spread across London and on multiple occasions. Extracted ion chromatograms for all analytes in a London tap water sample are available in Figure S6. These results indicate potential widespread occurrence of all analytes in London municipal waters. Given the impossibility to account for TBHCD differential degradation due to variable residence time in the pipeline, it is not possible at this stage to analyse spatiotemporal trends. In order to do so, sampling immediately after treatment should be attempted. A more comprehensive spatiotemporal study is recommended to understand occurrence on a much larger scale.

Stability and reactivity of TBHCD

A stability study conducted in laboratory tap water (pH=7.8, water treatment: chlorination) showed that frozen samples could only be considered stable for 24 hours (details in Table S4). Refrigerated tap water solutions showed a 12% peak area percent (A%) decrease over 24 hours and consequently could not be considered stable. Moreover, very significant transformation occurred at room temperature (RT) in tap water (85% decrease in 24 hours). These results shall be considered valid only for the drinking water composition tested. Refrigerated ultrapure water solutions were found to be stable for 48 hours.

Odd-electron fragments in electrospray ionisation are rare, mostly occurring in positive rather than negative mode, generated by stabilization of the radical ion.[27-31] All four HHCDs detected in samples shared a distinctive fragmentation pattern (see Figure 3A, 3D, 3E and 3F), which included $[M\text{-}H\text{-}Halogen^{\bullet}]^{\bullet-}$, $[M\text{-}H\text{-}Halogen^{\bullet}\text{-}CO]^{\bullet-}$ and $[C_4HalogenO]^{-}$ in all recorded spectra, strongly indicating the presence of an identical structure with different halogen substituents. As the radical formation is favoured on the sp_3-hybridised carbon, in BDCHCD $[M\text{-}H\text{-}Br^{\bullet}]^{\bullet-}$ is diagnostic for the position of the bromine, making it a stereocenter. This unique fragmentation pattern highlights the existence of relatively stable dihalogenated radical species, suggesting low C-halogen bond dissociation enthalpies (BDEs) for the trihalogenated species. The PIS of dibromo-HCD (DBHCD, previously tentatively identified in drinking water)[11] also revealed a fragment with low intensity generated by the neutral loss of radical bromine ($Br^{\bullet}$)(Figure 3B).

Other *beta*-dicarbonyl structures like *N*-bromosuccinimide (NBS, commonly employed as an organic synthesis reagent) are a source of $Br^{\bullet}$.[32,33] NBS undergoes slow thermal homolysis in nitrogen atmosphere and absence of light.[34] The BDEs of NBS and of all four trihalogenated-HCDs were calculated employing a computational protocol that well reproduces the experimental BDE of NBS ($BDE_{calculated}$=67.2 kcal mol^{-1} -Figure 4A- vs. $BDE_{experimental}$=66.0 ± kcal mol^{-1})[35]. The calculated C-Br bond BDE in TBHCD was 52.7 kcal mol^{-1} (Figure 4B), 14.5 kcal mol^{-1} lower than that of NBS. Pronounced delocalisation of the unpaired electron (Figures 4C and 5C) is in accordance with the favourable homolytic cleavage. C-Br BDEs were almost constant for the other brominated species: 52.5 and 52.3 kcal mol^{-1} for CDBHCD (Figure 4D) and BDCHCD (Figure 4E). These values are also very close to that of the hydrogen peroxide O-O bond (51.1 kcal mol^{-1}),[36,37] a well-known source of radical species in the body.[38] The calculated BDE for the C-Cl bond in TCHCD (Figure 4F) and BDCHCD

(Figure 4E) was 64.9 kcal mol^{-1}, and 65.2 kcal mol^{-1} for CDBHCD, implying that also the C-Cl bond cleavage is energetically favourable in comparison to NBS. Furthermore, in the mixed halogenated compounds, the C-Br bond is cleaved first, in agreement with the fragmentation observed in Figure 3. Calculated bond dissociation enthalpies and free energies in the gas phase and in water are reported in Table S5.

The negative charge in all HHCDs is delocalised between oxygens in positions 3 and 5, and an identical pK_a of 4.5 was calculated for all four species with ACD/Labs Percepta software (Toronto, Ontario). Therefore, they should present primarily in anionic form both in drinking water and blood. Figure 5B represents resonance and tautomeric structures of deprotonated DBHCD implying a symmetry axis and exchangeability of C-H hydrogen (the strongest pK_a for DBHCD was calculated at 2.4).

Standards in ultrapure water (UPW) exposed to daylight consistently exhibited degradation of TBHCD and formation of DBHCD, with a ratio between DBHCD formation and TBHCD degradation of 0.68 (n=12, %RSD=14%). When exposed to daylight at RT, TBHCD A% reduced to 73% on average of its initial area in 90 minutes, while an average 17 A% of DBHCD was formed at the same time (details in Table S6). By comparison, reference solutions left in the dark at RT during the same interval did not show any statistically significant reduction in TBHCD or formation of DBHCD (n=6). A compound tentatively identified as 4-bromo-5-hydroxycyclopent-4-ene-1,2,3-trione (BHCT) was also shown to form increasingly with daylight exposure, with a ratio between its area increase and TBHCD area decrease of 0.13 (n=12, %RSD=21%). In the absence of reference materials, A% of DBHCD and BHCT were calculated by assuming ionisation efficiencies identical to that of TBHCD. When 100 μg mL^{-1} of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO, a paramagnetic radical scavenger) were added to UPW, over 90 minutes of daylight exposure TBHCD degradation was decreased by

an average of 92%, while DBHCD and BHCT formation was diminished by 81 and 62% on average, respectively (n=6, %RSD of peak areas ≤15% for all compounds). In acetone and dimethyl sulfoxide (DMSO) exposed to daylight to investigate hydrogen source for DBHCD formation, no statistically significant formation of DBHCD was observed (n=6), while the average BHCT A% reached 4% in acetone and 22% in DMSO over 3 hours. In general, DBHCD was observed to degrade faster than TBHCD in tap water. In frozen tap water solutions, an average 47% A% drop was recorded for DBHCD in 24 hours (n=3, %RSD=3%), while no statistically significant degradation of TBHCD was observed. Therefore, degradation of DBHCD and TBHCD in drinking water is expected to occur at the same time, but at different rates, making semi-quantification of DBHCD more challenging and outside the scope of this initial work.

While no statistically significant degradation was observed at pH 4 and at a physiologically relevant pH of 7.4 during two hours at RT in the dark, at pH 8.5 and 9 the compound clearly degraded forming both DBHCD and BHCT, at a rate that increased with increasing pH (%RSD<15% over triplicate preparations for all compounds, details in Table S7). TBHCD average A% dropped to 81% within 2 hours at pH 8.5, and to 58% within 2 hours at pH 9. Acidification of a spiked tap water sample at pH 5 did not result in improved TBHCD sample stability at RT in the dark: in 24 hours the A% decreased by over 99% on average, ruling out the possibility of prolonged sample stability by acidification. When 100 μg mL^{-1} of TEMPO were added to UPW solutions adjusted at pH 9, over 2 hours of storage in the dark at RT, TBHCD degradation decreased by an average of 70%, while DBHCD and BHCT formation also decreased by 67 and 62% on average, respectively. (n=6, %RSD of the areas ≤15% for all compounds). These results clearly indicate that TBHCD degradation induced by both light and alkaline conditions involves the formation of a radical pair. Radical anions are well-known to

react with water with mechanisms involving proton and electron transfer.[39,40] As well as light-induced homolysis, light-induced heterolysis has been shown to proceed through the formation of geminate radical pairs.[41] Our results are in accordance with the notion that TEMPO promotes recombination of radical pairs via spin-exchange,[42] and scavenges free radicals with a rate is inversely proportional to the radical's stability.[43] The mechanisms of DBHCD and BHCT formation remain to be determined.

A small reduction in HHCD formation was reported in simulated drinking water by Pan *et al*[14] at pH 8.5 in comparison to pH 6.0 in presence of bromide. Our results suggest that the degradation rate is faster in alkaline conditions. Hypochlorous acid has a pK_a of 7.40, and is a much stronger oxidant in its protonated form.[44] Similarly, hypobromous acid is a much stronger oxidant in its protonated form, but has a higher pK_a of 8.55.[44] Hypochlorous acid oxidises bromide to hypobromous acid. Therefore, while at pH 6 we expect both hypochlorous and hypobromous acid to be primarily in their most oxidizing form, at pH 8.5 only a small quantity of hypobromous acid should be active. They also report an increase in HHCD formation by prolonging the contact time from 1 hour to 5 days with chlorinamination, and a decrease in HHCD formation over the same period with chlorination. However, at 5 days of exposure to chlorine, TBHCD was the only species visible. In ozonation/chlorination and ozonation/chloramination treatment, Han *et al* reported decreasing intensities of HHCD with increasing ozone does.[17] Increasing ozone doses are likely to augment the oxidation of hypobromous acid to bromate, limiting the formation of brominated HCDs. Ozone could also react with aromatic precursors of HHCDs, limiting their availability to form both chlorinated and brominated HCDs.[45] A much higher proportion of bromine HCD substitution was reported for ozonation/chlorination in comparison to ozonation/chloramination. It is important to highlight that TBHCD stability in test solutions must be taken into consideration to accurately

evaluate experimental formation/degradation. The reaction pathways and kinetic of degradation of TBHCD in drinking water remain to be demonstrated as part of future research projects, requiring synthesis on a much larger scale (including radioisotopes and/or stable isotope containing test materials). To date, it cannot be excluded that the TBHCD reactivity in treated water could derive from its electrophilicity rather than from its tendency to homolyse.

Bacterial mutagenicity tests (Salmonella/microsome assay)

The mutagenicity of TBHCD and DBMSA was investigated with a Salmonella/microsome assay, the most common procedure for routine mutagenicity testing of chemicals,[46] and widely employed for DBPs.[5] For this study the following strains were employed: TA98, sensitive to frameshift mutagens,[47] TA100, sensitive base substitutions, and TA102, developed to detect genetic damage caused by radicals.[48] We found that TBHCD causes induction of His^+ revertants at doses > 100/plate in all three tester strains (Figure 6, individual values in Table 8A), in absence of metabolic activation mix. The highest concentration (500 µg/plate) caused acute toxicity (dissolved background lawn) which was paralleled by a decline of the revertant numbers. The most pronounced effect was detected in TA100 (6.6) followed by TA98 (6.2) and TA102 (2.2) (numbers in parenthesis indicate mutant ratios, i.e. chemically induced revertants versus background rates). Addition of metabolic activation mix (rat liver S-9) led to a decrease of the mutagenic activity by ~50 % (Figure 6). No evidence of mutagenic activity of DBMSA under identical conditions was detected. (See Figure S4). Limited substance availability did not allow for DBMSA testing with metabolic activation in strains TA98 and TA102. The decline of formation of His^+ revertants after addition of the metabolic activation mix (which is added in in vitro mutagenicity tests to mimic the activation of certain indirectly-acting promutagens)[49] was also observed in earlier experiments with halogenated DBPs,[50,51] and is probably a

consequence of direct reactions of the test compound with molecules contained in the mix. However, certain brominated compounds are activated by the mix [52]. Most known halogenated DBPs cause base substitution mutations and are by far more active in TA100 than in other strains.[9] We found that TBHCD causes also mutations TA98 and TA102 under very similar conditions. This indicates that the compound causes DNA damage via molecular mechanisms which are different from other halogenated DBPs. It must be noted that, while mutagenic activity was detected at dozens/hundreds of μg/plate, concentrations in drinking water are several orders of magnitude lower in the ng L^{-1} range. Obviously, no risk can be inferred as this test is not quantitative, and comprehensive risk assessment should be performed.

It must be noted that, at this stage, the formation of $Br^{\bullet}$ cannot be assumed to be responsible for the observed bacterial mutagenicity and further work is certainly required, in particular to assess the electrophilic reactivity of TBHCD with DNA. However, formation of $Br^{\bullet}$ is expected with light exposure and could occur in the body.[38] Hypochlorous acid is also a known mutagen[53] and a potential source of radical chlorine with slightly higher BDE than the one here calculated for TBHCD.[36] However, hypochlorous acid quickly reacts as an electrophile in drinking water.[54] NBS, reported in this work as a reference for $Br^{\bullet}$ formation potential, is mutagenic in Salmonella/microsome tests (Source: European Chemicals Agency, http://echa.europa.eu/, consulted on the 30th August 2023). Furthermore, NBS was shown to generate genotoxic DNA interstrand cross-links connecting 7,8-dihydro-8-oxoadenine to an opposite base.[55] $Br^{\bullet}$ is scavenged by dissolved organic matter (DOM) and bicarbonate, forming hydroxylated species rather than brominated ones in micropollutants degradation studies,[56] and in general reacting with antioxidant DOM by electron transfer forming bromide, rather than brominated organic species.[57] The reaction of $Br^{\bullet}$ with phenol, a compound

representative of common DOM moieties, yields mainly *para*-benzoquinone,[58] a known mutagen[59] and carcinogen.[60,61] Furthermore, Br• is believed to be involved in the oxidation of guanine, responsible for renal carcinogenesis induced by bromate.[62]

Conclusions

The previously unassessed mutagenicity and the unique reactivity of an emerging class of DBPs in drinking water is reported for the first time. Following successful synthesis and purification of TBHCD, ^{13}C NMR and X-ray crystallography data were recorded, leading to the previously unavailable confirmation of the structure as 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione. We showed that TBHCD undergoes degradation induced by both light and alkaline pH, at rates that can be significantly reduced by addition of TEMPO, clearly indicating the formation of a radical pair. Furthermore, the computationally-derived BDE for C-Br was 14.5 kcal mol^{-1} lower for TBHCD than N-Br in NBS. Chlorinated HHCDs showed also lower BDEs than NBS, clearly indicating their potential to undergo homolysis in drinking water and in the body. A much larger-scale synthesis campaign is now required to comprehensively define the reactivity of trihalogenated-HCDs in drinking water and in the human body, e.g. employing radiolabelling and electron paramagnetic resonance spectroscopy experiments.

TBHCD was found to be unstable in frozen drinking water, and therefore constitutes a significant analytical challenge, as testing must be performed shortly after sample collection. Post-potabilisation conditions (residual chlorine, residence time etc.) are therefore likely to greatly influence the concentration of TBHCD at the point-of-use. For the first time, a sensitive method for TBHCD quantification was developed, validated and applied, revealing average levels above 100 ng L^{-1} in London tap water, an intermediate concentration for a DBP. CDBHCD,

BDCHCD and TCHCD were semiquantified at higher concencentrations on average. A much larger analytical campaign is now required to assess population exposure at scale.

TBHCD was shown herein to be a potent mutagen in the Salmonella/microsome assay in strains TA98, TA100 and TA102. Despite results of bacterial experiments cannot be employed directly for human risk assessment, genetic damage is one of the hallmarks of human cancer,[8] and therefore these observations indicate that human exposure may lead to adverse effects. No mutagenic activity was detected for DBMSA. However, in consideration of their predicted developmental toxicity, the widespread occurrence of DBMSA at hundreds of ng L^{-1}, and the estimated concentrations of DCMSA and BCMSA at µg L^{-1} levels should warrant further confirmatory toxicological investigations into these compounds.

This work is a multidisciplinary effort prompted by NTA results, where toxicity was predicted *in silico* for emerging chemical contaminants. Synthesis, exposure quantification and *in vitro* toxicity testing are resource-intensive but indispensable steps to bridge the water exposome knowledge gap and ultimately gain comprehensive control over exposure to harmful substance via drinking water.

Methods

Synthesis of 4-hydroxycyclopent-4-ene-1,3-dione

Potassium (2.0 g, 51 mmol) was added under nitrogen atmosphere to anhydrous ethanol (8.9 mL, 150 mmol) in diethyl ether (50 mL) at 0°C (not stirred until complete potassium solubilisation). Diethyl oxalate (3.4 mL, 25 mmol) was added, giving a yellow homogeneous solution, followed by diethyl-1,3-acetonedicarboxylate (4.7 mL, 26 mmol) which resulted in a deep orange solution. Within minutes, the entire reaction mixture solidified. After 18 h, the

solid jelly-like substance was dissolved in 2 M aqueous sulfuric acid (200 mL) and extracted with ethyl acetate (3 × 200 mL). The combined organics were dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to yield a crude orange oil of diethyl 2,4,5-trioxocyclopentane-1,3-dicarboxylate (6.6 g), that gradually solidified and was used without further purification. Crude diester (1.3 g) was dissolved in 12 M aqueous hydrochloric acid (100 mL) and the resulting orange solution was heated to 100°C for 90 min, during which it turned brown. The reaction mixture was allowed to cool and the solution was concentrated *in vacuo* to give a brown residue. This residue was then dried overnight in a vacuum desiccator at RT. Over the course of two days, the brown residue was sublimed at 120°C under a static vacuum of 0.1 mbar to give 4-hydroxycyclopent-4-ene-1,3-dione (134 mg, 1.09 mmol, 22%) as off-white crystals contaminated with oxalic acid (11%).

mp. 117.3–118.5°C (dec) (lit. 172–174°C);[18] IR (film) 1758, 1637, 1518 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.78 (br s, oxalic acid), 6.23 (s, 1H, CH), 3.43 (br s, 1H, OH), 2.93 (s, 2H, CH_2); $^{13}C\{^{1}H\}$ NMR (126 MHz, DMSO-d_6) δ 198.1 (CO), 197.1 (CO), 171.6 (COH), 161.0 (oxalic acid), 120.2 (CH), 41.8 (CH_2); HRMS (APCI+) calcd for $C_5H_5O_3$ $(M+H)^+$: 113.0233; found: 113.0237.

Synthesis of 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione

Bromine (0.37 mL, 7.2 mmol) was added under nitrogen atmosphere to a suspension of HCD (80.6 mg, 0.654 mmol, 11% oxalic acid) in anhydrous chloroform (2.2 mL). The resulting deep red suspension was heated to 60°C for 2 hours and allowed to cool. The reaction mixture was concentrated *in vacuo* to give a pale orange solid. Recrystallisation from chloroform, employing a hot filtration to remove sparingly soluble oxalic acid, yielded colourless white

crystals which were triturated with ice cold chloroform (5 × 1 mL) and dried *in vacuo*, providing pure 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione (95.6 mg, 42%).

mp. 190.0–195.3°C (dec); IR (film) 3191, 1765, 1710, 1626 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.38 (br s, 1H, OH); $^{13}C\{^{1}H\}$ NMR (126 MHz, DMSO-d_6) δ 190.0 (CO), 178.8 (CO), 168.3 (CO), 106.9 (CBr), 58.2 (CBr_2); HRMS (APCI–) calcd for $C_5{}^{79}Br_3O_3$ $(M–H)^–$: 344.7403; found: 344.7408. Anal. Calcd for $C_5HBr_3O_3$: C, 17.22; H, 0.29; Br, 68.74. Found: C, 17.35; H, 0.24; Br, 68.68.

Analytical method

A large-volume (50 µL) direct-injection method was developed on a Shimadzu LCMS9030 LC-QTOF with Nexera XR LC system and standard electrospray ionisation source (Shimadzu, Kyoto, Japan). A Waters Atlantis Premier BEH C_{18} AX column (2.5 µm, 2.1 x 100 mm) was employed for gradient LC, with a 5 mM ammonium bicarbonate solution in water (adjusted to pH 6.9 with acetic acid) as mobile phase A, and a 5 mM ammonium bicarbonate solution in acetonitrile:ultrapure water 9:1 (adjusted to pH 8.9 with diethylamine before organic solvent addition). Method performance was evaluated employing laboratory tap water (containing 132 and 560 ng L^{-1} respectively of TBHCD and DBMSA) and following procedure for sample preparation described below. Linearity was assessed in laboratory tap water spiked at 13 concentrations ranging from 10 to 10,000 ng L^{-1}. Repeatability was tested in a sample spiked at 50 and 1000 ng L^{-1} (n=10 for each concentration). Matrix interference was tested comparing background-corrected spiked laboratory tap water to a standard prepared in ultrapure water (solutions fortified at 500 ng L^{-1}, n=10). As no samples with low concentration of the analytes could be retrieved, ultrapure water spiked at 50 ng L^{-1} was employed to evaluate limits of quantification for both compounds in compliance with paragraph 6.2.2 of the Eurachem Guidelines[21] (n=10).

Samples were collected in Lightsafe 15 mL centrifuge tubes from seven household drinking water taps in London (GPS location in Table S2), frozen immediately after collection, and analysed within 24 hours. Immediately after thawing, tubes were centrifuged at 3000 rpm and 900 µL were transferred to amber glass vials. 100 µL of water:methanol 1:1 solutions containing the analytes at concentration ranging from 0 to 10000 ng L^{-1} were added, generating solutions fortified at 0, 50, 100, 200, 500 and 1000 ng L^{-1} for standard addition quantification of TBHCD and DBMSA. The amber vials were then immediately transferred to the LC autosampler at 4°C for analysis. BDCHCD, CDBHCD and TBHCD were semi-quantified on the basis of the ratio between their peak areas and that of TBHCD in unspiked samples.[26] An identical procedure was adopted to semi-quantify DCMSA and BCMSA employing DBMSA as a reference.

Stability and reactivity assessment

Compound stability was evaluated over 24 and 48 hours to observe potential degradation of TBHCD in municipal water samples at room temperature (20 °C), 4°C and -18°C, as well as in standards in ultrapure water at 4°C. Laboratory tap water and UPW were fortified with 500 ng L^{-1} and transferred to amber LC vials, immediately stored under each condition (n=3, injected in duplicate). Twenty four-hour stability at room temperature of unspiked tap water adjusted to pH 5 with acetic acid was later assessed with the same procedure. A 1000 ng L^{-1} TBHCD solution in UPW was employed to evaluate photostability of TBHCD, monitoring degradation in clear glass vials left in natural light at room temperature and sampled in duplicate at intervals of 15 minutes for 90 minutes. Reference vials in amber glass left at room temperature in the dark were sampled every 30 minutes during the same time interval (n=6) and employed to evaluate degradation not ascribable to photodegradation. For all experiments in this

section, variations lower than two times the area standard deviation of multiple injections of reference solutions (N≥6) were deemed to be not statistically significant. DBHCD and BHCT formation was approximated as A% in comparison to that of TBHCD at time zero, as their relative ionisation efficiencies could not be determined. Identical preparations containing TBHCD at 1000 ng L^{-1} with and without the addition of 100 μg mL^{-1} of TEMPO were compared after 90 minutes of exposure to natural light (n=6). Duplicate 20 μg L^{-1} solutions prepared in acetone and DMSO were exposed to natural light for 1, 2 and 3 hours and compared after 20-fold dilution in water (n=6 for each solvent).

To evaluate pH stability, triplicate 1000 ng L^{-1} solutions were prepared in ultrapure water adjusted to pH 4 with acetic acid, to pH 8.5 and 9 with ammonium hydroxide, and in a 10 mM phosphate buffer adjusted at pH 7.4 with ammonium hydroxide. Solutions were compared to standards in ultrapure water at the same concentration and injected after 1 and 2 hours of storage in the dark at RT (six preparations in total for each pH value). Identical preparations at pH 9 containing TBHCD at 1000 ng L^{-1}, with and without the addition of 100 μg mL^{-1} of TEMPO, were compared after 2 hours of storage at RT in the dark (n=6).

To calculate BDEs, geometry optimisations of the systems were carried out at the B3LYP/6-311++G(d,p) level of theory, using Gaussian16 (revision C.01).[63] Frequency calculation resulted in no imaginary frequencies for all the minima confirming the nature of the critical points. BDE calculations from the optimized structures were carried out using the G3B3 procedure.[64]

Salmonella/microsome assay

Plate incorporation assays were conducted as described in the protocol of Maron and Ames,[65] employing for both TBHCD and DBMSA strains TA98 and TA102 without metabolic activation

mix (S9), and TA100 (with and without S-9). Characterisation of the strains (uvrB, rfa, pKM101 and pAQ1) was performed before the main experiments.[59] Positive and negative controls were included in all individual series. A response was considered positive when the mean number of revertant colonies was two-fold or higher the one of the negative control ("two fold rule").[43] TBHCD and DBMSA were tested in plate incorporation assays and were dissolved in a mix of DMSO and water (1:2). Incubation was conducted in the dark. For each experimental point three plates were tested in parallel. 2,4,7-trinitro-9-fluorenone (0.1 µg/plate) was used as a positive control for TA98 and caused 1061±201 revertants per plate. Sodium azide (1.5 µg/plate) was used as a positive control for TA100 (without S9) and caused 636±62 revertants per plate. 2-aminoantracene (2.0 µg/plate) was used in experiments employing TA100 with metabolic activation and induced 1102.0±75 revertants per plate. Methyl methanesulphonate (2 µg/plate) was used as a positive control for TA102 and caused 2067±153 revertants per plate.

Acknowledgments

We are grateful to Ian Mudway, Curtis Gallagher, Alexandra Richardson, Michael Edges, Eric Auyang, Keng Tiong Ng, Oloyede Olukemi, Abdullah Shahid, Stuart Elliot, Peter Haycock, Fergus Long and Alberto Collauto for their support.

This study was funded by the National Institute for Health and Care Research (NIHR) Health Protection Research Units in Chemical and Radiation Threats and Hazards and Environmental Exposures and Health, both partnerships between the UK Health Security Agency and Imperial College London. The views expressed are those of the authors and not necessarily those of the NIHR, UK Health Security Agency or the Department of Health and Social Care.

We thank the EPSRC Centre for Dotoral Training in Next Generation Synthesis and Reaction Technology (Imperial College London EP/S022232/1) for a studentship to B. M. J. L.

Edoardo Jun Mattioli was supported by Fondazione Umberto Veronesi.

Raw data were generated at Imperial College. Derived data supporting the findings of this study are available from the corresponding author LB on request.

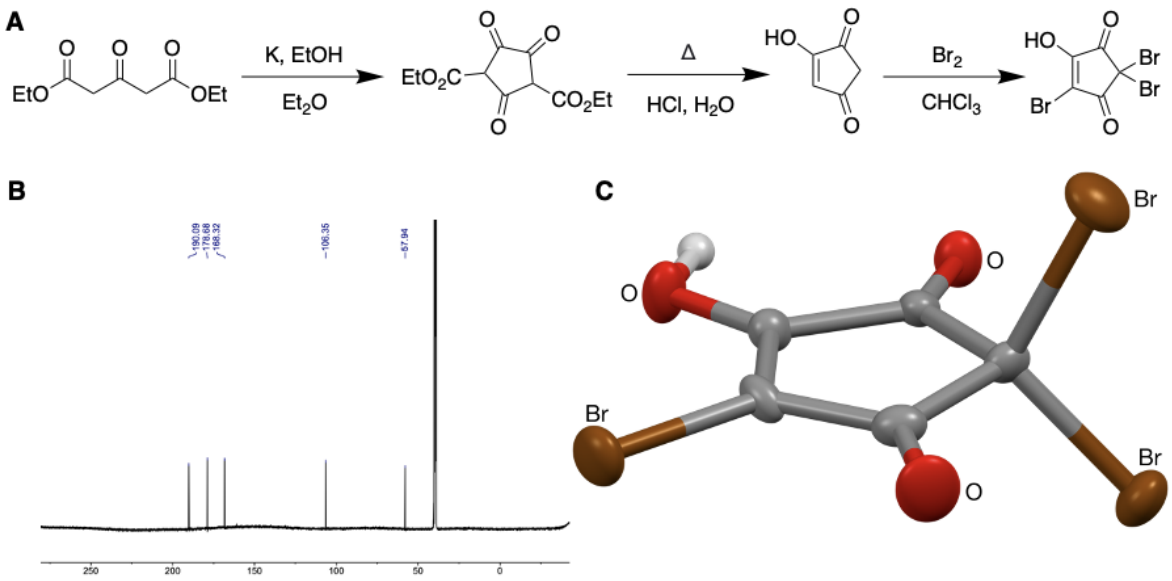

Figure 1. A) Synthetic route, B) $^{13}C\{^{1}H\}$ NMR inverse-gated spectrum and C) X-ray molecular structure of 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione (50% probability ellipsoids).

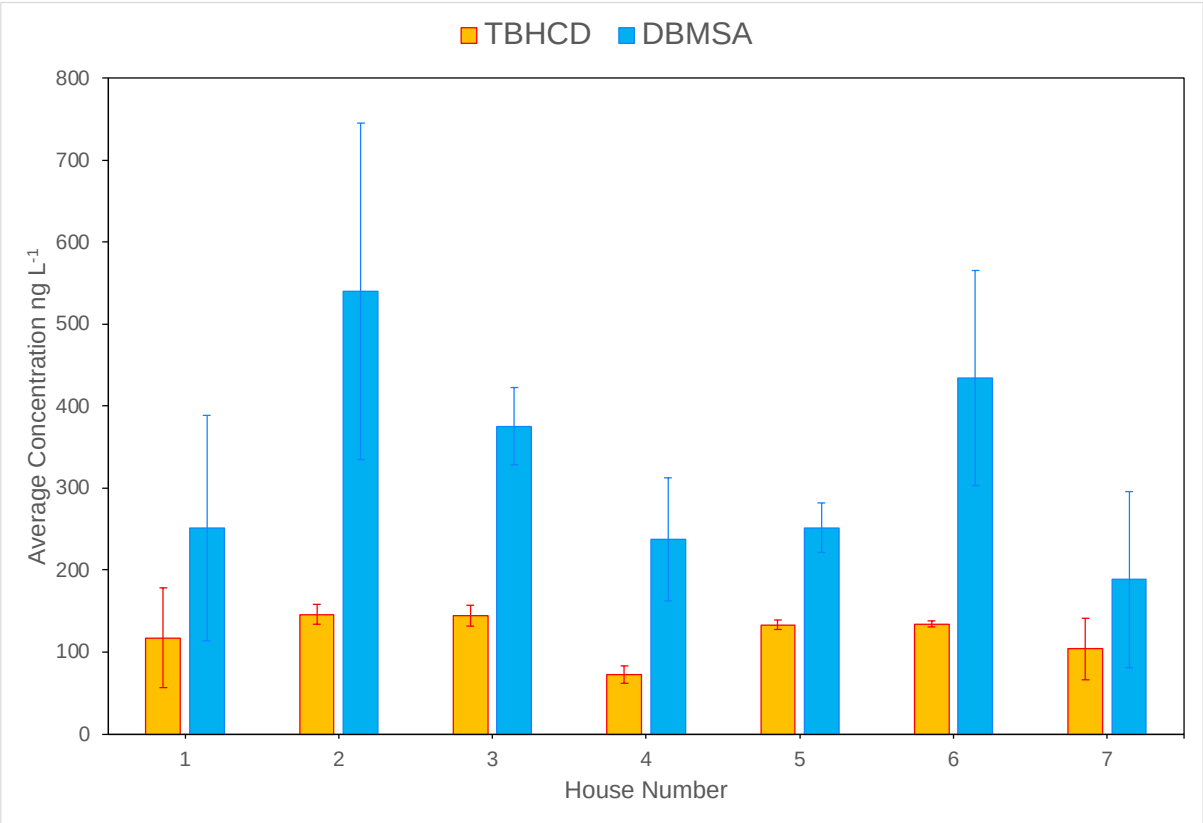

Figure 2. Bar chart representing average concentrations in ng L^{-1} of 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione (TBHCD) and dibromomethanesulfonic acid (DBMSA) in tap water from 7 London households, sampled in mid-August, end of August and mid-September 2023 (see also Tables S2 and S3). Error bars representing standard deviation over three timepoints and not error uncertainty over repeated measurement.

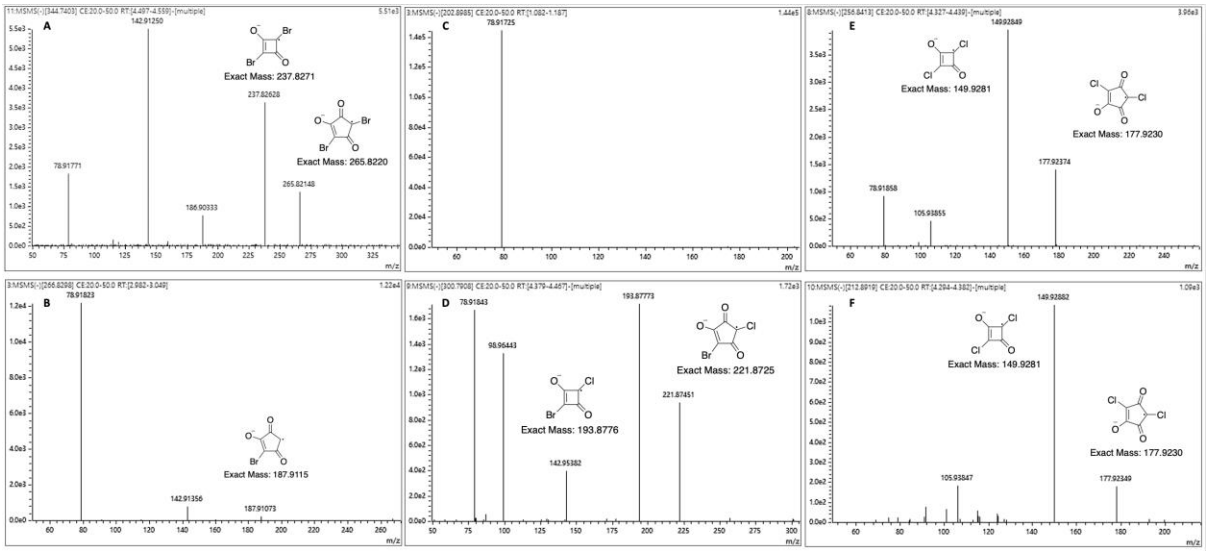

Figure 3. Product ion scan spectra of 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione (A), 2,4-dibromo-5-hydroxycyclopent-4-ene-1,3-dione (B), 4-bromo-5-hydroxycyclopent-4-ene-1,2,3-trione (C), 2,4-dibromo-2-chloro or 2,2-dibromo-4-chloro-5-hydroxycyclopent-4-ene-1,3-dione (D), 2-bromo-2,4-dichloro-5-hydroxycyclopent-4-ene-1,3-dione (E) and 2,2,4-trichloro-5-hydroxycyclopent-4-ene-1,3-dione (F), with structures and exact masses of odd-electrons species present in the spectra.

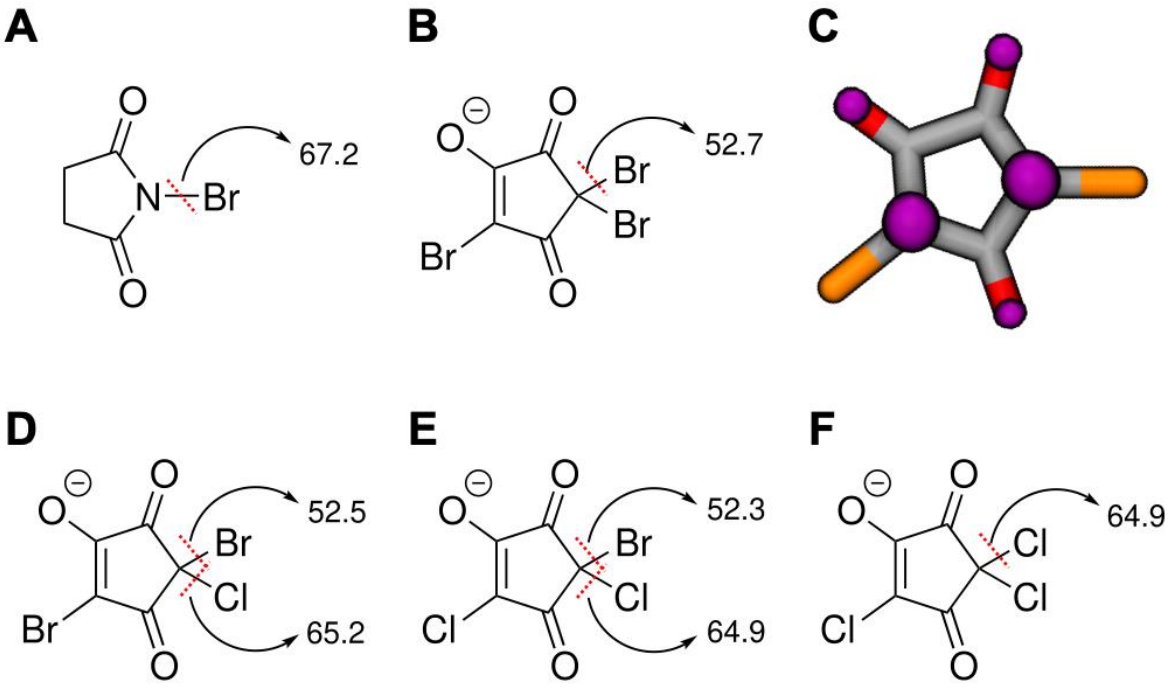

Figure 4. Calculation of C-Br and C-Cl homolytic cleavage BDEs (in kcal mol^{-1}) for the of the bonds for N-bromosuccinimide (A), 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione (TBHCD) (B), 2,4-dibromo-2-chloro-5-hydroxycyclopent-4-ene-1,3-dione (D), 2-bromo-2,4-dichloro-5-hydroxycyclopent-4-ene-1,3-dione (E), and 2,2,4-trichloro-5-hydroxycyclopent-4-ene-1,3-dione (F). Spin density distribution plot of the radical anion generated with C-Br homolysis of TBHCD is represented in (C), spin density in purple.

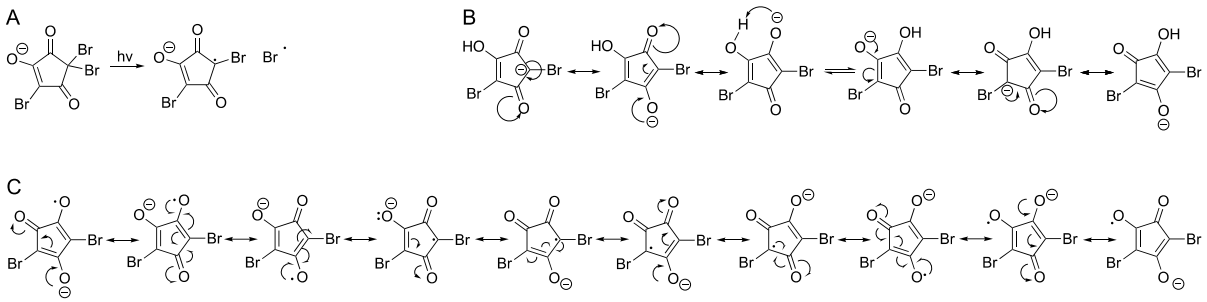

Figure 5. Homolysis of 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione (A); resonance and tautomeric structures for deprotonated 2,4-dibromo-5-hydroxycyclopent-4-ene-1,3-dione in drinking water (B); resonance structures for deprotonated 2,4-dibromo-5-hydroxycyclopent-4-ene-2-yl-1,3-dione (C). All structures are represented in anionic form reflecting ionization state at nearly-neutral pH.

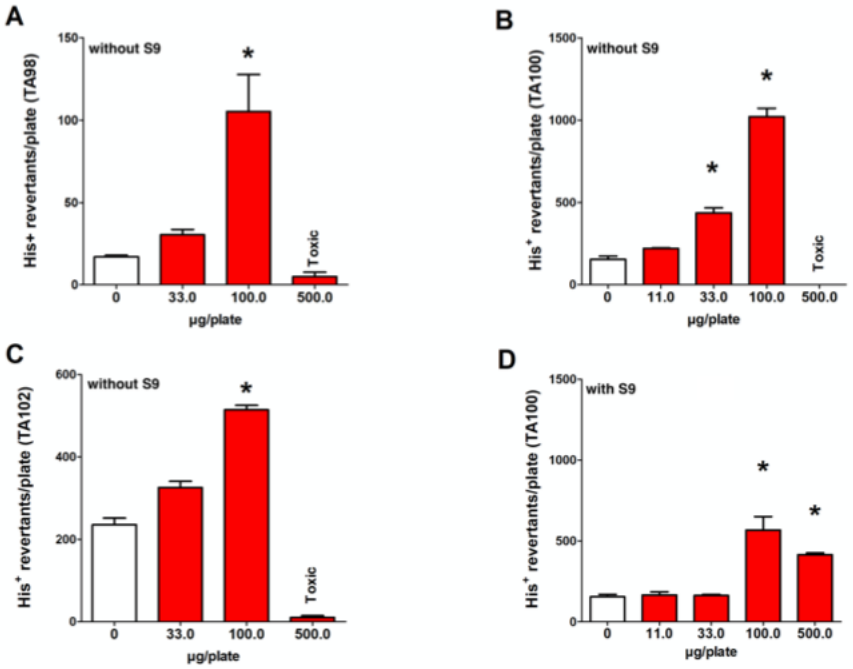

Figure 6: results of a representative experiment reporting mutagenic activities 2,2,4-tribromo-5-hydroxycyclopent-4-ene-1,3-dione with Salmonella strains TA98 (A), TA100 (B), and TA102 (C) without metabolic activation, and strain 100 with metabolic activation (D). Columns indicate mean ± SD from three plates. * positive according to the two-fold rule.

CCDC 2307874 contains the supplementary crystallographic data for this paper. This data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

References

1 Wang, Z., Walker, G. W., Muir, D. C. & Nagatani-Yoshida, K. Toward a global understanding of chemical pollution: a first comprehensive analysis of national and regional chemical inventories. *Environmental Science & Technology* **54**, 2575-2584 (2020).

2 Persson, L. *et al.* Outside the safe operating space of the planetary boundary for novel entities. *Environmental science & technology* **56**, 1510-1521 (2022).

3 Rappaport, S. M. & Smith, M. T. Environment and disease risks. *science* **330**, 460-461 (2010).

4 Muir, D. C., Getzinger, G. J., McBride, M. & Ferguson, P. L. How Many Chemicals in Commerce Have Been Analyzed in Environmental Media? A 50 Year Bibliometric Analysis. *Environmental Science & Technology* (2023).

5 Richardson, S. D., Plewa, M. J., Wagner, E. D., Schoeny, R. & DeMarini, D. M. Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutation Research/Reviews in Mutation Research* **636**, 178-242 (2007).

6 Grellier, J., Rushton, L., Briggs, D. J. & Nieuwenhuijsen, M. J. Assessing the human health impacts of exposure to disinfection by-products—A critical review of concepts and methods. *Environment International* **78**, 61-81 (2015).

7 Legay, C., Rodriguez, M. J., Sérodes, J. B. & Levallois, P. Estimation of chlorination by-products presence in drinking water in epidemiological studies on adverse reproductive outcomes: a review. *Science of the Total Environment* **408**, 456-472 (2010).

8 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011). https://doi.org/10.1016/j.cell.2011.02.013

9 DeMarini, D. M. A review on the 40th anniversary of the first regulation of drinking water disinfection by ‐ products. *Environmental and molecular mutagenesis* **61**, 588-601 (2020).

10 Reemtsma, T. *et al.* Mind the Gap: Persistent and Mobile Organic Compounds, Water Contaminants That Slip Through. *Environmental Science and Technology* **50**, 10308-11035 (2016).

11 Ciccarelli, D. *et al.* Enhanced selectivity for acidic contaminants in drinking water: From suspect screening to toxicity prediction. *Journal of Hazardous Materials* **448**, 130906 (2023).

12 Zahn, D., Frömel, T. & Knepper, T. P. Halogenated methanesulfonic acids: a new class of organic micropollutants in the water cycle. *Water research* **101**, 292-299 (2016).

13 Zahn, D., Meusinger, R., Frömel, T. & Knepper, T. P. Halomethanesulfonic acids—a new class of polar disinfection byproducts: standard synthesis, occurrence, and indirect assessment of mitigation options. *Environmental Science & Technology* **53**, 8994-9002 (2019).

14 Pan, Y. *et al.* A new group of disinfection byproducts in drinking water: trihalo-hydroxy-cyclopentene-diones. *Environmental science & technology* **50**, 7344-7352 (2016).

15 Gonsior, M. *et al.* Changes in dissolved organic matter during the treatment processes of a drinking water plant in Sweden and formation of previously unknown disinfection byproducts. *Environmental science & technology* **48**, 12714-12722 (2014).

16 Li, J. *et al.* Identification of unknown disinfection byproducts in drinking water produced from Taihu Lake source water. *Journal of Environmental Sciences* **113**, 1-11 (2022).

17 Han, J., Zhai, H., Zhang, X., Liu, J. & Sharma, V. K. Effects of ozone dose on brominated DBPs in subsequent chlor (am) ination: A comprehensive study of aliphatic, alicyclic and aromatic DBPs. *Water Research*, 121039 (2023).

18 Hesse, G. & Moell, H. Cyklopentantrion. *Naturwissenschaften* **40**, 411-411 (1953).

19 Chickos, J. S. Synthesis of 3, 5-dicarbethoxy-1, 2, 4-cyclopentanetrione. Correction. *The Journal of Organic Chemistry* **38**, 1231-1232 (1973).

20 Samarian, C. & Wanzlick, H.-W. Zur darstellung, chemie und konstitution des 1, 2, 4-cyclopentantrions. *Tetrahedron Letters* **15**, 2125-2128 (1974).

21 Magnusson, B. & Örnemark, U. Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, (2nd ed. 2014). ISBN 978-91-87461-59-0. Available from www.eurachem.org. (2014).

22 Thompson, M. Standard additions: myth and reality. *Analytical Methods Committee, Royal Society of Chemistry, AMCTB No 37, March 2009* (2009).

23 European Union Reference Laboratories, EURL Guidance Document on standard addition in the field of the analysis of residues of pharmaco- logically active substances, Version 1.1, 29 November 2022. (2022).

24 European Medicines Agency, Committee for Medicinal Products for Human Use, ICH guideline M10 on bioanalytical method validation and study sample analysis. *EMA/CHMP/ICH/172948/2019, 25 July 2022* (2022).

25 EU Reference Laboratories for Residues of Pesticides, Analytical quality control and method validation procedures for pesticide residues analysis in food and feed. *SANTE 11312/2021* (2021).

26 Pieke, E. N., Granby, K., Trier, X. & Smedsgaard, J. A framework to estimate concentrations of potentially unknown substances by semi-quantification in liquid chromatography electrospray ionization mass spectrometry. *Analytica chimica acta* **975**, 30-41 (2017).

27 Fornal, E. Formation of odd‐electron product ions in collision‐induced fragmentation of electrospray‐generated protonated cathinone derivatives: aryl α‐primary amino ketones. *Rapid Communications in Mass Spectrometry* **27**, 1858-1866 (2013).

28 Justesen, U. Collision‐induced fragmentation of deprotonated methoxylated flavonoids, obtained by electrospray ionization mass spectrometry. *Journal of Mass Spectrometry* **36**, 169-178 (2001).

29 Jin, W. *et al.* Simultaneous analysis of multiple bioactive constituents in Rheum tanguticum Maxim. ex Balf. by high‐performance liquid chromatography coupled to tandem mass spectrometry. *Rapid Communications in Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up‐to‐the‐Minute Research in Mass Spectrometry* **21**, 2351-2360 (2007).

30 Jeilani, Y. A., Cardelino, B. H. & Ibeanusi, V. M. Density functional theory and mass spectrometry of phthalate fragmentations mechanisms: modeling hyperconjugated carbocation and radical cation complexes with neutral molecules. *Journal of the American Society for Mass Spectrometry* **22** (2011).

31 Cardozo, K. H., Carvalho, V. M., Pinto, E. & Colepicolo, P. Fragmentation of mycosporine‐like amino acids by hydrogen/deuterium exchange and electrospray ionisation tandem mass spectrometry. *Rapid Communications in Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up‐to‐the‐Minute Research in Mass Spectrometry* **20**, 253-258 (2006).

32 Incremona, J. H. & Martin, J. C. N-Bromosuccinimide. Mechanisms of allylic bromination and related reactions. *Journal of the American Chemical Society* **92**, 627-634 (1970).

33 Chow, Y. L. & Zhao, D. C. Photodecomposition of N-bromosuccinimide. Radical chain carriers and their interrelations. *The Journal of Organic Chemistry* **52**, 1931-1939 (1987).

34 Dauben Jr, H. J. & McCoy, L. L. N-Bromosuccinimide. I. Allylic Bromination, a General Survey of Reaction Variables1-3. *Journal of the American Chemical Society* **81**, 4863-4873 (1959).

35 Luo, Y.-R. *Comprehensive handbook of chemical bond energies*. (CRC press, 2007).

36 Cottrell, T. L. The strengths of chemical bonds. *(No Title)* (1954).

37 Halliwell, B., Clement, M. V. & Long, L. H. Hydrogen peroxide in the human body. *FEBS letters* **486**, 10-13 (2000).

38 Hrycay, E. G. & Bandiera, S. M. Involvement of cytochrome P450 in reactive oxygen species formation and cancer. *Advances in Pharmacology* **74**, 35-84 (2015).

39 Bank, S. & Bockrath, B. Reactions of aromatic radical anions. VI. Kinetic study of the reaction of sodium naphthalene with water. *Journal of the American Chemical Society* **93**, 430-437 (1971).

40 Hayon, E., Ibata, T., Lichtin, N. & Simic, M. Electron and hydrogen atom attachment to aromatic carbonyl compounds in aqueous solution. Absorption spectra and dissociation constants of ketyl radicals. *The Journal of Physical Chemistry* **76**, 2072-2078 (1972).

41 Dreyer, J. & Peters, K. S. Picosecond Kinetic Study of the Photoinduced Homolysis and Heterolysis of Diphenymethyl Bromide. 1. The Nature of the Conversion from Radical Pairs to Ion Pairs. *The Journal of Physical Chemistry* **100**, 15156-15161 (1996).

42 Step, E. N., Buchachenko, A. L. & Turro, N. J. Paramagnetic interactions of triplet radical pairs with nitroxide radicals: an" Antiscavenging" effect. *Journal of the American Chemical Society* **116**, 5462-5466 (1994).

43 Chateauneuf, J., Lusztyk, J. & Ingold, K. Absolute rate constants for the reactions of some carbon-centered radicals with 2, 2, 6, 6-tetramethyl-1-piperidinoxyl. *The Journal of Organic Chemistry* **53**, 1629-1632 (1988).

44 Lide, D. R. *CRC handbook of chemistry and physics*. Vol. 85 (CRC press, 2004).

45 Önnby, L., Walpen, N., Salhi, E., Sander, M. & von Gunten, U. Two analytical approaches quantifying the electron donating capacities of dissolved organic matter to monitor its oxidation during chlorination and ozonation. *Water research* **144**, 677-689 (2018).
46 Corvi, R. & Madia, F. In vitro genotoxicity testing–Can the performance be enhanced? *Food and Chemical Toxicology* **106**, 600-608 (2017).
47 Levy, D. D. *et al.* Recommended criteria for the evaluation of bacterial mutagenicity data (Ames test). *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **848**, 403074 (2019).
48 Levin, D. E., Hollstein, M., Christman, M. F. & Ames, B. N. in *Methods in Enzymology* Vol. 105 249-254 (Elsevier, 1984).
49 Frantz, C. & Malling, H. The quantitative microsomal mutagenesis assay method. *Mutation Research/Environmental Mutagenesis and Related Subjects* **31**, 365-380 (1975).
50 Stalter, D., O'Malley, E., Von Gunten, U. & Escher, B. I. Fingerprinting the reactive toxicity pathways of 50 drinking water disinfection by-products. *Water Research* **91**, 19-30 (2016).
51 Giller, S., Le Curieux, F., Erb, F. & Marzin, D. Comparative genotoxicity of halogenated acetic acids found in drinking water. *Mutagenesis* **12**, 321-328 (1997). https://doi.org/10.1093/mutage/12.5.321
52 Richardson, S. D., Plewa, M. J., Wagner, E. D., Schoeny, R. & Demarini, D. M. Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat Res* **636**, 178-242 (2007). https://doi.org/10.1016/j.mrrev.2007.09.001
53 Van Rensburg, C., Van Staden, A., Anderson, R. & Van Rensburg, E. Hypochlorous acid potentiates hydrogen peroxide-mediated DNA-strand breaks in human mononuclear leucocytes. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **265**, 255-261 (1992).
54 Fisher, I., Kastl, G. & Sathasivan, A. A suitable model of combined effects of temperature and initial condition on chlorine bulk decay in water distribution systems. *Water research* **46**, 3293-3303 (2012).
55 Rozelle, A. L., Cheun, Y., Vilas, C. K., Koag, M.-C. & Lee, S. DNA interstrand cross-links induced by the major oxidative adenine lesion 7, 8-dihydro-8-oxoadenine. *Nature communications* **12**, 1897 (2021).
56 Guo, K. *et al.* Roles of bromine radicals and hydroxyl radicals in the degradation of micropollutants by the UV/bromine process. *Environmental Science & Technology* **54**, 6415-6426 (2020).
57 Lei, Y. *et al.* Bromine Radical (Br• and Br2•–) Reactivity with Dissolved Organic Matter and Brominated Organic Byproduct Formation. *Environmental Science & Technology* **56**, 5189-5199 (2022).
58 Lim, S., Barrios, B., Minakata, D. & von Gunten, U. Reactivity of bromine radical with dissolved organic matter moieties and monochloramine: Effect on bromate formation during ozonation. *Environmental Science & Technology* (2023).
59 Hakura, A., Mochida, H., Tsutsui, Y. & Yamatsu, K. Mutagenicity of benzoquinones for Ames Salmonella tester strains. *Mutation Research Letters* **347**, 37-43 (1995).
60 Gold, L. S., Manley, N. B., Slone, T. H. & Rohrbach, L. Supplement to the Carcinogenic Potency Database (CPDB): results of animal bioassays published in the general

literature in 1993 to 1994 and by the National Toxicology Program in 1995 to 1996. *Environmental Health Perspectives* **107**, 527-600 (1999).

61 Gold, L. S., Manley, N. B., Slone, T. H., Rohrbach, L. & Garfinkel, G. B. Supplement to the Carcinogenic Potency Database (CPDB): results of animal bioassays published in the general literature through 1997 and by the National Toxicology Program in 1997–1998. *Toxicological Sciences* **85**, 747-808 (2005).

62 Murata, M. *et al.* Requirement of glutathione and cysteine in guanine-specific oxidation of DNA by carcinogenic potassium bromate. *Chemical research in toxicology* **14**, 678-685 (2001).

63 Frisch, M. J. T., G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A. V.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Sonnenberg, J. L.; Williams-Young, D.; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J. J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Keith, T. A.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; Fox, . Gaussian 16, Revision C.01. *D. J. Gaussian, Inc., Wallingford CT* (2016).

64 Baboul, A. G., Curtiss, L. A., Redfern, P. C. & Raghavachari, K. Gaussian-3 theory using density functional geometries and zero-point energies. *The Journal of chemical physics* **110**, 7650-7657 (1999).

65 Maron, D. M. & Ames, B. N. Revised methods for the Salmonella mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects* **113**, 173-215 (1983).