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Article

Characteristic Fragment-Based Nontarget Identification of Thiol-Reactive Compounds in Disinfected Wastewater Using High-Resolution Mass Spectrometry: Focus on α,β-Unsaturated Carbonyls

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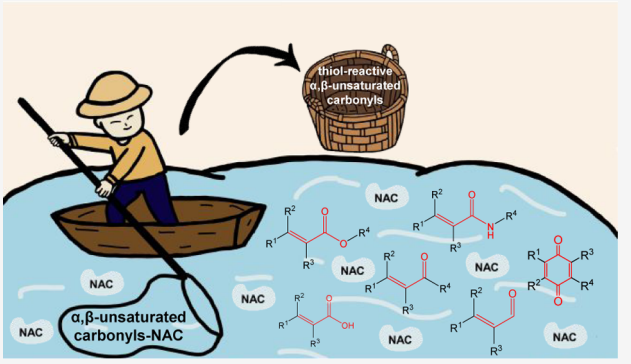
ABSTRACT: Thiol-reactive compounds, byproducts of wastewater chlorination, are under scrutiny for their high potential toxicity in water reuse applications. To date, a limited number of compounds have been identified in disinfected wastewater. In this study, we proposed a framework for comprehensively screening and identifying thiol-reactive compounds using a thiol probe combined with a nontarget high-resolution mass spectrometry (HRMS) analysis workflow, in which we monitored a fragment ion ($C_5H_8NO_3S^-$). To specifically target α,β-unsaturated carbonyls, this approach was developed and validated for 15 model α,β-unsaturated carbonyl compounds. Then, the technique was applied to analyze chlorinated municipal wastewater effluent, and 58 tentative thiol-reactive compounds were detected. We predicted the formulas of the 30 highest abundance α,β-unsaturated carbonyls. Among these, we used commercially available standards to validate our approach and quantify seven α,β-unsaturated carbonyls, namely, acrylic acid, 2,6-di*tert*-butyl-1,4-benzoquinone, *N*-acetyl-*p*-benzoquinone imine, *trans*-2-hexenal, 3-methyl-2-cyclopenten-1-one, dibutyl maleate and parthenolide, with concentrations ranging from <0.017−4.28 μg/L. These seven α,β-unsaturated carbonyls were identified as contributors to the toxicity of chlorinated wastewater, making up 4% of its overall cytotoxicity. This study demonstrated the characteristic fragment-based nontarget analysis as an effective approach to screen and identify thiol-reactive α,β-unsaturated carbonyls in treated wastewater.



KEYWORDS: *α,β-unsaturated carbonyls, N-acetyl-l-cysteine, high-resolution mass spectrometry, nontarget analysis*

INTRODUCTION

With nearly one-third of the global population living in countries experiencing high water stress, water utilities across the world are increasingly considering the reuse of treated wastewater to mitigate water scarcity.[1] In potable water reuse, where wastewater is treated to drinking water quality, treatment plants are responsible for protecting public health from pathogens, as well as from anthropogenic[2] or neo-formed compounds[3] that are either present in the wastewater or generated during treatment processes. Disinfection (e.g., chlorination, ozonation), designed to remove pathogens, is a key step in this process. As an unintended consequence of disinfection, disinfection byproducts (DBPs) may form from the reactions of disinfectants with organic matter.[4] DBPs are associated with adverse health effects, including cancer, and have been identified as priority pollutants in water reuse trains by the National Research Council.[5] To date, more than 700 DBP compounds have been identified, and 11 DBPs, including trihalomethanes (THM_4) and haloacetic acids (HAA_5) are regulated in U.S. drinking waters.[6] Over the past decade, cytotoxicity-based assessments have shifted the attention toward certain classes of identified unregulated DBPs, such as haloacetonitriles (HANs)[7] and carbonyl compounds,[8] as potential DBPs for further prioritization given their cytotoxicity and occurrence in treated water. Despite this, recent studies have shown that unidentified DBPs may be cytotoxicity drivers in reuse waters,[9] requiring more robust frameworks for identifying DBPs that should be prioritized.

Recently, α,β-unsaturated carbonyl compounds, a group of thiol-reactive compounds, have received attention due to their relatively high toxicity (i.e., 2 orders of magnitude more toxic than their saturated analogs).[10,11] Exposure to α,β-unsaturated carbonyls is a toxicological concern because their electrophilic

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character leads to cellular damage through reactions with nucleophilic groups in protein and DNA.[12−14] Still, only a few α,β-unsaturated carbonyls (e.g., acrolein, crotonaldehyde) have been identified and quantified in waters, in part due to the insufficient sensitivity of previously developed nontarget analysis method.[8,15−17]

Recently, a combination of effect-directed and reactivity-directed analysis (EDA/RDA) and nontargeted analysis has been proposed to identify and prioritize DBPs in treated waters.[18,19] A key focus of these strategies is the detection of electrophilic compounds, which can form covalent adducts with biomolecules such as DNA and proteins—a molecular initiating event associated with adverse health outcomes including cancer and cardiovascular diseases.[20,21] To support the identification of such reactive species, *in chemico* screening methods have been developed to assess electrophilic candidate chemicals based on their ability to form covalent adducts with nucleophilic biomolecular sites.[22,23] For example, electrophilic oxidation products from model compounds have been successfully identified through their reaction with thiol groups, such as those in *N*-acetyl-L-cysteine (NAC).[15] Building on these developments, a recent study introduced the "Thiol Reactome" strategy, which uses a glutathione (GSH)-based probe combined with nontargeted mass spectrometry to selectively capture and identify thiol-reactive DBPs from complex water mixtures.[24] This acellular assay has proven effective for detecting toxicologically relevant DBPs, aiding chemical prioritization for further study.[24] However, among the predominant classes of thiol-reactive DBPs, only four α,β-unsaturated carbonyls were identified among the 181 tentative DBP-GSH adducts detected.[24] Thus, complementary screening approaches are still needed to broaden the identification of α,β-unsaturated carbonyl compounds in treated waters.

High-resolution mass spectrometry (HRMS) with data-independent acquisition (DIA) is a powerful method for the identification of unknown chemicals in complex mixtures. HRMS-DIA can broadly acquire MS^2 spectra and identify precursors in predetermined isolation windows, with high resolution and mass accuracy.[25,26] NAC adducts have been successfully characterized in different fields, ranging from metabolomics to food safety applications.[27−33] These applications rely on a characteristic neutral loss (CNL) of 129 Da when the thioether bond is cleaved from NAC adducts. However, this fragmentation could miss NAC adducts that do not undergo a loss of 129 Da or have a weak relative abundance of resulting fragment ions.[32,34] Instead, monitoring for high-abundance characteristic fragments generated by NAC adducts could increase sensitivity in the nontarget identification of NAC adducts of unknown thiol-reactive compounds, including α,β-unsaturated carbonyls.

In this study, we introduce a novel framework for the comprehensive screening and identification of thiol-reactive compounds with a specific focus on α,β-unsaturated carbonyls. Here, we developed a nontarget analysis workflow based on monitoring a novel characteristic fragment ion ($C_5H_8NO_3S^-$) for NAC adducts using HRMS-DIA. We applied this workflow screening for NAC adducts in disinfected secondary effluents treated by NAC. Subsequently, we pinpointed the structures of potential α,β-unsaturated carbonyls by examining untreated real disinfected waters and annotating the MS/MS spectra in data-dependent acquisition (DDA) mode, which is more conducive to structural elucidation. We then assessed the biotoxicity of our newly identified compounds using a *Vibrio fischeri* based bioluminescence inhibition assay. This assay has been used to assess the biotoxicity of thiol-reactive DBPs[35] and is sensitive to a broad range of organic chemicals.[36] Our approach brings us one step closer to identifying which DBPs should be prioritized in water reuse treatment.

MATERIALS AND METHODS

Sample Collection and Preparation. The water sample was collected from the undisinfected effluent of a municipal activated sludge wastewater treatment plant (WWTP) in Foshan, South China. Six mg-Cl_2/L of chlorine, yielding a 1 mg of Cl_2/L as free chlorine residual after 24 h, was applied to the water. The sample was then left to react, headspace-free, in a glass bottle for 24 h in the dark at ∼25 °C. The free chlorine was quenched with sodium sulfite.

Solid phase extraction (SPE) using HLB cartridges (1000 mg, 6 mL; Bioland) was used to concentrate the sample. The sample was split into a 1 L aliquot (for chemical analyses) and 4 L aliquot (for toxicity analyses). The 1 L aliquot was loaded onto a preconditioned cartridge (10 mL of dichloromethane followed by 10 mL of methanol and 10 mL of Milli-Q water), and eluted with 5 mL of dichloromethane followed by 5 mL of methanol.[37] The resulting concentrate was evaporated to near dryness under a gentle stream of nitrogen, and reconstituted with 1 mL methanol. Then, the extract was centrifuged at 12,000 rpm for 10 min to remove insoluble impurities, and the supernatant was stored at 4 °C until analysis. The 4 L aliquot was prepared in a similar manner, but was reconstituted with 200 μL of dimethyl sulfoxide (DMSO) rather than methanol.[38] For the procedure blank, 4 L of Mili-Q water was passed through the SPE protocol and reconstituted with 200 μL of DMSO. It should be noted that low-molecular-weight compounds may be partially lost during the SPE pretreatment process, and the evaporation step under nitrogen can further lead to the loss of highly volatile carbonyl compounds.[39,40]

NAC Adducts Formation Assay. After solid-phase extraction, derivatization was performed on an aliquot to achieve an optimal balance between extract cleanliness and fragmentation quality. We selected NAC, instead of GSH, as the probe primarily due to its stability and suitability for analysis in complex matrices, such as wastewater, compared to GSH.[41] Our data showed that NAC mainly formed single adducts with α,β-unsaturated carbonyls, while literature showed that GSH could form double and triple adducts.[41] In addition, the acetylated N-terminus of NAC can prevent the formation of Schiff bases with carbonyl groups. Thus, NAC was selected as the probe compound for nontarget screening of α,β-unsaturated carbonyls.

To benchmark the method, each of the 15 α,β-unsaturated carbonyl standards (10 μM for each chemical) was incubated with freshly prepared NAC (1 mM) in phosphate-buffered saline solution (PBS; pH 8, 10 mM) at 25 °C for 24 h in the dark.[24] The model compounds were chosen to span different structural subclasses commonly reported in disinfected waters.[16,42] All incubations were done in triplicate. The concentration used for NAC was in excess to ensure fully reacted α,β-unsaturated carbonyl compounds prior to HRMS analysis. NAC and PBS blanks were used as controls. These NAC adducts remain stable over a one-month period, with the exception of crotonic acid (Figure S1). For water extracts (500 relative enrichment factors [REF]; 2× dilution factor from the original extract), the NAC concentration was increased to 10 mM to ensure the NAC concentration was in excess of the

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carbonyls. Water extracts were subjected to HRMS analysis both before and after NAC addition for comparison.

Nontarget Screening and Identification of α,β-Unsaturated Carbonyls. Nontarget screening and identification of α,β-unsaturated carbonyls treated with NAC were performed by an UPLC system coupled to a hybrid Q-Exactive Orbitrap mass spectrometer (UPLC-Q-Orbitrap HRMS, Thermo Fisher). A Hypersil GOLD C18 column (100 mm × 2.1 mm, 1.9 μm) was used to separate the analytes with a sample injection volume of 5 μL. The mobile phases in the UPLC system were 0.02% acetic acid in ultrapure water (A) and 0.02% acetic acid in acetonitrile (B). The LC gradient for all samples was as follows: 5% B for 0−2 min; 5%−95% B in 2−12 min; 95% B in 12−16 min; 5% B at 16.1 min; 5% B in 16.1−20 min. The flow rate was held at 0.3 mL/min and the column temperature was held at 40 °C. The Q-Exactive mass spectrometer was operated in negative electrospray ionization (ESI) mode and data were acquired in the DIA mode. Each sample was injected 5 times, covering isolation windows of m/z 200−300, 300−400, 400−500, 500−600, and 600−700, respectively. A full scan was performed between m/z 200 and m/z 700 at resolution R = 140,000 (at m/z 200) with an automatic gain control (AGC) of 1×10^6 and a maximum injection time of 100 ms. For each injection, one full scan (the same mass range as the DIA scan) was followed by five DIA MS^2 scans with each isolation window of 20 m/z at a resolution R = 70,000 (at m/z 200). The DIA analysis was performed with normalized collision energies of 20, 40, and 60 eV acquired in stepped mode. The maximum injection time was set at 50 ms and the AGC was set at 1×10^6.

To confirm the structures of identified α,β-unsaturated carbonyl compounds in the original water extracts, DDA MS/MS spectra of the water extracts were obtained in positive ESI mode with an inclusion list containing candidate ions of α,β-unsaturated carbonyl compounds identified from the DIA data. The sheath gas and auxiliary gas were set at 35 and 10 arbitrary units (a.u.), respectively. The spray voltage was 3 kV, and the capillary and auxiliary gas heater temperatures were 320 and 350 °C, respectively.

Screening Workflow for α,β-Unsaturated Carbonyls. Identification of unknown α,β-unsaturated carbonyls in disinfected water included two steps: (1) nontarget screening of NAC adducts and (2) structural identification of α,β-unsaturated carbonyls.

Step 1. Multiple successive MS^2 windows during the DIA were used in the nontarget analysis workflow to analyze the NAC-treated samples. The raw DIA data were transformed to mzXML files using "MSConvert" from ProteoWizard (Version: 3.0.21303)[43] and the files were imported into R (Version 4.2.2, R Development Core Team). The diagnostic fragment ion of m/z 162.0231 ($C_5H_8NO_3S^-$) was extracted from the multiplexed MS^2 spectra by an R-script with a tolerance of 10 ppm. The MS^1 ions corresponding to the apex of each fragment peak in the MS^2 chromatogram were extracted from the MS^1 spectrum. The similarity of the chromatograms between the diagnostic fragment ion and the MS^1 ion was calculated by Pearson correlation coefficients (r_{XY}, eq 1) as used previously in metabolomic and proteomic studies.[25,44]

$$r_{XY} = \frac{\sum_{i=1}^{n}(X_i - \overline{X})(Y_i - \overline{Y})}{\sqrt{\sum_{i=1}^{n}(X_i - \overline{X})^2} \times \sqrt{\sum_{i=1}^{n}(Y_i - \overline{Y})^2}} \quad (1)$$

Here, X and Y are the MS^2 and MS^1 ion intensity, respectively; and $\overline{X}$ and $\overline{Y}$ correspond to the sample mean; i labels the ith scan. The MS^1 ion with the highest correlation coefficient was chosen as a potential precursor of the NAC adduct. Once the potential precursor of the NAC adduct was selected, we confirmed that it was not present in the procedure blank, NAC blank and PBS blank.

Step 2. In our workflow, the mass of an NAC adduct is the mass of an thiol-reactive compound and the mass of NAC (163.0303 Da). The exact mass of candidate thiol-reactive compounds were obtained by subtracting 163.0303 from the exact mass of candidate NAC adducts. These were profiled into an inclusion list of the DDA method for obtaining the MS/MS spectra. Elemental compositions of candidate thiol-reactive compounds were calculated from these m/z values using Xcalibur 4.0 (Thermo Fisher Scientific, San Jose, CA) with a mass error of 5 ppm. Chemical formulas were set to contain up to 100 C, 200 H, 18 N, 36 O, 6 S and 4 Cl per molecule. All assigned formulas were required to meet previously published basic chemical criteria.[24]

DDA MS/MS spectra of the m/z values of candidate thiol-reactive compounds were obtained for untreated water extract samples. To selectively identify α,β-unsaturated carbonyls among these thiol-reactive compounds, MS/MS spectra were annotated and interpreted using the mzCloud database[45] (http://www.mzcloud.org/) and *in silico* fragmentation platforms (Mass Frontier,[46] CFM-ID[47]) to find candidate structures, with a specific emphasis on candidate structures bearing α,β-unsaturated carbonyl functionalities. Compounds with commercially available standards were confirmed by comparing the retention time (RT) and MS/MS spectrum between the sample and the standard. Five levels of confidence were used to identify the detected compounds according to the previous report.[48] Level 5 annotations matched only the compound's accurate mass, while Level 4 used both accurate mass and isotopic pattern for further identification. Level 3 possible annotations matched proposed α,β-unsaturated carbonyl structures in a curated library within ±5 mDa. In addition to these criteria, Level 2 probable annotations were confirmed using a published database. Finally, Level 1 annotations achieved the highest confidence, matching retention time (RT) and MS/MS spectra with corresponding reference standards.

Quantification of α,β-Unsaturated Carbonyls. The α,β-unsaturated carbonyls identified from nontarget screening were quantified directly in disinfected water samples without NAC derivatizations, since this process may introduce uncertainties regarding whether the compounds are fully derivatized to form single adducts. Quantification was performed using a TSQ Quantiva triple-quadrupole mass spectrometer connected to a UltiMate 3000 UPLC system (Thermo Fisher Scientific) in the positive electrospray ionization mode (ESI+). A Hypersil GOLD C18 column (100 mm × 2.1 mm, 1.9 μm) was used for separation of analytes, and the elution gradient was the same as the one used UPLC-Q-Orbitrap HRMS analysis. The flow rate was 0.3 mL/min and injection volume was 20 μL. The column compartment temperature was maintained at 40 °C. The sheath gas and auxiliary gas were set to 35 and 10 arbs, respectively. The spray voltage was 3 kV, and the capillary temperature and the auxiliary gas heater temperature were 320 and 350 °C, respectively. The scan was performed in the multiple reaction monitoring (MRM) mode with peak widths

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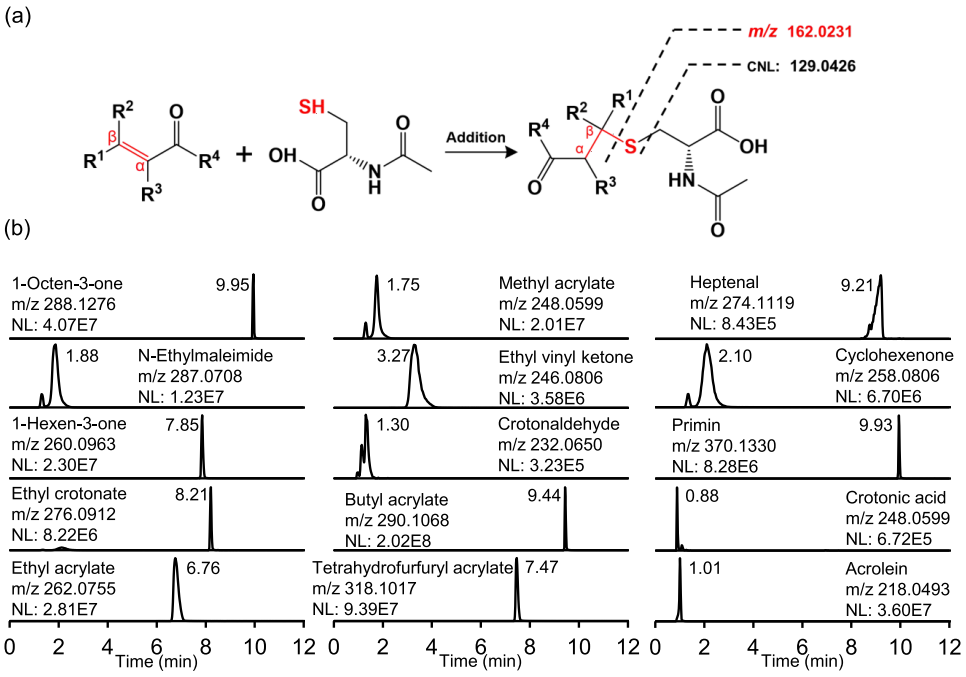

Figure 1. Validation of the formation of α,β-unsaturated carbonyls with NAC via addition. (a) The reaction route between NAC and α,β-unsaturated carbonyls via addition. (b) Chromatograms of the adducts of 15 model α,β-unsaturated carbonyls formed via addition.

of Q1 (fwhm) and Q3 122 (fwhm) being 0.70 and dwell time of 0.05 s per transition.

Bioluminescence Inhibition Test with *V. fischeri*. The *V. fischeri* bioassay was performed according to the ISO standard method 11348−3,[49] modified to a 96-well plate format.[35] Zinc sulfate was used as a positive control. Briefly, the extracts and standards redissolved in DMSO were pipetted in a 96-well plate using a geometric dilution series in a 2% NaCl solution and added to the reconstituted freeze-dried bacteria. The bioluminescence was measured prior to an addition of the sample and after a 15 min incubation. The inhibition of bioluminescence was calculated as described in ISO standard method 11348−3:2007.[49] The effect concentration EC_p was calculated using eq 2, where s is the slope of the concentration-effect curve, and EC_{50} is the median effect concentration.

$$\text{inhibition [\%]} = \frac{100\%}{1 + 10^{s\cdot(\log EC_{50} - \log \text{concentration})}} \quad (2)$$

The EC_p values of individual compounds are given in units of mg/L and sample EC_p is reported in dimensionless relative enrichment factors (REF).

Toxicity Equivalent Concentrations. For disinfected water samples containing multiple chemicals at low concentrations, concentration addition (which assumes toxicity is additive) has been suggested as a simplified approach to evaluate the mixture toxicity.[50] The toxicity equivalent concentration (TEQ) is the calculated concentration of a reference compound that elicits an equivalent response in a particular assay as the sample, determined from both bioassays and chemical analysis.[35,51] By comparing the TEQ of a water sample (TEQ_{water}) to the TEQ of the detected compounds (TEQ_{chem}), we can assess the contribution of detected chemicals to the sample toxicity. As the water sample investigated in the present study showed only a very low effect level, the end point used for reporting was the EC_{10}, the concentration causing 10% of the maximum effect.[52] The concentration of the water sample was expressed in REF units. The REF is the product of the sample concentration factor through the SPE process and the dilution of the extract in the bioassay. A REF > 1 means that the sample is enriched in the bioassay, a REF < 1 means it was diluted in the bioassay, and a REF of 1 is equivalent to the organic micropollutants in the undiluted and unconcentrated sample (excluding constituents not captured by SPE). The TEQ_{water} was calculated by dividing the EC_{10} of the reference compound by the EC_{10} of the water sample. As described in previous research, a "virtual" baseline toxicant with a 300 g mol^{-1} molecular weight was chosen as the reference compound,[35,53] with an EC_{10} of 1.37 mg L^{-1}. The TEQ_{water} is the ratio of the EC_{10} for the reference compound to the water sample (eq 3).

$$TEQ_{water} = \frac{EC_{10}\ (\text{reference compound})}{EC_{10}\ (\text{water sample})} \quad (3)$$

To calculate the TEQ_{chem}, it was necessary to determine the relative potency (RP_i) of each detected chemical (i), which is defined as the ratio of the EC_{10} for the reference compound to the detected chemical (eq 4).

$$RP_i = \frac{EC_{10}\ (\text{reference compound})}{EC_{10}\ (i)} \quad (4)$$

The TEQ of a given compound can be expressed as the product of the RP_i and the concentration of the compound C_i (mg L^{-1}). The overall TEQ_{chem} can be defined as the sum of the TEQ_i of each compound of interest (eq 5).

$$TEQ_{chem} = \sum_{i=1}^{n} TEQ_i = \sum_{i=1}^{n} RP_i \cdot C_i \quad (5)$$

Data Analysis. Xcalibur Qual Browser was used to extract ion chromatograms and MS^1 and MS/MS spectra throughout the study. All statistical analyses were performed using

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Table 1. Information of α,β-Unsaturated Carbonyls in the Disinfected Water Sample Detected by the Nontarget Analysis Workflow

no.	compound	formula	molecular weight	RT (min)	CAS number	confidence level
1	acrylic acid	$C_3H_4O_2$	72.0211	12.71	79−10−7	1
2	cyclobutenedione	$C_4H_2O_2$	82.0054	2.32	32936−74−6	3
3	*trans*-2-hexenal	$C_6H_{10}O$	98.0732	10.60	6728−26−3	1
4	3-methyl-2-cyclopenten-1-one	C_6H_8O	96.0575	6.41	2758−18−1	1
5	(2*E*)-2-octenal	$C_8H_{14}O$	126.1045	10.28	2548−87−0	3
6	2-oxo-2*H*-indole-6-carbonitrile	$C_9H_4ON_2$	156.0324	1.48	199327−63−4	3
7	2,5-diamino-4*H*,7*H*-[1,2,4]triazolo[1,5-A] pyrimidin-7-one	$C_5H_6ON_6$	166.0603	0.87	-	3
8	*N*-acetyl-*p*-benzoquinone imine	$C_8H_7O_2N$	149.0477	0.97	50700−49−7	1
9	(2*E*)-3-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-propenal	$C_{12}H_{18}O$	178.1358	14.87	4951−40−0	3
10	5-amino-6-hydrazino-1,5-dihydro-4*H*-imidazo[4,5-*c*]pyridin-4-one	$C_6H_8ON_6$	180.0760	13.55	91713−21−2	3
11	methyl 4-methoxycinnamate	$C_{11}H_{12}O_3$	192.0786	10.44	3901−07−3	3
12	ethylene dimethacrylate	$C_{10}H_{14}O_4$	198.0892	14.92	97−90−5	3
13	resorufin	$C_{12}H_7O_3N$	213.0426	12.17	635−78−9	2
14	primin	$C_{12}H_{16}O_3$	208.1099	15.72	15121−94−5	3
15	7-methoxycoumarin-4-acetic acid	$C_{12}H_{10}O_5$	234.0528	10.97	62935−72−2	3
16	dibutyl maleate	$C_{12}H_{20}O_4$	228.1362	14.02	105−76−0	1
17	5,5-epoxymethano-2,2,6-trimethyl-7-oxa-bicyclo[4.3.2]non-9-en-8-one	$C_{14}H_{20}O_3$	236.1412	0.9	-	3
18	2-(3-hydroxy-4-methylphenyl)-5-methyl-4-hexen-3-one	$C_{14}H_{18}O_2$	218.1307	13.55	-	3
19	2,6-di*tert*-butyl-1,4-benzoquinone	$C_{14}H_{20}O_2$	220.1463	15.25	719−22−2	1
20	5,8-dimethyl-1-methylene-4,5,5a,6,9,9a-hexahydro-3a*H*-azuleno[6,5-*b*] furan-2,7-dione	$C_{15}H_{18}O_3$	246.1256	12.82	-	2
21	parthenolide	$C_{15}H_{20}O_3$	248.1412	12.27	20554−84−1	1
22	1,2,2,6,6-pentamethyl-4-piperidyl methacrylate	$C_{14}H_{25}O_2N$	239.1885	13.62	68548−08−3	3
23	3-methyl-7-[(5-methyl-1,2,4-oxadiazol-3-yl)methyl]purine-2,6-dione	$C_{10}H_{10}O_3N_6$	262.0814	0.88	115779−20−9	3
24	(2*E*)-2-(2,3,4-trimethylbenzylidene)-1-benzothiophen-3(2*H*)-one	$C_{18}H_{16}OS$	280.0922	0.9	331724−45−9	3
25	12-oxo phytodienoic Acid	$C_{18}H_{28}O_3$	292.2038	14.35	85551−10−6	2
26	3-(4-methoxyphenyl)-1-phenylpyrazole-4-carbaldehyde	$C_{17}H_{14}O_2N_2$	278.1055	13.29	36640−42−3	3
27	*S*-benzyl (2*E*)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-butenethioate	$C_{20}H_{26}OS$	314.1704	12.06	-	3
28	diethyl 4-cyclohexyl-2,6-dimethyl-1,4-dihydro-3,5-pyridinedicarboxylate	$C_{19}H_{29}O_4N$	335.2097	12.09	1539−59−9	3
29	theophylline, 7-(3-nicotinamidopropyl)-	$C_{16}H_{18}N_6O_3$	342.1440	11.34	70454−27−2	3
30	(6*Z*)-6-(2-oxo-4-tridecyl-3-oxetanylidene)hexanamide	$C_{22}H_{39}O_3N$	365.2930	15.19	1646795−59−6	3

Microsoft Excel or GraphPad Prism (v 8.0.2, GraphPad Software inc., San Diego, CA). Linear regression analyses were performed using Microsoft Excel, and the EC_{10} value was calculated using the nonlinear fit function in GraphPad Prism.

RESULTS AND DISCUSSION

Nontarget Analysis Workflow Development and Validation with Model α,β-Unsaturated Carbonyls. To screen for unknown thiol-reactive α,β-unsaturated carbonyls, we detected NAC adducts produced after they reacted with the thiol functional group in NAC via Michael addition (Figure 1a). To demonstrate the feasibility of the method, we spiked NAC into a mixture of 15 commonly used model α,β-unsaturated carbonyl standards (10 μM/standard), encompassing six structural classes, and measured the formation of their corresponding NAC adducts (Table S1). All 15 NAC adducts were detected in the reaction mixtures after 24 h of incubation (Figure 1b).

Previous studies have documented that NAC adducts display a characteristic neutral loss of 129 Da ($C_5H_7NO_3$) in MS/MS experiments,[27] which was leveraged by recent studies to screen for NAC adducts using a DIA.[32] We used this fragmentation pattern to detect NAC adducts formed from the 15 model carbonyl compounds. Of the 15 compounds, only 13 model NAC adducts displayed a neutral loss of $C_5H_7NO_3$ (129.0426 Da) with the relative abundances of generated ions ranging from 3−82%. For the remaining two compounds (heptenal-NAC and primin-NAC), the fragment ion abundance resulting from the neutral loss of 129.0426 Da was too low to be observed. Effectively, the sensitivity of the screening method is lower for certain structures, which may bias results.

Interestingly, we found a highly abundant *m*/*z* 162.0231 fragment ion ($C_5H_8NO_3S^-$) for all the 15 model NAC adducts, with a relative abundance of 100% for 14 of them and 28% for the rest (Table S2). Previously, thiol-Michael adducts have been found to be reversible, especially under thermal treatment.[54] Thus, it is not surprising that NAC adducts tend to dissociate at the newly formed thioester bond under collision-induced dissociation, forming NAC fragments ($C_5H_8NO_3S^-$). Meanwhile, the dissociation of the thioester bond within the NAC structure is not favored, resulting in the low abundances of fragment ions generated by the neutral loss of 129.0426 Da. We established our nontargeted analysis workflow by monitoring the characteristic fragment ion ($C_5H_8NO_3S^-$) to screen for NAC adducts and identify thiol-reactive compounds (Figure S2). In our workflow, we extracted the characteristic fragment ions and identified potential precursors, as shown in Table S3. For example, in the case of 1-octen-3-one-NAC adduct detected at a retention time of 9.95 min, the characteristic fragment ion ($C_5H_8NO_3S^-$) was observed at 9.94 min. Corresponding to the characteristic fragment ion, we identified its potential precursor at *m*/*z* 288.1279 ($[C_{13}H_{22}O_4NS^-]$, *m*/*z* 288.1276, mass error = 1.04 ppm). In parallel, we analyzed the 15 model

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α,β-unsaturated carbonyls with HPLC-Orbitrap in DDA mode and profiled an inclusion list of their accurate m/z value. Figure S3 shows that all the model compounds and their DDA MS/MS spectra could be detected. These were used for annotating the structure of thiol-reactive compounds with specific focus on potential α,β-unsaturated carbonyls. Specifically, while $C_5H_8NO_3S^-$ is a reliable fragment for detecting primary Michael adducts, secondary modifications may produce different fragmentation patterns for α,β-unsaturated aldehydes,[55] which could require complementary fragments to achieve comprehensive detection.

Application of the Nontarget Analysis Workflow to Disinfected Water. We then identified thiol-reactive α,β-unsaturated carbonyls in chlorine-disinfected secondary effluent using the developed workflow. When extracted with 10 ppm mass error, multiple characteristic peaks for the characteristic fragment ion ($C_5H_8NO_3S^-$, m/z 162.0231) were detected in multiple precursor isolation windows. An intensity cutoff of 10,000 was used in the DIA analysis, and peaks exceeding this threshold were identified as NAC adducts for subsequent data analysis. A total of 178 characteristic peaks were detected in the NAC-treated sample, and the corresponding precursor ions were identified using an R-script. First, 118 compounds were labeled as potential NAC adducts after confirming that they were not present in corresponding controls. Then, the NAC-treated sample was analyzed in DDA mode to obtain the MS/MS spectra of the potential NAC adducts to verify that the characteristic peak ($C_5H_8NO_3S^-$, m/z 162.0231) was produced. NAC adducts form through the Michael addition reaction, where NAC is added to the carbonyls, and we obtained the exact mass of thiol-reactive compounds by subtracting the exact mass of NAC ($C_5H_9NO_3S$, 163.0303) from the exact mass of candidate NAC adducts. As an additional control, the untreated water sample extract was analyzed to detect the presence of these potential thiol-reactive compounds to confirm that all these potential NAC adducts were formed through the Michael addition reaction. As a result, 58 compounds were confirmed as NAC adduct candidates (Table S4).

The m/z values of potential thiol-reactive compounds calculated from the m/z values of NAC adducts were profiled into an inclusion list of the DDA method, which was then applied to the untreated water extract sample. These compounds showed a wide range of retention times (0.8−15.7 min), peak intensities (6.11×10^4−1.67×10^8), and m/z values (72.0211−538.8020). We focused on these compounds with high-abundance (>2×10^5) to predict their molecular formulas and structures. To screen out α,β-unsaturated carbonyls from the 58 potential thiol-reactive compounds, we used four confidence levels as described in the Method section, with our highest confidence levels in Level 1.[34] Among 58 candidates, 30 met Level 3 or higher confidence criteria (Table 1). Twenty matched the curated library and were possibly annotated as α,β-unsaturated carbonyls (Level 3), while ten structures matched the published databases[45−47] and were annotated as probable α,β-unsaturated carbonyls (Level 2). Standards were commercially available for seven of the α,β-unsaturated carbonyls, which were further confirmed at confidence Level 1. The MS^2 chromatograms of the common characteristic fragment ($C_5H_8NO_3S^-$, ±10 ppm) for these seven α,β-unsaturated carbonyls (Peaks 1−7) are illustrated in Figure 2. The mass error was set to 10 ppm to make sure that all characteristic fragments could be extracted. The remaining

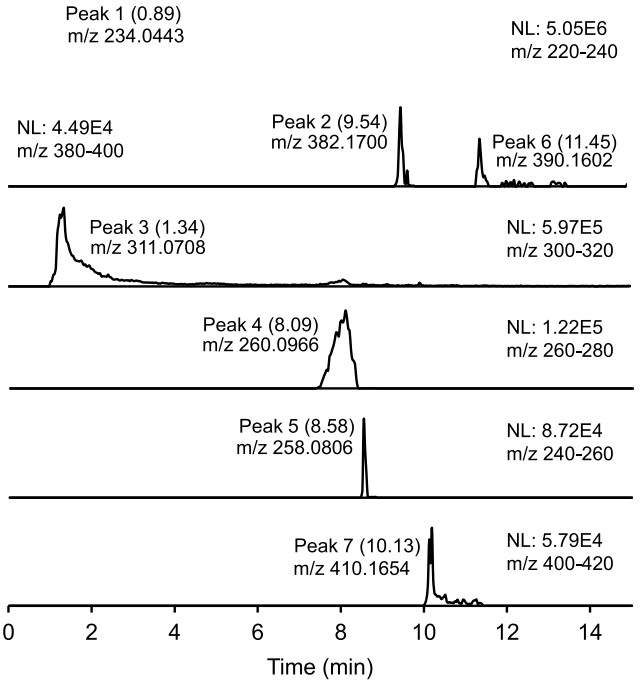

Figure 2. Nontarget peaks in the MS^2 chromatogram of the characteristic fragment ($C_5H_8NO_3S^-$, ±10 ppm) from the water sample extract.

28 unannotated features are likely to correspond to other unsaturated organic byproducts (e.g., nitriles and esters) that can undergo the Michael addition reaction.

Peak 1 was detected in the DIA window of m/z 220−240 with a 0.89 min retention time (RT). We observed MS^1 ions in the MS^1 spectrum corresponding to the apex of $C_5H_8NO_3S^-$ peak in the MS^2 chromatogram. Chromatogram similarities between $C_5H_8NO_3S^-$ and all the MS^1 ions, evaluated using Pearson correlation coefficients, revealed that the precursor was m/z 234.0443. The NAC-treated sample was then analyzed in DDA mode to acquire the MS/MS spectra of the potential precursor, confirming the presence of the characteristic peak. The m/z value of the candidate unsaturated carbonyl compound was determined by subtracting 163.0303 from the m/z value of the candidate NAC adduct. MS/MS spectra collected using the DDA method analysis of the untreated sample were used to identify the potential α,β-unsaturated carbonyl compound as acrylic acid. This matched our expectation, as the NAC adduct of acrylic acid formed through a Michael addition pathway (Figure 3a) was detected at m/z = 234.0443, 0.43 ppm and RT = 0.88 min (Figure 3b). The identity of acrylic acid in the water extract was confirmed by matching the RT (12.70 min) and MS/MS spectrum to a commercially available standard (Level 1, Figure 3c). Within its MS/MS spectrum, two fragment ions at m/z 55.0182 and 73.0285 were identified, with relative abundances of 100 and 22%, respectively, mirroring those of the commercial standard. Peak 2 was detected in the DIA window of m/z 380−400 with the precursor ion of m/z = 382.1700 at a 9.54 min RT. Following a procedure similar to that described for Peak 1, we identified the compound as 2,6-di*tert*-butyl-1,4-benzoquinone (BHT-Q). The identification was validated by comparing the RTs and MS/MS spectra of the sample and the corresponding standard (Level 1, Figure 3). The NAC adduct of BHT-Q was detected at 9.56 min, and BHT-Q was detected

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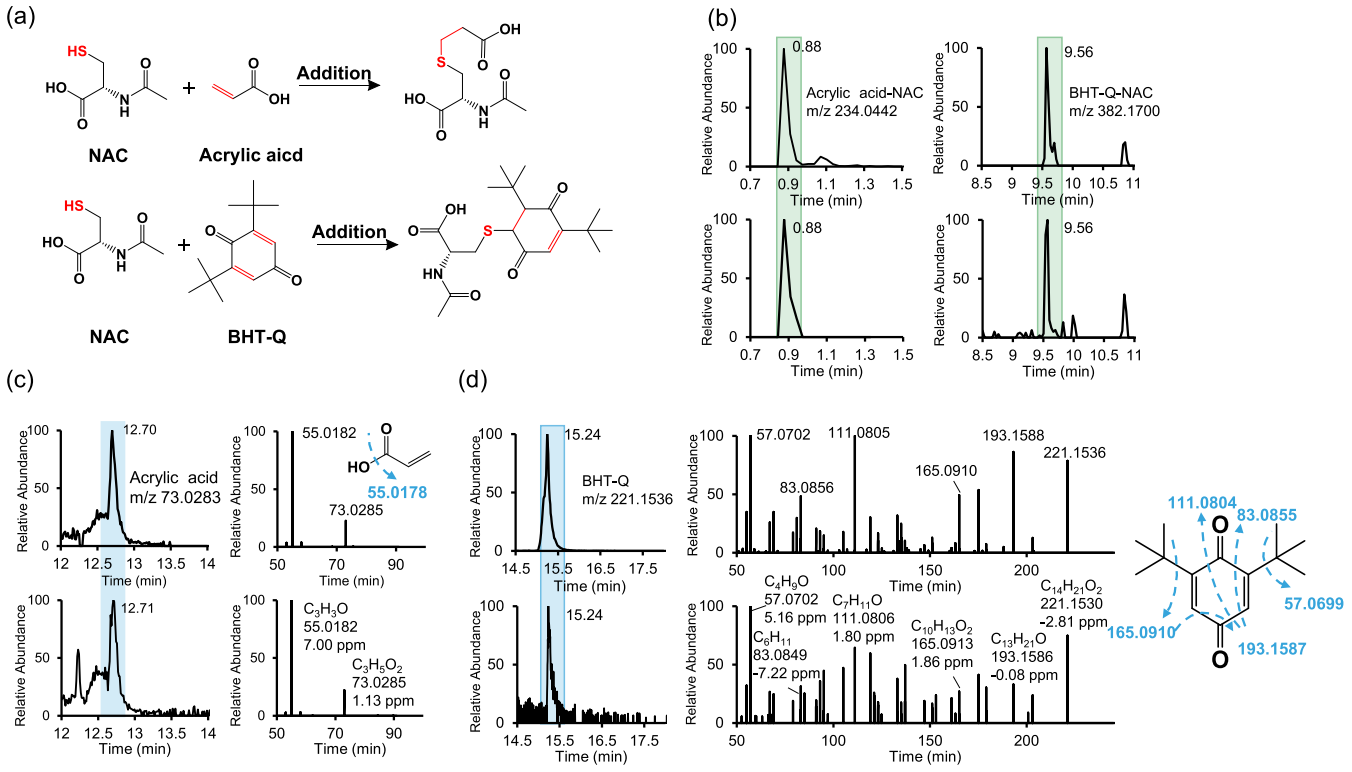

Figure 3. Confirmation of the identities of two representative α,β-unsaturated carbonyls using authentic standards. (a) Proposed reaction schemes between NAC and acrylic acid (top) and BHT-Q (bottom). (b) Confirmation of the NAC adducts of acrylic acid (bottom left) and BHT-Q (bottom right) by matching their retention time to the NAC adducts found after the reaction of authentic standards (top) with NAC. (c) Confirmation of acrylic acid (bottom) by matching the retention time and MS/MS spectrum to the authentic standards (top). (d) Confirmation of BHT-Q (bottom) by matching the retention time and MS/MS spectrum to the authentic standards (top).

at m/z 221.1536 and RT = 15.25 min, matching the RT and fragmentation pattern in the water-extracted sample, allowing for BHT-Q identification at Level 1 confidence. Quinones are recognized as a class of highly reactive compounds known to react quickly with NAC via adduct formation in treated wastewater,[56,57] indicating that this approach can be utilized to identify NAC adducts. Peak 3 was detected in the DIA window of m/z 300−320 (Figure 2), with a 1.34 min RT, and m/z 311.0708 precursor ion. Then, the NAC-treated sample was analyzed in DDA mode to confirm the production of the characteristic peak, and the MS/MS spectrum of the untreated sample was analyzed to obtain the potential α,β-unsaturated carbonyl. The candidate was proposed as N-acetyl-p-benzoquinone imine (NAPQI). The RT of the m/z 311.0708 (1.9 ppm) ion was the same as the NAC adduct of NAPQI (Figure S4a). We found that the RT (Figure S4b) and the fragment ions of the DDA MS/MS spectrum (Figure S4c) in the untreated water extract sample matched those in the MS/MS spectrum of commercially available NAPQI standards. Based on these data, we confirmed the compound as NAPQI, a chlorination product of acetaminophen (Level 1). The binding of NAPQI with NAC has been reported in previous studies,[58,59] demonstrating that this framework can be used to discover NAC adducts. Peak 4 was observed at 8.09 min RT in the DIA mode of m/z 260−280 (Figure 2), and its precursor was identified as m/z 260.0966. Similar to the previously discussed peaks, trans-2-hexenal was tentatively identified. The retention time of ion at m/z 260.0966 (1.15 ppm) matched the NAC adduct of *trans*-2-hexenal (Figure S5a). The DDA MS/MS spectrum (shown in Figure S5c) revealed fragment ions at m/z 57.0338 ($C_3H_5O^+$, 5.2 ppm), m/z 81.0699 ($C_6H_9^+$, 0.4 ppm) and m/z 55.0546 ($C_4H_7^+$, 6.9 ppm). RT data (Figure S5b) and spectra for a commercially available standard collectively confirmed this compound as trans-2-hexenal (Level 1). Peak 5, observed at 8.58 min within the DIA scan range of m/z 240−260, had its precursor ion pinpointed at m/z 258.0806, and the candidate was identified as 3-methyl-2-cyclopenten-1-one. A comparison of both the RT and the MS/MS spectra between the NAC adduct and the standard reference, confirmed the compound as 3-methyl-2-cyclopenten-1-one (Figure S6). The precursor of peak 6 (RT = 11.45 min) with a precursor ion of m/z 390.1602 was identified as dibutyl maleate (Figure S7a). The DDA MS/MS spectrum (Figure S7c) contained fragment ions of m/z 57.0702, m/z 99.0077 and m/z 117.0182, as well as the matching RT confirmed compound identity (Level 1, Figure S7b). Similarly, peak 7 (RT = 10.13 min) with a precursor ion of m/z 410.1654 was identified as parthenolide, and verified using a reference standard (Level 1, Figure S8).

Overall, we identified seven α,β-unsaturated carbonyls in the water sample extract by utilizing a nontarget screening method based on characteristic fragments and authentic standards. These compounds are acrylic acid, BHT-Q, NAPQI, *trans*-2-hexenal, 3-methyl-2-cyclopenten-1-one, dibutyl maleate, and parthenolide. Among them, 3-methyl-2-cyclopenten-1-one, dibutyl maleate, and parthenolide were discovered for the first time. The identification of these compounds significantly enhances our understanding of the species of α,β-unsaturated carbonyls present in chlorine-disinfected wastewater.

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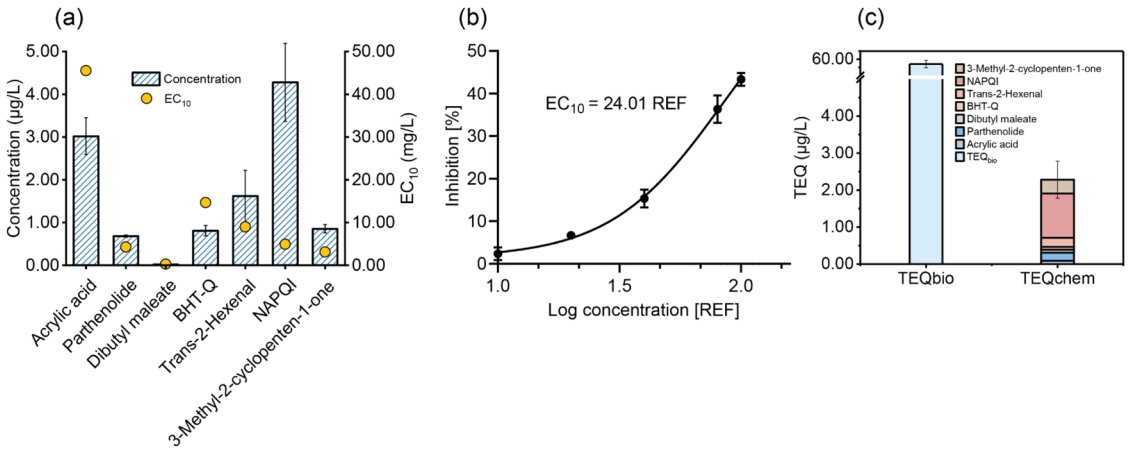

Figure 4. Toxicity assessment of confirmed chemicals to the water extract sample. (a) The concentration and EC_{10} values of 7 confirmed chemicals detected in the water extract sample. (b) The concentration-effect curve of the water extract sample. (c) Toxicity equivalent concentrations (TEQ) determined for the water extract sample (TEQ_{bio}) and the relative contribution of each chemical (TEQ_{chem}) tested individual.

Occurrence of Emerging α,β-Unsaturated Carbonyls in Chlorine-disinfected Secondary Effluent. Using commercially available standards, 7 thiol-reactive α,β-unsaturated carbonyls, including acrylic acid, BHT-Q, NAPQI, *trans*-2-hexenal, 3-methyl-2-cyclopenten-1-one, dibutyl maleate, and parthenolide, were quantified in the water sample at concentrations ranging from 0.0170 to 4.28 μg/L (Figure 4a). NAPQI was most abundant, with a 4.28 ± 1.6 μg/L (mean ± standard derivation, n = 3) concentration, followed by acrylic acid (3.02 ± 0.43 μg/L), *trans*-2-hexenal (1.62 ± 0.60 μg/L), 3-methyl-2-cyclopenten-1-one (0.854 ± 0.096 μg/L), BHT-Q (0.810 ± 0.12 μg/L), parthenolide (0.682 ± 0.030 μg/L) and dibutyl maleate (0.0170 ± 0.0010 μg/L).

Several of the quantified compounds have been detected in environmental samples in the past. NAPQI is a byproduct of acetaminophen (a widely used analgesic) chlorination.[60] At a 4 mg-Cl_2/L chlorine dose, NAPQI can form from acetaminophen at an observed 25% yield after 1 h.[61] NAPQI can also form within the human body during acetaminophen decomposition, where it depletes hepatic glutathione levels and accumulates, potentially inducing oxidative stress and hepatocellular liver damage.[58] The significant concentrations of NAPQI in disinfected water raise concerns about its potential health implications, particularly given its established hepatotoxic effects. The concentration of BHT-Q was also reported in different water samples such as raw wastewater (20−35 ng/L), treated wastewater (48−63 ng/L), and tap water (10−90 ng/L),[62] at concentrations 10−80 times lower than in the current study. BHT-Q is a transformation product of butylated hydroxytoluene (BHT), a synthetic phenolic antioxidant prevalent in various products, including rubber, elastomers, plastics, cosmetics, pharmaceuticals, and food products.[63−65] Rodil et al. showed that BHT could be oxidized to considerably stable BHT-Q through chlorination.[62] Both BHT-Q and BHT, have been detected in two activated sludge WWTPs in Albany, New York.[65] BHT additionally biotransforms to BHT-Q in the human body, and could pose health risks by reacting with nucleophilic sites in biomolecules.[66] A recent study also identified acrylic acid in disinfected drinking water, observed at relatively high concentrations ranging from 2.18 to 23.42 mg/L in water extracts (20 REF).[24] *Trans*-2-hexenal has been detected in potable water reuse facilities and conventional drinking water treatment plants, with the highest recorded concentration of 0.205 μg/L.[8]

While compounds such as NAPQI, BHT-Q, acrylic acid, and trans-2-hexenal have been detected in water in the past, 3-methyl-2-cyclopenten-1-one, dibutyl maleate, and parthenolide have not been reported previously, showing that our framework can identify previously undetected compounds that could pose a health risk. The emergence of these α,β-unsaturated carbonyls underscores the critical need for a thorough assessment of their potential toxicity and their collective impact on the safety of disinfected wastewater.

Toxicity Contribution of Confirmed Chemicals to the Water Extract Sample. To determine the toxicity contribution of thiol-reactive α,β-unsaturated carbonyls to the water sample, the total bacterial toxicity of the water extract sample and identified α,β-unsaturated carbonyls was tested using *V. fischeri*. For the bioassay of the water extract sample, the measured response was plotted against the sample concentration expressed as the relative enrichment factor (REF) (Figure 4b and Table S5). The ideal case would be a full concentration-inhibition curve covering 0 to 100% of the effect, which typically has a sigmoidal shape that can be described with a log−logistic equation, from which an EC_{50} can be calculated. The EC_{50} is often used in toxicology as a sensitivity indicator of a sample or a compound. As the water sample investigated in this study showed a low effect level, the EC_{10} value for the water extract sample derived from the log concentration-inhibition curve was chosen instead. The EC_{10} value was converted to a toxicity equivalent concentration for the water sample, TEQ_{water}. TEQ_{water} converts the toxicity into the concentration of reference compound that would elicit the same response as the sample mixture, simplifying mixture-toxicity modeling.[36,67] In this study, the TEQ_{water} was calculated at 57.1 μg/L.

The EC_{10} values of the identified substances were derived by measuring the dose−response relationships of all individual compounds, which were determined at 0.290−45.5 mg/L (Figure 4a). As predicted, acrylic acid showed weak bacterial cytotoxicity, consistent with a previous study.[24] On the other hand, the most potent bacterial cytotoxicity was observed for dibutyl maleate, which was found at the lowest concentration. The more potent cytotoxicity of dibutyl maleate is consistent with its greater thiol reactivity. The contribution of the

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individual chemicals to the mixture toxicity was calculated using the measured concentrations and relative potencies of each of the seven identified chemicals, the sum thereof was converted to a toxicity equivalent concentration of the chemicals, TEQ_{chem}. The seven compounds explain 4% of the bacterial toxicity in the water extract sample (TEQ_{water} = 57.1 μg/L and TEQ_{chem} = 2.28 μg/L, Figure 4c) and NAPQI was the predominant contributor to the total explained toxicity (52%, respectively) (Figure 4c and Table S5). Our results are able to explain a fairly high fraction of the total sample toxicity. In an earlier study, ten water samples were analyzed, and up to 64 chemicals regulated by the U.S. Environmental Protection Agency (EPA) that were detected from a target list of over 200 chemicals explained less than 1% of toxicity in the environmental water samples.[36] Here, the seven confirmed α,β-unsaturated carbonyls included for toxicity assessment in the current study explained 4% of the toxicity, highlighting the importance of unregulated compounds for total sample toxicity. Our framework can be used to unveil the contribution of the remaining identified compounds to toxicity when standards become available.

ENVIRONMENTAL IMPLICATIONS

This study has several limitations. First, the proposed method primarily targets NAC adducts formed via Michael addition with α,β-unsaturated carbonyls, which may limit the detection of other electrophilic species capable of reacting with thiols. Compounds such as nitriles, esters, or halogenated DBPs may also form adducts with NAC, but are not fully captured by the current analytical workflow. Second, relatively low recoveries were observed for certain volatile α,β-unsaturated carbonyls, including acrylic acid and *trans*-2-hexenal, indicating that improvements in sample pretreatment (e.g., pH and temperature) are needed to minimize analyte loss. Third, chromatographic separation of highly polar or volatile compounds was suboptimal, with issues such as peak broadening or splitting. These challenges highlight the need for further optimization of LC conditions, including the selection of alternative stationary phases or mobile phase compositions. Finally, although NAC adducts were used to infer chemical identities, some of the observed cytotoxicity in the water sample likely stems from unidentified compounds or alternative DBPs not reacting through the same mechanisms. Additionally, the analyzed secondary-effluent samples were collected from a single WWTP, the observed environmental concentrations and 4% explained cytotoxicity should not be taken as representative of all reuse waters.

Despite these limitations, our study demonstrates the utility of NAC as a thiol probe for the nontarget identification of electrophilic contaminants in treated wastewater. A total of 58 thiol-reactive compounds were tentatively identified, among which 3-methyl-2-cyclopenten-1-one, dibutyl maleate, and parthenolide were reported in disinfected water for the first time. Seven compounds, including NAPQI and acrylic acid, were confirmed using authentic standards. However, the identified α,β-unsaturated carbonyls accounted for only 4% of the observed bacterial cytotoxicity, underscoring the complexity and unknown risks associated with disinfection byproducts. Future research should aim to broaden the chemical coverage of thiol-reactive species by incorporating complementary analytical strategies, such as alternative thiol-based probes, DIA methods, and expanded adduct mass searches. Development of next-generation derivatization agents with improved selectivity, reactivity, and compatibility with complex matrices would enhance screening performance. Furthermore, structural confirmation of newly identified compounds through synthesis and toxicity evaluation using more relevant bioassays is essential. Lastly, the environmental health implications of these compounds should be further investigated, and alternative disinfection approaches that minimize the formation of reactive DBPs should be considered to ensure both microbial safety and chemical safety in treated water.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.5c04392.

Detailed information on chemicals and reagents, methods of quality control and compound quantification, potential precursors of model α,β-unsaturated carbonyls-NAC adducts and their MS/MS fragmentation, candidate compounds identified in the NAC-treated sample, instrumental parameters, nontarget analysis workflow, chromatogram and MS/MS spectra of α,β-unsaturated carbonyls-NAC adducts (PDF)

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Notes

The authors declare no competing financial interest.

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