A Quantitative Toxicogenomics Assay Reveals the Evolution and Nature of Toxicity during the Transformation of Environmental Pollutants

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Supporting Information

ABSTRACT: The incomplete mineralization of contaminants of emerging concern (CECs) during the advanced oxidation processes can generate transformation products that exhibit toxicity comparable to or greater than that of the original contaminant. In this study, we demonstrated the application of a novel, fast, and cost-effective quantitative toxicogenomics-based approach for the evaluation of the evolution and nature of toxicity along the electro-Fenton oxidative degradation of three representative CECs whose oxidative degradation pathways have been relatively well studied, bisphenol A, triclosan, and ibuprofen. The evolution of toxicity as a result of the transformation of parent chemicals and production of intermediates during the course of degradation are monitored, and the quantitative toxicogenomics assay results revealed the dynamic toxicity changes and mechanisms, as well as their association with identified intermediates during the electro-Fenton oxidation process of the selected CECs. Although for the three CECs, a majority (>75%) of the parent compounds disappeared at the 15 min reaction time, the nearly complete elimination of toxicity required a minimal 30 min reaction time, and they seem to correspond to the disappearance of identified aromatic intermediates. Bisphenol A led to a wide range of stress responses, and some identified transformation products containing phenolic or quinone group, such as 1,4-benzoquinone and hydroquinone, likely contributed to the transit toxicity exhibited as DNA stress (genotoxicity) and membrane stress during the degradation. Triclosan is known to cause severe oxidative stress, and although the oxidative damage potential decreased concomitantly with the disappearance of triclosan after a 15 min reaction, the sustained toxicity associated with both membrane and protein stress was likely attributed at least partially to the production of 2,4-dichlorophenol that is known to cause the production of abnormal proteins and affect the cell membrane. Ibuprofen affects the cell transporter function and exhibited significantly high membrane stress related to both membrane structure and function. Oxidative degradation of ibuprofen led to a shift in its toxicity profile from mainly membrane stress to one that exhibited not only sustained membrane stress but also protein stress and DNA stress. The information-rich and high-resolution toxicogenomics results served as “fingerprints” that discerned and revealed the toxicity mechanism at the molecular level among the CECs and their oxidation transformation products. This study demonstrated that the quantitative toxicogenomics assay can serve as a useful tool for remediation technology efficacy assessment and provide guidance about process design and optimization for desired toxicity elimination and risk reduction.

INTRODUCTION

Great scientific and engineering challenges exist in addressing the water quality problems associated with contaminants of emerging concern (CECs) in terms of understanding their harmful impact and risk and developing cost-effective remediation technologies.1 Traditional water and wastewater treatment processes are not designed to eliminate most of the CECs, especially at the trace levels present in source water and the aquatic environment.2,3 Various treatment technologies have been explored, and advanced oxidation processes (AOPs) such as photocatalysis,4 ozonation,5 dioxide chlorine,6 a Fenton-based process,7−11 and use of other strong oxidants12 have been reported to be promising for the effective degradation of CECs. Considering the energy and cost, the mineralization rates (i.e., TOC removal) by AOPs are generally

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low, generating byproducts often with polarity and solubility higher than those of the parent compounds. The oxidative transformation pathways of organic contaminants are rather complex and dynamic, and the degradation intermediate mixtures might exhibit toxicity comparable to or greater than that of the original contaminant(s). Some recognized toxic byproducts include hydroxylamines, phenols, quinones, aldehydes, and chlorinated or brominated compounds. Although it is recognized that monitoring and understanding the evolution of toxicity during the remediation process are greatly important for risk reduction assessment, studies that comprehensively investigate the temporal evolution of toxicity during remediation processes and their association with transformation intermediates are very rare because of the lack of a generally accepted and feasible toxicity evaluation approach.

The challenges in the required resources and time participated to handle the toxicity testing efforts for the large and ever-increasing number of contaminants in various environmental samples. Techniques developed for toxicity testing and their association with the identification of transformation intermediates are still required a substantial amount of time and effort. In addition, most of them provide only specific toxicity endpoints without more comprehensive and detailed information about the toxicity profiles and mechanisms. Furthermore, isolated assays that cover only one biomarker or a few or specific effects cannot be directly translated to an integrated adverse outcome or phenotypic endpoints.

Recently, we reported a toxicogenomics-based toxicity assessment method that allows a fast, yet informative, mechanistic and quantitative toxicity evaluation of pollutants. Compared with the traditional cytotoxicity approach, or the current battery of bioassays, this quantitative toxicogenomics method in combination with bioinformatics computation detects the overall toxicity level and reveals potential toxicity profiles and mechanisms with a single assay within hours (2–4 h), which greatly reduces the test time, amount of resources, and cost. In addition, the measurement is sufficiently sensitive to capture the subcytotoxic impact at concentrations much lower than those that can lead to detectable phenotype effects; therefore, it requires fewer or even possibly eliminates the sample extraction and concentration steps. The proposed method may serve as an alternative or complementary approach to the current toxicity assays for environmental applications.

In this study, we, for the first time, demonstrate the application of a quantitative toxicogenomics-based approach for the evaluation of the evolution and nature of toxicity along the electro-Fenton oxidative degradation of triclosan and ibuprofen. These chemicals were selected because their toxicity and oxidative degradation pathways have been relatively well-studied. The temporal changes in toxicity level and profile as the results of the transformation of parent chemicals and production of intermediates during the course of remediation were monitored to reveal the dynamic toxicity changes and mechanisms, as well as their association with specific intermediate intermediates during the oxidative transformation process.

**Materials and Methods**

**Chemicals and Wastewater Samples.** Bisphenol A, 4-chlorocatechol (97%), 4-chlororesorcinol (98%), chloro-p-benzoquinone (95%), and 4-isobutylacetophenone were purchased from Sigma-Aldrich. Ibuprofen (99%), 2,4-dichlorophenol (99%), 4-ethylbenzadehyde (98%), resorcinol (98%), chlorohydroquinone (90%), catechol (99%), and benzoquinone (99%) were supplied by Acros. Triclosan (99%) was purchased from Calbiochem, and hydroquinone was from Fischer Scientific. Deionized (DI) water (18.0 mΩ cm) obtained from a Millipore Milli-Q system was used in all the experiments. All chemicals used in this study were above analytical grade.

**Electro-Fenton Degradation of CECs.** A 600 mL acrylic cell was used for the electro-Fenton degradation of CECs at ambient temperature (25 ± 1 °C). Two pieces of mixed metal oxides (MMO, IrO2/Ta2O5 coated on titanium mesh type, 3N International) were selected to provide a 42 mm spacing in parallel position. A more detailed description and characterization of the electro-Fenton system was described previously. A single in vitro cell-based bioassay or a battery of them using cell lines or biomarkers have been successfully applied to environmental water samples. These in vitro bioassays are more sensitive to early warning signs, because cellular responses are activated before the actual harm occurs, and they indicate the presence of associated stressors. However, these batteries of bioassays consisting of a number of separated assays with different species still require a substantial amount of time and effort. The proposed method may serve as an alternative or complementary approach to the current toxicity assays for environmental applications.

In this study, we, for the first time, demonstrate the application of a quantitative toxicogenomics-based approach for the evaluation of the evolution and nature of toxicity along the electro-Fenton oxidative degradation of triclosan and ibuprofen. These chemicals were selected because their toxicity and oxidative degradation pathways have been relatively well-studied. The temporal changes in toxicity level and profile as the results of the transformation of parent chemicals and production of intermediates during the course of remediation were monitored to reveal the dynamic toxicity changes and mechanisms, as well as their association with identified intermediates during the oxidative transformation process.

**Identification and Chemical Analysis of Transformation Products.** The detailed methods for the identification and chemical analysis of transformation products were described previously. Briefly, bisphenol A, triclosan, and ibuprofen were measured by a 1200 Infinity Series high-performance liquid chromatography (HPLC) system (Agilent) equipped with both a 1260 diode array detector (DAD) and a 1260 fluorescence detector (FLD) and with two columns, a Thermo ODS Hypersil C18 column (4.6 mm × 50 mm) and an Agilent Eclipse AAA C18 column (4.6 mm × 150 mm). The key transformation intermediates at different time points during the remediation process were identified by HPLC and gas chromatography coupled to mass spectrometry and are listed in Table S1 of the Supporting Information. The proposed possible transformation pathways for the three CECs based on the identified key intermediates are summarized in Figure S1 of the Supporting Information. The TOC concentration was measured with a TOC analyzer (TOC-L CPH, Shimadzu).

**Sample Preparation for Toxicity Assessments.** The samples taken during the oxidation process at 0, 15, 30, and 60 min were subjected to the toxicity assessment. All samples were neutralized to pH ~7 prior to enrichment by lyophilization (Freezone 4.5, LABCONCO, Kansas City, MO) at −42 °C under a 1 Torr vacuum. The lyophilization procedure was applied because it has been reported to yield higher rates of...
recovery of nonvolatile compounds than solid phase extraction (SPE)."\textsuperscript{37}

**Microtox Assay.** A photobacterial *Vibrio fisheri* (ATCC 49387) specific photosynthesis inhibition test (Microtox) was used for the evaluation of nonspecific toxicity according to ISO standard method 11348-2.\textsuperscript{38} The assay was performed in a solid black 96-well microplate (Costar, Bethesda, MD). The inhibition of luminescence was calculated as the difference between the light intensity at time 0 and 15 min measured with a microplate reader (Synergy Multi-Mode, Biotek, Winooski, VT). A 1 M NaCl solution was used as a negative control, and ZnSO\textsubscript{4} was used as a positive control. The results from Microtox tests were expressed as the inverse of EC\textsubscript{50}, which was the concentration causing a 50% photosynthesis inhibition effect. The concentration is reported in units of relative enrichment factor (REF), which is defined as the ratio of the enrichment factor from the sample concentration (i.e., extraction) step and the dilution factor of the water sample concentrated for the toxicity bioassay.

**Toxicogenomics Assay and Endpoint Derivation.** A high-throughput mechanistic toxicity assay method was employed that uses a GFP-fused whole-cell array of *Escherichia coli* K12, MG1655.\textsuperscript{31,39,40} The selected assay library monitors the promoter activities of an assembly of genes involved in different known cellular stress response pathways, such as oxidative stress, DNA stress, protein stress, membrane stress (including membrane transporter, efflux pump, energy metabolism, flagella metabolism, and lipopolysaccharide metabolism), etc. (Table S2 of the Supporting Information).

The detailed protocol for the toxicogenomics assay was described previously.\textsuperscript{31,32} In brief, *E. coli* was cultivated in 384-well plates (Costar) in the dark to prevent GFP photobleaching until the early exponential growth stage was reached (OD\textsubscript{600} ∼ 0.2). The enriched (via freeze-dry) water samples were resuspended in minimal medium (M9) containing *E. coli* culture strains at various concentrations. The plate was then placed into a microplate reader (Synergy Multi-Mode, Biotek) for the measurement of cell growth (absorbance, 600 nm) and fluorescence (excitation at 485 nm, emission at 528 nm) every 5 min over a period of 2 h. All tests were conducted in triplicate.

The alteration of gene expression, also called induction factor \(I = P_e / P_c\), for a given gene at each time point due to sample exposure, was represented by the ratio of the cell-normalized gene expression GFP level (normalized over cell concentration) under the experimental condition with water sample exposure \((P_e)\) to that under the control condition without any chemical exposure \((P_c)\). Then the natural log of \(I = \ln(I)\) at every time point was compiled for further analysis. All data were corrected for various controls, including blank with medium control (with and without water samples) and promoterless bacterial controls (with and without water samples). The TELI (transcriptional effect level index) value, a recently developed index for the interpretation of toxicogenomics data, was employed to quantify the magnitude of the altered gene(s) expression level upon the exposure to toxicants. TELI is calculated by integrating induction factor values over time and indicates the cumulative transcriptional effect of a given gene over a certain exposure period.\textsuperscript{32,41} TELI values can be determined for a single gene or for a number of genes representing a specific pathway or the entire stress response library.\textsuperscript{32} TELI exhibited dose–response patterns for both a single gene and a gene ensemble.\textsuperscript{32} The specific or overall toxicity obtained by the toxicogenomics-based approach was represented by the corresponding REF that causes the TELI value to reach 1.5 (termed EC-TELI\textsubscript{1.5}) based on the dose–response curves, similar to the approach that has been applied for the umuC genotoxicity assay by Escher et al.\textsuperscript{42} In addition, the corresponding oxidative stress and genotoxicity for each sample were also calculated as the toxic equivalents, termed oxidative_TEQ and geno_TEQ, as concentrations of reference compounds H\textsubscript{2}O\textsubscript{2}\textsuperscript{43} and mitomycin\textsuperscript{44} respectively.

**Gene Enrichment Analysis.** To evaluate the activities of a certain pathway or assembly of genes, gene set enrichment analyses (GSEA) were performed by ranking a list of genes based on their TELI values according to the work of Aravin.\textsuperscript{45} For each pathway or stress response category, GSEA calculated the enrichment score by examining the ranked gene list from the high-score end to the low-score end, giving a rewarding score if a gene belongs to the pathway of interest and a penalizing score otherwise. The significance \((p < 0.05)\) of each pathway was determined by comparing their ranking scores to the corresponding empirical distributions. The null distributions were generated by randomly permuting the specific pathway and all others 1000 times.

**RESULTS AND DISCUSSION**

**Temporal Toxicity Evolution and Profile Changes during the Degradation of CECs.** Toxicity changes during the electro-Fenton oxidation of the CECs were evaluated via both the toxicogenomics assay and commonly used Microtox test for comparison. Figure 1 and Figure S2 of the Supporting Information showed the toxicity results during the electro-Fenton transformation of the three chemicals at 0, 15, 30, and 60 min based on the toxicogenomics assay endpoints and the Microtox results, respectively. The Microtox assay detected the toxicity of only triclosan and not that of two other chemicals at time zero, indicating that triclosan exhibits a toxicity higher than those of bisphenol A and ibuprofen at the same concentration. Bisphenol A and ibuprofen are known to be nontoxic to Gram-negative bacteria such as *V. fisheri*.\textsuperscript{46} The Microtox results indicated a decrease in the toxicity of triclosan after 15 min oxidative transformations. However, the changes in toxicity of bisphenol A and ibuprofen could not be detected and discerned by the Microtox assay at the detection limit under our study condition [relative enrichment factor (REF) = 10]. In comparison, the more sensitive toxicogenomics assay clearly revealed the trends of toxicity reduction of the three CECs during the transformation process.

For the three CECs tested, the parent compound was reduced rapidly during the initial 15 min, with 73, 96, and 83% reduction for bisphenol A, triclosan, and ibuprofen, respectively. However, toxicity evaluation, based on molecular endpoint 1/EC-TELI\textsubscript{1.5} values, indicated that the toxicity did not always decrease proportionally and concomitantly with the disappearance of the parent contaminant (Figure 1). For example, although 83% of ibuprofen disappeared after 15 min, the overall toxicity did not seem to decrease and actually slightly increased (Figure 1c). The sustained or elevated toxicity was likely associated with the production of toxic intermediates (see Table S1 of the Supporting Information and a detailed discussion below). The nearly complete elimination of toxicity (1/EC-TELI\textsubscript{1.5} values of <1 are considered nontoxic according to the EPA WET and ISO water quality determination methods\textsuperscript{47,48}) for the three CECs required a minimal reaction time of 30 min, and they seemed to correspond to the
disappearance of identified aromatic intermediates (see the pathway of degradation and intermediate identification in Table S1 and Figure S1 of the Supporting Information). The TOC measurements also suggested limited mineralization even after degradation for 60 min, with 49.2, 74.1, and 64.3% TOC remaining for bisphenol A, triclosan, and ibuprofen, respectively (Figure S3 of the Supporting Information). This implies that practical AOP applications will unlikely achieve complete mineralization with economical considerations. Therefore, system design, optimization, and operation should incorporate remediation efficacy assessment to ensure the desired toxicity and risk reduction.49,50

The Quantitative Toxicogenomics Assay Reveals the Nature and Dynamics of Toxicity Evolution during the Transformation of CECs. The changes in the temporal toxicity profile during the transformation of individual CECs are shown in Figure 2, and the significantly altered stress response category (p < 0.05) based on gene set enrichment analysis is highlighted. The magnitude of gene expression changes was indicated by the absolute TELI value of each gene after a 2 h exposure (that considers both up- and down-regulation as altered expression).32 These profiles revealed the dynamic nature of toxicity resulted from the individual CECs as well as from a mixture of transformation products during the course of the degradation process. To further quantitatively illustrate the nature of toxicity changes during the transformation of the CECs, molecular endpoint 1/EC-TELI1.5 values for the individual stress response categories were calculated and are plotted in Figure 3. The potential oxidative stress and genotoxicity were also calculated as the equivalent concentrations of reference compounds H2O243 and mitomycin,44 respectively (Figure 3). The concept of a toxic equivalent concentration was a widely applied method for expressing the toxicity of complex mixtures of compounds that act via receptor-mediated mechanisms such as induction of the aryl hydrocarbon or estrogen receptors. Escher et al. expanded this concept to a molecular assay such as the umuC assay for genotoxicity.42,51 Here, we further expanded the TEQ concept for quantifying toxicogenomics-based molecular assays. As shown in Figures 2 and 3, bisphenol A seemed to impact the cell across all stress response categories. Bisphenol A is known to cause DNA damage as it activates the recA gene that serves as a regulatory protein to initiate the SOS response to DNA damage.52,53 Most of the identified intermediates from bisphenol A degradation at 15 min contained a phenolic or quinone group, and detected quinone electrophilic reactive intermediates such as 1,4-benzoquinone and hydroquinone could cause DNA damage (Table S1 of the Supporting Information), therefore leading to or at least contributing to the sustained DNA stress.54−56 Bisphenol A also induced sustained
dichlorophenol can cause protein stress by the production of oxidant H$_2$O$_2$. The phenol group in 2,4-bisphenol A (transformation process). The left Y-axis shows 1/EC-TELI1.5 values for individual stress response categories were determined from dose–response curves and revealed the magnitude as well as specific toxicity mechanism and nature. The right Y-axis shows the genotoxicity, measured as Geno_TEQ, determined as the equivalent concentration of reference model genotoxic compound MMC (mitomycin) that yields a TELI value of 1.5. The oxidative damage potential, measured as oxidative-TEQ, was determined as the equivalent concentration of reference oxidant H$_2$O$_2$ that yields a TELI value of 1.5.

membrane stress at 15 min, which might be contributed by one of the intermediates identified, 1,4-benzoquinone. As a bioactive quinone, 1,4-benzoquinone affects the electron transport chain and transporter activity on the membrane.56

Triclosan is known to cause severe oxidative stress as well as DNA stress by permeating the bacterial cell wall and targeting multiple cytoplasmic and membrane sites.34,57 This was consistent with the significant oxidative stress and DNA stress exhibited by triclosan at time zero (Figures 2 and 3). Both the oxidative damage potential and the genotoxicity (oxidative_-TEQ and geno_TEQ, respectively) decreased concomitantly with the disappearance of triclosan after a 15 min reaction. The sustained toxicity associated with both membrane and protein stress (Figure 3b) at 15 min was likely attributed at least partially to the production of 2,4-dichlorophenol (Table S1 of the Supporting Information). The phenol group in 2,4-dichlorophenol can cause protein stress by the production of abnormal proteins and membrane stress when entering the cell through the outer and inner membranes.58

Ibuprofen affects the transporter function as influx and efflux of the cell membrane;59 thus, it exhibited significantly high membrane stress at the initial stage (Figures 2c and 3c). Oxidative degradation of ibuprofen led to a shift in the toxicity profile from mainly membrane stress to one that exhibited not only membrane stress but also protein stress and DNA stress at 15 min. The sustained or even slightly increased toxicity corresponding to membrane and protein stresses at 15 min was likely associated with the identified intermediate, 4-isobutylacetoophenone, which was known to be toxic to cell walls and the membrane.60 Both ibuprofen and 4-isobutylacetophenone can strongly bind to proteins, affecting protein metabolism and causing protein dysfunction, thus inducing protein stress.35 The slightly elevated DNA stress at 15 min indicated that either the identified transformation byproduct 4-isobutylacetophenone exhibits genotoxicity or there was another unidentified genotoxic intermediate(s). Oxidative stress genes yielded a relatively weak response for both ibuprofen and its intermediates, in agreement with previous reports.35,59

For the three CECs evaluated, a minimum of 30 min seemed to be required for more complete toxicity reduction, although varying levels of residual toxicity remained. The residual toxicity exhibited as general stress, membrane stress, and oxidative stress after 30 and 60 min electro-Fenton transformations of bisphenol A indicated the potential production of persistent toxic products. These results demonstrated that oxidative transformation pathways of organic contaminants were rather complex and may lead to dynamic toxicity evolutions and changes as a result of the formation of degradation intermediates. The toxicogenomics-based assay provided detailed information about and insights into the dynamic toxicity changes and mechanisms, as well as their association with identifiable intermediates during the transformation processes.

Insights into the Distinct Toxicity Mechanisms and Profiles during the Transformation of Three CECs. More in-depth examination of gene activities disclosed genetic level information and distinction among the toxic effects and the mechanism of the parent testing chemicals, and their intermediates evolved during the oxidative transformation process. Figure 4 shows the detailed gene activation (TELI value for a single gene of >1.5) for oxidative stress, DNA stress, and membrane stress response pathways.

Oxidative Stress. Figure 4a illustrates the altered gene expression in selected essential oxidative stress biomarker genes, which are involved in defending against oxidative stress and scavenging oxidative radicals in the form of enzymes that can detoxify reactive oxygen species (ROS).33 Three of them are ROS sensors, oxyR, soxR, and soxS. Two alkyl hydroperoxide reductases, ahpC and ahpF, are the primarily scavenging enzymes for endogenously produced organic hydroperoxide and H$_2$O$_2$. Two catalase/hydroperoxidases, katG and katE, are the primary scavengers at high H$_2$O$_2$ concentrations. Three superoxide dismutases (SODs), sodA, sodB, and sodC, represent the first line of defense against ROS, converting superoxide radicals to hydrogen peroxide and water.

For all three CECs, although the parent chemical as well as the resultant mixture of residual parent compound and intermediates exerted oxidative stress, the distinct activation profiles of specific oxidative stress biomarkers revealed the discernible and varying molecular level oxidative damaging pathway activities evoked by the different chemicals. Both
bisphenol A and triclosan altered more than half of the selected oxidative stress genes at time zero and 15 min, indicating the oxidative nature of these two chemicals and their transformation intermediates. Both bisphenol A and triclosan led to upregulation of the master hydrogen peroxide stress regulator and sensor oxyR at 0 and 15 min, indicating the presence of ROS as a hydrogen peroxide radical. In comparison, ibuprofen led to overexpression of fewer oxidative stress biomarkers and

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**Figure 4.** Stress response pathways and biomarker genes that showed altered expression (TELI gene > 1.5) during the electro-Fenton transformation of the three CECs: (a) oxidative stress pathways, (b) DNA damage and repair pathways, and (c) membrane stress response pathways. The genes were clustered into subcategories on the basis of their functions and involvement in various pathways (Table S2 of the Supporting Information). Different colors indicate different times during the electro-Fenton treatment: blue for 0 min, red for 15 min, green for 30 min, and purple for 60 min.
did not activate oxyR, suggesting a weaker extent of oxidative stress. Varying superoxide dismutases (SODs) were upregulated during the degradation of the three CECs. All of the catalase/hydroperoxidases and alkyl hydroperoxide reductases showed upregulation during the degradation of triclosan, while only part or none of them were upregulated with the other two CECs. This indicated that triclosan and its intermediates likely cause more H$_2$O$_2$-related oxidative stress.

**DNA Stress.** Impacts on DNA damage pathways of the tested chemicals and their transformation products during the electro-Fenton reaction are shown in Figure 4b. Genes recA and lexA, which control the transcription of several genes involved in the cellular response to DNA damage, are involved in SOS regulation. The ada gene, one of the two separate direct repair mechanisms in *E. coli*, controls the transcription of the genes involved in the process of repair of alkylated DNA. Three genes are involved in base excision repair (BER), and they are mutT, nfo, and polB. Nucleotide excision repair (NER) is a generalized DNA repair process that can repair a wide diversity of DNA lesions, and uvrA and uvrD are indicative of this repair pathway. The uvrD gene, which is a superfamily I DNA helicase, is also involved in mismatch repair (MMR). Another gene involved in MMR, sbi, is a highly stable single-stranded DNA binding protein that also functions in double-strand break repair (DBR) and DNA replication (Figure 4b).

Bisphenol A activated lexA, recA, and other genes involved in multiple DNA damage and repair pathways during the 30 min reaction (0, 15, and 30 min), especially those genes involved in double-strand break repair that repair those breaks that occur at two or more locations in the chromosome, indicating relatively severe DNA damage caused by BPA. This indicated that bisphenol A and its intermediates led to the most severe DNA stress among the three CECs, and these DNA-damaging products were relatively persistent and could be eliminated only after 60 min. Triclosan resulted in changes in the gene expression level for most of the DNA repair pathway, including BER, NER, MMR, and DBR. The transformed intermediates of triclosan still exhibited some DNA stress at 15 min. The number of upregulated DNA repair genes increased after degradation of ibuprofen for 15 min, indicating the generation of potentially unknown genotoxic intermediates.

**Membrane Stress.** Membrane stress is less characterized than oxidative and DNA stress in this study. Genes were classified into several subcategories on the basis of the functions related to membrane structure and function, including those related to energy (sdhC and cyoA), multidrug efflux (emrA, emrE, and marR), the cell wall and membrane structure (san, bacA, and dacB), the cell envelope (amiC and clS)A, and flagella (motA and fliG).62

Many genes related to multidrug efflux and the cell membrane structure and envelope were upregulated during the degradation of three CECs, but with distinct activation profiles as the results of different modes of action (Figure 4c). Multidrug efflux is a universal mechanism responsible for the extrusion of toxic substances and antibiotics outside the cell. Most of these genes are often induced by the chemicals with phenolic rings or quinone-mediated bactericidal activity. As discussed earlier, most of the parent compounds and intermediates of the three CECs contained a phenolic or quinone group. Ibuprofen affected many genes related to multidrug efflux, as well as the cell wall and membrane structure, which were believed to be linked to drug resistance.62 Triclosan oxidation seemed to generate membrane-damaging intermediates as suggested by the increased magnitude and number of altered expressions in membrane stress-related genes at 15 min, which were further degraded after 30 min. For bisphenol A, genes related to multidrug efflux, emrA and marR, showed altered expression at 60 min, indicating the potential presence of toxic products (i.e., phenolic or quinone-containing chemicals) even after a 60 min process.

**Evaluation of the Toxicity during the Transformation of Environmental Pollutants.** There are limited reports that evaluated toxicity changes during the degradation of CECs by AOPs. Chiang et al. observed an increase in toxicity in the initial stage of photocatalytic oxidation of bisphenol A and a gradual decrease afterward using the luminescent bacterial *V. fischeri*.25 Dirany et al. noted a sudden increase in the level of inhibition of luminescence of *V. fischeri* for electro-Fenton degradation of sulfachloropyridazine.7 Zhao et al. observed a slight increase in estrogenic activity in the heterogeneous photo-Fenton degradation of 17β-estradiol in simulated drinking water using yeast-based enzyme expression.24 These previous studies reported the changes in nonspecific phenotype toxicity endpoints, with no or limited insights into the temporal dynamics of the nature of toxicity mechanisms associated with transformation products that evolved during the degradation. In comparison, the high-throughput mechanistic toxicogenomics-based toxicity assay used in this study allows a more comprehensive and detailed evaluation of toxicity during the remediation process, which can be linked with key intermediates, thereby improving our understanding of underlying toxicity changes during a degradation process. Therefore, the quantitative toxicogenomics assay may serve as a useful tool for the assessment of remediation technology efficacy and provides guidance for process design and optimization for desired toxicity elimination and risk reduction.

**ASSOCIATED CONTENT**

 Supporting Information

Additional descriptions of the identification of degradation intermediates for bisphenol A, triclosan, and ibuprofen, genes included in the stress assay library, Microtox results, and TOC results (three figures and two tables). This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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