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Water Impact Statement

In this work, we investigated the toxicological responses of 1,4-dioxane – a trace organic solvent widely present in recycled water – during UV-based advanced oxidation processes (UV/AOPs) using cyto- and geno-toxicity bioassays. This is a novel approach to apply quick screening tools to minimize the toxicity response of recycled water, which is critical to portable reuse implementation. This is a timely study considering the occurrence of small and neutrally charged organic molecules in recycled water prior to UV treatment. The manuscript will be of interest to scientists, engineers and practitioners concerned with validation of UV/AOPs for wastewater recycle and potable reuse.

Cyto- and Geno-Toxicity of 1,4-Dioxane and Its Transformation Products during Ultraviolet-Driven Advanced Oxidation Processes

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Abstract

Ultraviolet-driven advanced oxidation processes (UV/AOP) are an integral step in the water reuse treatment train. The toxicity of trace organic transformation products during UV/AOP is critical to its implementation. This study examined the cyto- and geno-toxicity of transformation products of 1,4-dioxane (1,4-D), a trace organic contaminant commonly found in secondary wastewater, in extracts using the CellSensor p53RE-bla HCt-116 cell assay following UV photolysis at 254 nm with three oxidants of hydrogen peroxide (H_2O_2), persulfate ($S_2O_8^{2-}$) and monochloramine (NH_2Cl). 1,4-D was transformed into six major oxidation byproducts, including ethylene glycol diformate, formaldehyde, glycolaldehyde, glycolic acid, formic acid, and methoxyacetic acid. Formaldehyde and glycolaldehyde were the most geno- and cyto- toxic, while 1,4-D had weak genotoxicity and no cytotoxicity. The order for cytotoxicity on the basis of EC50 values followed: glycolaldehyde > formaldehyde > formic acid > glycolic acid > 1,4-D > ethylene glycol diformate ≈ methoxyacetic acid, with glycolaldehyde and formaldehyde showing high genotoxicity. With the three UV/AOPs, genotoxicity expressed as Mitomycin equivalency (MEQ) increased significantly by 10 to 100 fold with a UV dosage of 720 $mJ \cdot cm^{-2}$, mainly due to the formation of glycolaldehyde. UV/$S_2O_8^{2-}$ reduced the MEQ with an extended UV dosage of 1440 $mJ \cdot cm^{-2}$, due to the transformation of toxic aldehydes to less toxic organic acids. In contrast, UV/H_2O_2 increased MEQ with UV dosage, resulting from the accumulation of aldehyde products. UV/NH_2Cl showed the lowest MEQ due to its slow removal of 1,4-D. This study suggests that oxidant and UV dosage can affect the toxicological responses of treatments for recycled water.

Introduction

Reuse of treated wastewater effluent is critically needed to mitigate fresh water shortages.[1-4] Treatment processes typically involve membrane-based pretreatment and reverse osmosis followed by an advanced oxidation process (AOP).[5] Ultraviolet-driven advanced oxidation processes (UV/AOPs) for potable water reuse have been increasingly implemented to remove a variety of trace organic contaminants including pharmaceuticals and personal care products.[6-9] However, the formation of oxidation products with potential high toxicity is of increasing concern. Recently, investigation of toxic UV/AOPs byproducts has been reported and has received increasing attention.[10-14] It is likely that UV/H_2O_2 produces a suite of products that still pose toxicological responses when the parent contaminants are not fully mineralized.[10-12]

Hydrogen peroxide (H_2O_2) is the most commonly used UV/AOP oxidant in potable water reuse, with persulfate ($S_2O_8^{2-}$) and monochloramine (NH_2Cl) also being relevant.[15-17] NH_2Cl is frequently used as a membrane anti-foulant in water reuse treatment trains and can be used as a carry-over oxidant.[18] Each oxidant produces a unique set of reactive radicals in UV photolysis. For instance, H_2O_2 produces $HO^{\bullet}$, $S_2O_8^{2-}$ produces $SO_4^{\bullet-}$ and $HO^{\bullet}$, and NH_2Cl generates $Cl^{\bullet}$, $Cl_2^{\bullet-}$ and $HO^{\bullet}$.[19]

1,4-Dioxane (1,4-D) is ubiquitously present in municipal wastewater effluent, and it is not well rejected by RO membranes because it is a small and neutral molecule. Listed as a class B carcinogen by the USEPA with a notification level of 1 µg/L in California,[20,21] 1,4-D is used as a surrogate to validate UV/AOPs efficiency.[22,23] Efforts have been taken to remove 1,4-D and other neutral molecules in RO permeate using UV/AOPs.[18,21]

However, less attention has been paid to the formation of oxidation products of 1,4-D during water reuse treatment and the associated toxicity implications. There is an urgent need for a better understanding the occurrence of oxidation products and comparing their toxicity levels with 1,4-D. Therefore, the objectives of this research are to identify the formation and distribution of oxidation products of 1,4-D during the treatment by UV photolysis of H_2O_2, $S_2O_8^{2-}$ and NH_2Cl, and compare the toxicity of the transformation products to the original contaminant using human cell-based bioassays.

Materials and Methods

UV/AOP treatments. All chemicals used in this study were reagent grade or higher and obtained from Sigma Aldrich or Fisher Scientific. All cell culture supplies and chemicals were obtained from Life Technologies or Fisher Scientific and stored in appropriate conditions as instructed. The working solution contained 5 mM of an oxidant (*e.g.* H_2O_2, $S_2O_8^{2-}$ or NH_2Cl) and 1 mM 1,4-D at pH 8. This pH was typical of recycled water and all oxidants were chemically stable at this pH. To avoid potential interference of background chemicals on toxicity response, all experiments were conducted in Milli-Q water. A 50-mM NH_2Cl stock solution was prepared daily by adding a NaOCl stock solution to $(NH_4)_2SO_4$ with a N:Cl molar ratio of 1.2 and buffered at pH 8 using borate. Solutions were then transferred to multiple 8-mL quartz tubes and placed in a carousel in a UV chamber (ACE Glass). The samples were illuminated with a low-pressure monochromatic mercury UV lamp (λ=254 nm) at an intensity of 1.2 mW/cm^2 (Phillips TUV6T5) which was cooled by circulated water. The UV fluence was measured by a multimeter equipped with a thermopile 919P sensor (Newport Power meter). Samples

were collected every 5 mins, quenched with 10 mM sodium bisulfite, and followed with chemical analysis.

Analytical Methods. Concentrations of H_2O_2 and $S_2O_8^{2-}$ were measured by potassium iodine colorimetric method,[24] and NH_2Cl were determined using DPD titration.[25] Concentrations of 1,4-D and ethylene glycol diformate were directly measured with an Agilent 1200 liquid chromatography (Text S1). Formaldehyde and glycolaldehyde were derivatized with 2,4-dinitrophenylhydrazine (DNPH) and analyzed by HPLC-UV.[26] Concentrations of glycolic acid, formic acid and methoxyacetic acid were quantified by a Dionex 1000 Ion Chromatography (Text S1).

Toxicity Assays. HCT-116 Human colorectal carcinoma cells were cultured in a 5% CO_2 humidified incubator at 37 ^oC and collected after the fourth passage. Cyto-toxicity assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) and geno-toxicity assay (CellSensor p53RE-bla geno-toxicity assay) were subsequently conducted. These two assays were chosen because they were the most popular and robust toxicity assays to examine DNA damage and cell viability.[27] Details on both bioassays are provided in Text S2. The chemicals employed to treat the cells were prepared from known standards to avoid toxicity interference from other potential toxins including oxidants and quenching reagents. Standards of 1,4-D and its transformation products were mixed based on concentration distribution observed in the UV/AOP experiments (Table S1). Although concentrations of chemicals used in the bioassay were higher than the concentrations detected in recycled water, the data provided insight into the toxicity comparison among 1,4-D and its oxidation products. For the mixture toxicity, the chemical standards were mixed together based on the experimentally determined product compositions after

UV/AOP treatment with a UV dosage up to 1440 mJ·cm^{-2}, which is within the typical UV dosage in water reuse. After chemical exposure, fluorescence of the mixture in the bioassay was recorded using a Victor 2 plate reader (Perkin Elmer, Shelton, CT). Concentration response curves were then plotted and the EC50 values were calculated using GraphPad Prism 7. Both theoretical and experimentally observed genotoxicity Mitomycin equivalency quotients (MEQ) were calculated to determine the evolution of toxicity from 1,4-dioxane during UV/AOP treatments (Text S3).

Results and Discussion

Oxidation Products of 1,4-Dioxane in UV/$S_2O_8^{2-}$, UV/H_2O_2 and UV/NH_2Cl

UV/$S_2O_8^{2-}$ exhibited the fastest kinetics with respect to 1,4-D removal (Figure S3), because $S_2O_8^{2-}$ had a higher quantum yield than H_2O_2 (0.7 *vs*. 0.5), and produced both $SO_4^{\bullet-}$ and $HO^{\bullet}$ as reactive radicals.[19] Although UV/NH_2Cl produced $HO^{\bullet}$ through the transformation of $Cl^{\bullet}$ and $Cl_2^{\bullet-}$, the major radicals of $Cl^{\bullet}$ and $Cl_2^{\bullet-}$ are less reactive with 1,4-D as compared to $SO_4^{\bullet-}$ and $HO^{\bullet}$.[18,19] The three UV/AOPs produced a variety of 1,4-D oxidation products, which included ethylene glycol diformate, formaldehyde, glycolaldehyde, glycolic acid, formic acid, and methoxyacetic acid (Figure 1 and Table S1). Similar products have been identified in other oxidation processes.[28-31] $SO_4^{\bullet-}$, $HO^{\bullet}$, and $Cl_2^{\bullet-}$ likely oxidized 1,4-D through H atom abstraction to initially form 1,4-dioxanyl radical, and then reacted with O_2 to generate peroxyl radical (Scheme S1).[28] Peroxyl radicals were finally terminated to produce tetroxide. Study suggested that the decomposition of tetroxide led to the formation of oxyl radical, which was the precursor for formaldehyde and ethylene glycol diformate.[28] Formaldehyde was then oxidized by

radicals to formic acid. $SO_4^{\bullet-}$ favored the generation of methoxyacetic acid, and $HO^{\bullet}$ favored the formation of glycolic acid. In UV/NH_2Cl, only aldehyde products were observed, because an insufficient number of radicals were produced to degrade aldehyde products to carboxylic acids. The identified chemicals accounted for a majority of the transformation products, adding up to 80%-90% of the initial 1,4-dioxane dosage.

Cytotoxicity and genotoxicity of 1,4-dioxane and its oxidation products

Figure 2A presents a concentration-response curve of cell viability for 1,4-D, formaldehyde, and glycolaldehyde in HCT-116 cells. Other compounds with negligible cytotoxicity are shown in Figure S4A. The EC50 concentrations were reported in Table 1, with glycolaldehyde being the most cytotoxic (EC50 = 155 mM) followed by formaldehyde (EC50 = 613 mM). The rank order for cytotoxicity of 1,4-D and its oxidation products based on their EC50 values was: glycolaldehyde > formaldehyde > formic acid > glycolic acid > 1,4-dioxane > ethylene glycol diformate ≈ methoxyacetic acid. This trend showed that aldehydes in general exhibited a high cytotoxicity. Glycolaldehyde was highly cytotoxic to HK-2 cells and caused depletion of adenosine triphosphate (ATP), a release of lactate dehydrogenase (LDH), and degradation of enzymes as well as selected phospholipids.[32] Glycolaldehyde also induced growth inhibition and oxidative stress in human breast cancer cells.[33] Formaldehyde is known to be highly reactive with proteins and DNA that induces cytotoxicity.[34] Formaldehyde-induced cytotoxicity inhibited mitochondrial respiration, decreased ATP depletion, and generated reactive oxygen species which contributed to oxidative stress and cell lysis in isolated rat hepatocytes.[35] Ethylene glycol diformate has two carbonyl groups; however, when applied to HCT-116 cells, cell viability was not reduced in the present study.

Although studies on the toxicity of Ethylene glycol diformate have not been reported, our data suggests ethylene glycol diformate might be quickly metabolized to downstream products that are not cytotoxic.

Prior liturature reported that aldehydes are highly reactive electrophilic molecules that damage DNA through the formation of aldehyde-derived DNA adducts.[36,37] The genotoxicity assay using P53-GeneBLAzer Assay indicated that aldehyde compounds were highly genotoxic (Figure 4B) compared to 1,4-D and its carboxylic acid oxidation products (Figure S4B). The EC50 concentrations were 71 μM for glycolaldehyde and 395 μM for formaldehyde (Table 1). 1,4-D showed relatively low genotoxicity with an EC50 > 20000 μM. Glycolaldehyde has been reported to cause DNA-protein crosslinks and DNA single-strand breaks in human peripheral ononulcear blood cells.[38] Similarly, formaldehyde formed adducts with DNA and proteins, and resulted in chromosome loss due to formaldehyde-induced defects in the mitotic apparatus.[34,39] In agreement with our observation, 1,4-D produced negative genotoxicity responses in several in vitro assays.[40,41] A few studies reported that 1,4-dioxane elevated chromosomal breaks and DNA repairs in rats or mice with chronical injection of 1,4-D.[42,43] Our results indicated that 1,4-D is a weak genotoxicant to human cells. In contrast, its aldehyde oxidation products were extremely toxic to human cells.

Genotoxicity comparison among UV/$S_2O_8^{2-}$, UV/H_2O_2 and UV/NH_2Cl

Genotoxicity expressed as Mitomycin equivalency units (MEQ) was compared for the three oxidants with UV exposures of 0, 720 and 1440 mJ·cm^{-2}, respectively (Figure 3). At the beginning of the treatment, 1,4-D was the only chemical present in the system with a MEQ of 2.4×10^{-4}. After 720 mJ·cm^{-2} of irradiation, UV/$S_2O_8^{2-}$, UV/H_2O_2, and UV/NH_2Cl

increased the MEQs from 2.4×10^{-4} to 1.9×10^{-2}, 1.6×10^{-2} and 4.0×10^{-3}, respectively (Figure 3). Glycolaldehyde was consistently the major contributor to the MEQs in all three UV/AOPs (Table 1 and S1). After 1440 mJ·cm^{-2} of irradiation, the MEQ in UV/$S_2O_8^{2-}$ decreased from 1.9×10^{-2} to 1.2×10^{-2}, mainly due to the oxidation of glycolaldehyde to non-toxic carboxylic acids (Table S1). In contrast, H_2O_2 and NH_2Cl further increased the MEQs 2 to 3 times at a UV dosage of 1440 mJ·cm^{-2} (Figure 3), which was consistent with the 23-fold increase of glycolaldehyde concentrations. The results suggest that UV/$S_2O_8^{2-}$ oxidizes 1,4-D and further degrades its partial oxidation products, while UV/H_2O_2 and UV/NH_2Cl degrade 1,4-D at slower rates, resulting in an accumulation of toxic glycolaldehyde. The observed MEQ and theoretical MEQ did not statistically differ from each other in all three UV/AOP treatments, indicating that the mixture of the analytes produced an additive effect rather than synergistic effect. In addition, the data suggested that the identified transformation products (*i.e.*, accounted for > 80% of the product distribution) are the major contributors to the observed overall toxicity.

Engineering Implications

Our study addressed the concerns over the formation of more toxic oxidation products from UV/AOP treatment of recycled wastewater for potable water reuse. The degradation of 1,4-dioxane by UV/AOPs can generate glycolaldehyde and formaldehyde which induce toxicological responses 100 times higher than 1,4-dioxane itself. Validation of UV/AOP for water reuse applications requires at least 0.5-log of 1,4-D removal,[44] which corresponds to the extent of removal achieved after 15 minutes of UV/AOP under

experimental conditions in this study. For all three UV/AOPs, glycolaldehyde remained as the major product species. For many aromatic compounds, the initial oxidation steps usually lead to the formation of aldehydes as intermediates with higher geno- and cyto-toxicity. Despite the low-level existence of oxidation products in highly treated wastewater, an accurate assessment of potential human health risks from long-term exposure to these products is needed. Although human health risk assessment of oxidation product mixtures is complex, our study demonstrates that risk may be evaluated using cost-effective bioassay screening tools to identify causative agents in the mixture. Although additional treatment steps such as groundwater infiltration for indirect potable reuse may remove oxidation products, results from this study are important for prioritizing future toxicological assessment for potable water reuse, preparing the water industry for additional chemical detection methods and assisting the design of effective UV/AOPs that minimize the formation of toxic products.

Acknowledgement

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Supporting Information Section

Additional texts, figures and tables on analytical methods, cell bioassays and 1,4-D degradation and product distribution are provided in the Supporting Information Section.

Table 1 EC50 and relative effect potency (REP) values of 1,4-dioxane and its degradation products in P53RE-bla CT-116 cell line after 16 hours of exposure.

Toxicity-level Ranking	Chemicals	Cytotoxicity EC_{50} (μM)	Genotoxicity EC_{50} (μM)	REP*
1	Glycolaldehyde	$(1.6\pm0.2)\times10^{2}$	$(7.1\pm1.0)\times10^{1}$	6.7×10^{-2}
2	Formaldehyde	$(6.1\pm0.9)\times10^{2}$	$(4.0\pm2.5)\times10^{2}$	1.2×10^{-2}
3	Formic acid	$(1.3\pm3.0)\times10^{14}$	-	-
4	Glycolic acid	$(7.8\pm3.1)\times10^{15}$	-	-
5	1,4-dioxane	$(1.1\pm5.2)\times10^{29}$	$> (2.0\pm7.9)\times10^{4}$	2.4×10^{-4}

* REP is Relative effect potency, $REP=EC_{50(mitomycin)}/EC_{50(i)}$, where *i* is a specific degradation product. Details of the calculation is provided in Text S3 in the SI.

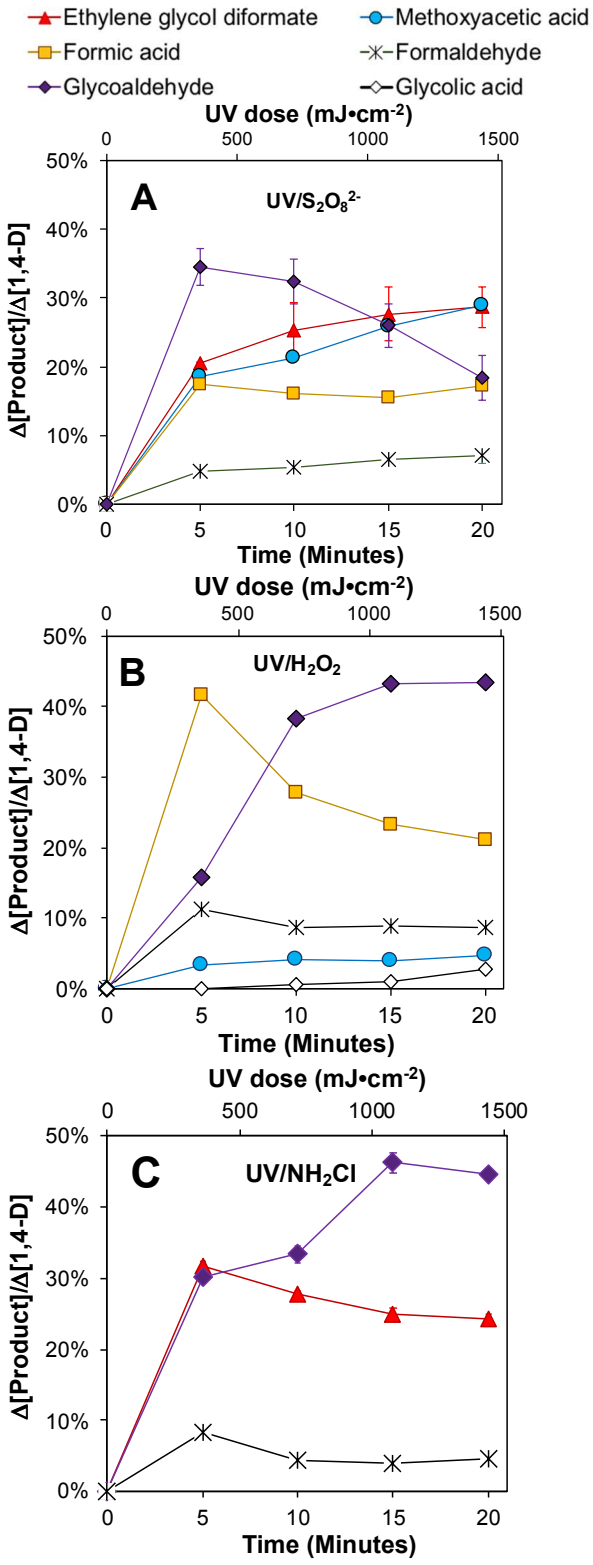
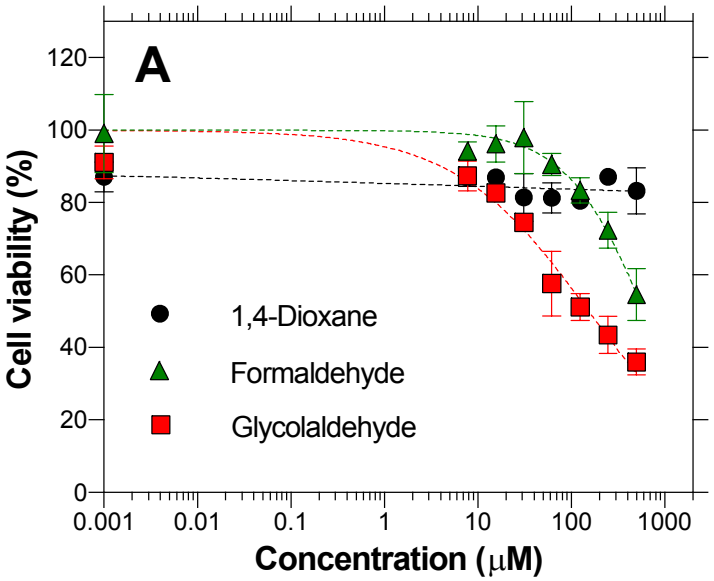


Figure 1 1,4-D degradation products evolution during the UV photolysis of (A) $S_2O_8^{2-}$, (B) H_2O_2 and (C) NH_2Cl as the oxidant. Initial [oxidant]=5 mM, initial [1,4-D]=1 mM, pH=8. The standard deviation of each data point was based on triplicate measurements.



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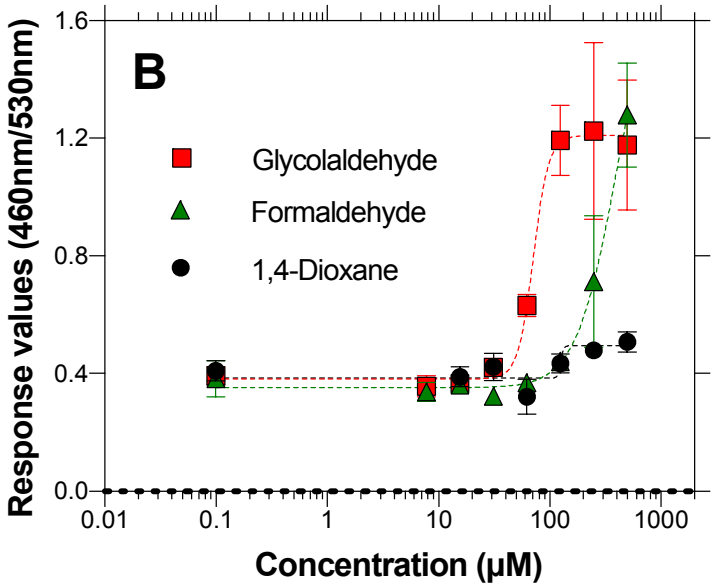

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230 **Figure 2** (A) Cytotoxicity and (B) Genotoxicity dose response curves of 1,4-dioxane,
231 formaldehyde and glycolaldehyde. Cell viability represented percent of viable cells compared to
232 the controls based on the MTT assay. Response values was calculated based on the ratio of
233 stimulated cells *vs*. unstimulated cells obtained from CellSensor p53RE-bla HCT-116 assay.
234 Each value represents the mean of replicates ± standard deviation.

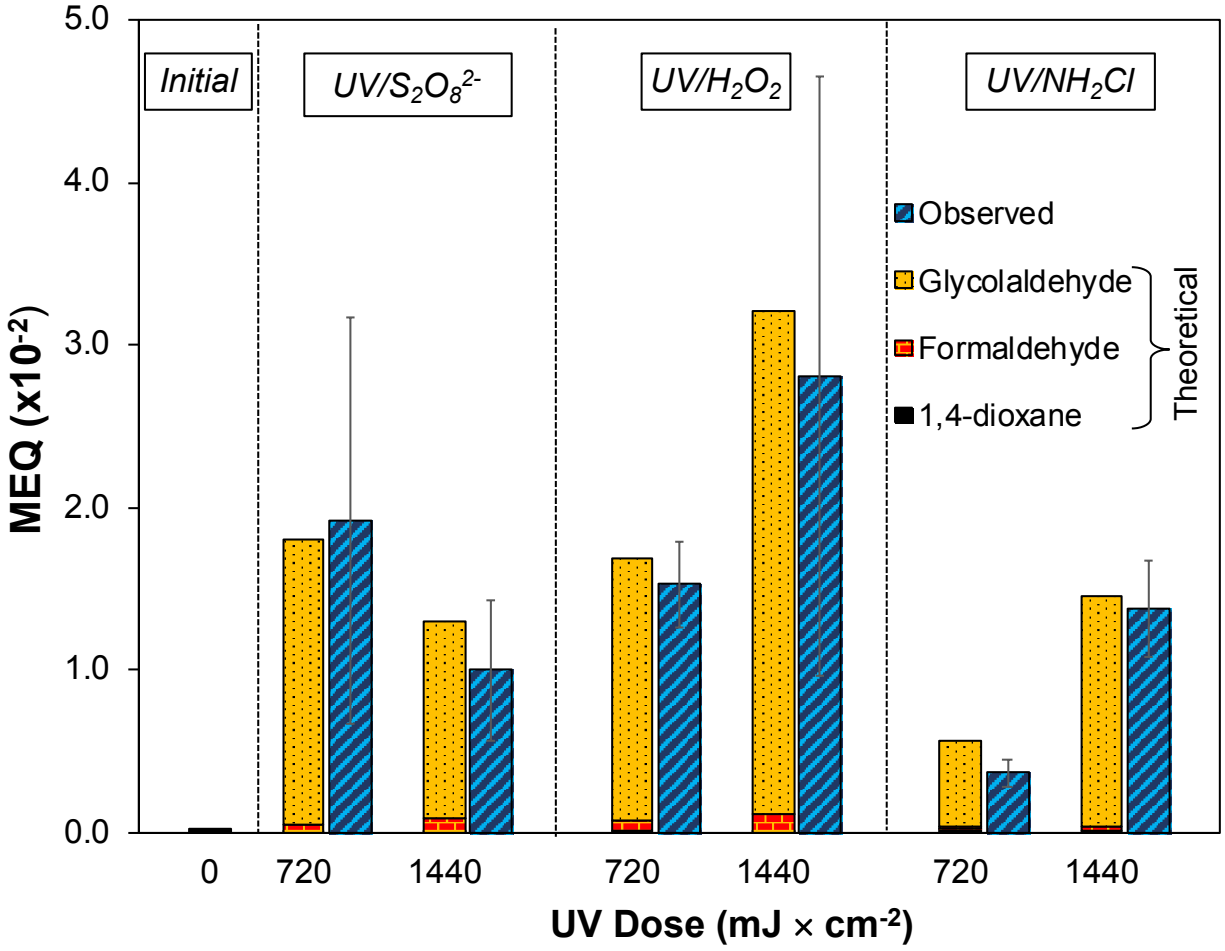

Figure 3. Mitomycin equivalent quotient of genotoxicity (MEQ) evolution during UV/AOPs treatment. The observed MEQ was calculated based on the EC50 of the mixture of 1,4-dioxane and six identified transformation products (Figure S5B). The concentration of each analyte was determined based on the experimental observations (Table S1). The theoretical MEQ was calculated based on the EC50 of each individual analyte (Figure 2B, Figure S4B and Text S3). Error bars represent one standard deviation. UV/$S_2O_8^{2-}$: two-way ANOVA test showed no difference between calculated and observed MEQ (P=0.677); and a significant difference between MEQ with different UV dose of 0, 720 and 1440 mJ×cm^{-2} (P=0.014). UV/H_2O_2: two-way ANOVA test showed no difference between calculated and observed MEQ (P=0.254); and a significant difference between MEQ with different UV dose of 0, 720 and 1440 mJ×cm^{-2} (P=0.0043). UV/NH_2Cl: two-way ANOVA test showed no difference between calculated and observed MEQ (P=0.250); and a significant difference between MEQ with different UV dose of 0, 720 and 1440 mJ×cm^{-2} (P=0.005).

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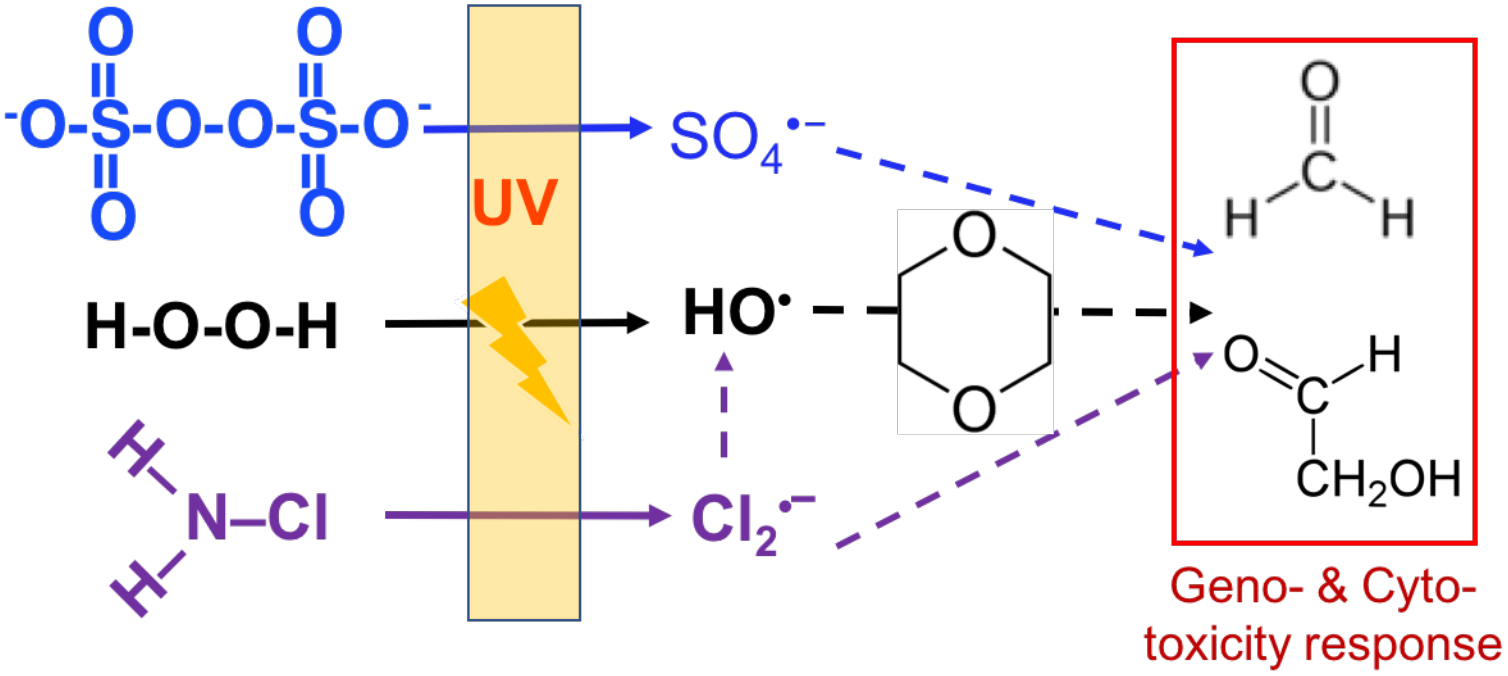
UV
H-O-O-H
N–Cl
HO•
Geno- & Cyto-
toxicity response