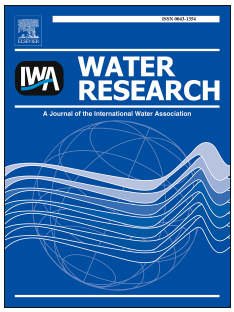
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ADVANCED OXIDATION
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Transformation of Contaminant Candidate List (CCL3) compounds during ozonation and advanced oxidation processes in drinking water: assessment of biological effects

Hana Mestankova[1], Austa M. Parker[2*], Nadine Bramaz[1], Silvio Canonica[1], Kristin Schirmer[1,3,4], Urs von Gunten[1,3,4^], and Karl G. Linden[2^]*

[1] Eawag, Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, P.O. Box 611, CH-8600 Dübendorf, Switzerland

[2] Department of Civil, Architectural, and Environmental Engineering, University of Colorado Boulder, Boulder, Colorado 80309, United States

[3] School of Architecture, Civil and Environmental Engineering (ENAC), Ecole Polytechnique Fédérale de Lausanne (EPFL), GR A1 465, Station 2, CH-1015 Lausanne, Switzerland

[4] Institute of Biogeochemistry and Pollutant Dynamics, ETH Zentrum, CH-8092 Zürich, Switzerland

* Co-primary authors contributed equally to this work.

^ Corresponding Authors: vongunten@eawag.ch and karl.linden@colorado.edu

ABSTRACT

The removal of emerging contaminants during water treatment is a current issue and various technologies are being explored. These include UV- and ozone-based advanced oxidation processes (AOPs). In this study, AOPs were explored for their degradation capabilities of 25 chemical contaminants on the US Environmental Protection Agency's Contaminant Candidate List 3 (CCL3) in drinking water. Twenty-three of these were found to be amenable to hydroxyl radical-based treatment, with second order rate constants for their reactions with hydroxyl radicals ($^{\bullet}$OH) in the range of 3–8×10^{9} $M^{-1}s^{-1}$. The development of biological activity of the contaminants, focusing on mutagenicity and estrogenicity, was followed in parallel with their degradation using the Ames and YES bioassays to detect potential changes in biological effects during oxidative treatment. The majority of treatment cases resulted in a loss of biological activity upon oxidation of the parent compounds without generation of any form of estrogenicity or mutagenicity. However, an increase in mutagenic activity was detected by oxidative transformation of the following CCL3 parent compounds: nitrobenzene ($^{\bullet}$OH, UV photolysis), quinoline ($^{\bullet}$OH, ozone), methamidophos ($^{\bullet}$OH), *N*-nitrosopyrolidine ($^{\bullet}$OH), *N*-nitrosodi-*n*-propylamine ($^{\bullet}$OH), aniline (UV photolysis), and *N*-nitrosodiphenylamine (UV photolysis). Only one case of formation of estrogenic activity was observed, namely, for the oxidation of quinoline by $^{\bullet}$OH. Overall, this study provides fundamental and practical information on AOP-based treatment of specific compounds of concern and represents a framework for evaluating the performance of transformation-based treatment processes.

KEYWORDS: Ultraviolet; ozone; hydroxyl radical; mutagenicity; estrogenicity

INTRODUCTION

Advanced Oxidation Processes

The presence of micropollutants in water resources is an issue of concern in the drinking water industry (Schwarzenbach et al. 2006). To keep their concentrations in drinking waters as low as possible, treatment processes for removal of micropollutants based on either separation (e.g., membranes, activated carbon) or transformation (e.g., oxidation) may be employed (Virkutyte et al. 2010). To this end, conventional and advanced oxidation processes (AOPs) have been demonstrated to be able to degrade the majority of micropollutants (Huber et al. 2003, Rosario-Ortiz et al. 2010, Lee and von Gunten 2010). During oxidation, micropollutants are typically not mineralized and transformation products are formed, often with similar structures to the parent compounds (von Sonntag and von Gunten 2012). Therefore, an assessment of the success of such processes should not only be based on a measurement of the abatement of parent compounds but also on an assessment of the remaining effects and even the potential formation of new biological activity (von Sonntag and von Gunten 2012).

Conventional ozonation and AOPs are transformation-based treatment processes which rely on the *in situ* production of the hydroxyl radical ($^{\bullet}OH$) that can quickly react with a wide range of micropollutants (Buxton et al. 1988). The most commonly used AOPs in water treatment are the combination of ozone with H_2O_2 and UV with H_2O_2. Ozone- and UV-based AOPs have both been utilized to provide treatment of regulated and emerging contaminants at full-scale facilities (Virkutyte et al. 2010). In addition to the $^{\bullet}OH$ driven oxidation processes, ozone-based AOPs include direct ozone reactions and UV-based AOPs also include an inherent direct photolysis component (Oppenlander 2007, von Sonntag and von Gunten 2012). Therefore,

in these cases the effect of direct ozone reactions or direct photolysis has to be considered as well.

Conventional ozonation and AOPs generally lead to the removal of the intended biological activity associated with micropollutants, as shown previously for estrogenic compounds, antibiotics, antiviral compounds and herbicides (Dodd et al. 2009, Lee et al. 2008, Mestankova et al. 2011, Mestankova et al. 2012, Rosenfeldt et al. 2007). However, in a few cases the oxidation of specific parent compounds leads to transformation products with similar or alternative modes of biological activity. For instance the oxidation of dimethylsulfamide, a harmless metabolite of the fungicide tolylfluanide, led to the formation of the mutagenic *N*-nitrosodimethylamine (NDMA) during ozonation of bromide- and dimethylsulfamide-containing waters (Schmidt and Brauch 2008, von Gunten et al. 2010). NDMA was also formed during ozonation of dyes containing dimethylamino functional groups (Yang et al. 2003). The identification of transformation products, with a subsequent evaluation of their biological effects for an overall risk assessment of micropollutant abatement during AOPs, is a tedious and expensive process. Therefore, the application of biological assays to samples taken during degradation is a cost efficient and useful approach to elucidate the formation of transformation products with increased biological effects or new modes of action (Escher and Fenner, 2011).

US EPA Contaminant Candidate List

The US EPA's Contaminant Candidate List (CCL) includes compounds that are being explored for possible regulation. The 1996 Amendments to the Safe Drinking Water Act require the EPA to publish a revised CCL every 5 years (USEPA 1996). This process ends in a regulatory determination for some contaminants. The most recent CCL is the CCL3, which contains 104 chemical and 12 microbial contaminants (USEPA October 8, 2009). The CCL3 list

provides insight into potential future regulatory activity, and therefore is a helpful framework to design an emerging contaminant study.

In this study, 25 compounds from the 104 chemical contaminants on the US EPA CCL3 were selected for evaluation (see below for explanation, Table 1 and supporting information (SI) Table S1). The rate of oxidative transformation of selected CCL3 compounds was tested for oxidation by $^{\bullet}OH$, ozone or UV photolysis by measuring the kinetics of the abatement of the parent compounds. The mixtures of transformation products formed during oxidation were then tested by bioassays for mutagenic and estrogenic activity. Contaminant degradation was followed using a parallel evaluation of abatement of the parent compound and biological activity, which enables the discovery of unknown transformation products of importance.

MATERIALS AND METHODS

Rationale of Contaminant Selection

104 chemicals from the CCL3 list were grouped into 8 classes (Table 1 and SI Table S1) based on their chemical structures and/or biological modes of action. The chemical structures and available information from literature enabled an estimate of the potential for oxidative transformation of the selected compounds. The criteria for the selection of 25 out of the 104 contaminants from the CCL3 were based on: (1) being treatable by AOP (i.e., high second order rate constant for the reaction with O_3 and/or $^{\bullet}OH$), (2) likelihood of being in the AOP influent (i.e., low octanol-water partition coefficient (K_{ow}) relative to other compounds in the class), (3) established analytical methods, and (4) regulatory relevance (i.e., based on health and occurrence data from the US EPA) (USEPA October 8, 2009). Halogenated compounds (group 1) were excluded from this study because they generally have low second order rate constants for the reaction with ozone and $^{\bullet}OH$ (von Gunten 2003). Olefines (group 2) were selected based on their

high reactivity towards ozone and •OH (Onstad et al. 2005, von Sonntag and von Gunten 2012). Nitro compounds (group 3) typically have low reactivities with ozone, however, depending on other moieties of the structure they may be efficiently transformed by •OH. Furthermore, nitro compounds are toxic and therefore interesting compounds for an assessment of biological effects during oxidative transformation (Cronin et al. 1998, Shinoda et al. 1998). Ethers, alcohols, phenols, aldehydes and other oxygen containing compounds (group 4) were chosen based on high rate constants for the reaction with •OH, thus likely to be candidates for treatment by AOPs (Buxton et al. 1988, Stefan and Bolton 1998). The selected nitrogen-containing compounds (group 5) were expected to have high reactivities with •OH, and in some cases (anilines) high reactivity with ozone. Organophosphorous compounds (group 6) were expected to react readily with •OH and possibly lead to toxicologically relevant degradation products (Nordmeyer and Dickson 1990). Amides (group 7) were also selected based on their reactivity towards •OH (de Laat et al. 1996). *N*-nitroso compounds (group 8) were selected based on their known biological effects (Magee and Barnes 1967, Mestnakova et al. 2014) and their potential to be photolysed (Sharpless et al. 2003, Plumlee and Reinhard 2007). Metalorganic compounds were not selected in the current study because they are expected to be mostly adsorbed to organic particles and sediments based on their high K_{ow} values (Gschwend and Wu 1985).

Procedure for the Assessment of the Selected CCL3 Compounds

Figure 1 outlines the experimental procedure for this study, with the following goals: (1) Assessment of the feasibility of treatment for the selected CCL3 compounds by oxidation processes, (2) determination of the removal of potential biological effects of the parent compounds and (3) investigation of the potential formation of toxic degradation products with similar or different modes of action than the parent compounds.

The evaluation of second-order rate constants for the reaction of the selected micropollutants with ozone and $^{\bullet}OH$ (and the efficiency of photolysis) provided a framework for selecting compounds for further study. Compounds with second order rate constants for the reaction with $^{\bullet}OH$ below 5×10^{8} $M^{-1}s^{-1}$ were not selected because a very low removal efficiency for such compounds can be expected in AOPs. Therefore, AOPs would not be a treatment process of choice, except for *N*-nitrosamines, which also undergo efficient transformation via direct photolysis (Sharpless et al. 2003). The selected compounds underwent biological effects testing following oxidative transformation, including both mutagenicity (Ames II) and estrogenicity (YES) tests. Overall, 45 individual treatment scenarios (via $^{\bullet}OH$, ozone, or direct UV photolysis) were evaluated across the compounds with both bioassays. The details of the treatments applied to each chemical are presented in SI, Table S2.

Figure 1 here

Oxidation Experiments with Hydroxyl Radical and Ozone

Each compound was evaluated individually in unique experiments. All chemicals used as the parent compounds were standards of analytical grade of ≥ 99% purity (suppliers and purity indicated in SI, Table S1). All aqueous solutions were prepared using deionized water obtained from a Milli-Q water purification system (Millipore). Chemicals used for preparation of media or reaction mixtures were ≥ 95% purity. Oxidation experiments for compounds from groups 3, 5, and 7 were carried out as previously described (Mestankova et al. 2011). Hydroxyl radicals for the degradation of the parent compounds in aqueous solutions (pH = 7, 5 mM phosphate buffer) were generated in systems based on the photolysis of hydrogen peroxide (1 mM, 34 mg/L or higher) by a medium pressure mercury vapor UV lamp filtered to emit between 308-410 nm to minimize UVC direct photolysis. The initial concentration of the parent compound was typically

100 µM, where solubility permitted. Oxidation experiments for compounds from groups 1, 2, 4, and 6 were carried out based on photolysis of hydrogen peroxide by a low pressure UV mercury vapor lamp (near-monochromatic at 254 nm: LPUV/H_2O_2) in a quasi-collimated beam system. Four LPUV lamps (ozone free, 15 W, General Electric #G15T8) were housed above a 4-inch aperture equipped with a manual shutter. Incident UV irradiance was measured by a calibrated radiometer (International Light Inc., Model 1700/SED 240/W). UV fluence (i.e. UV dose) was calculated by multiplying the average irradiance by the exposure time in seconds including factors based on Bolton and Linden (2003).

Ozonation experiments were carried out with solutions containing 100 µM or lower concentrations of the parent compound at pH 7 (5 mM phosphate) and in the presence of 10 mM *t*-butanol to scavenge $^{\bullet}OH$ such that ozone was the only oxidant. The solutions were stirred in borosilicate glass vials and spiked with various volumes of an ozone stock solution via a gas tight glass syringe to achieve the desired ozone dose and extent of degradation (Mestankova et al. 2011). An ozone stock solution (~ 1.3 mM) was produced by sparging O_3-containing oxygen through ice-cooled Milli-Q water (Bader and Hoigné 1981).

Direct Photolysis Experiments

Direct photodegradation experiments were performed using near-monochromatic light at 254 nm (Mestankova et al. 2011). More details are given in SI, Text S1.

Analytical Methods

Samples from degradation experiments were analyzed by HPLC with UV detection, HPLC-MS, or GC-MS (SI, Table S1 for specific analytical methods). Standard analytical methods were used, and parameters including compound purity and column sizing are given in

158 the SI, Table S1. Quantification of the parent compound and both bioassays were performed
159 within 12 hours of parent compound degradation. The standard deviations of the HPLC
160 measurements were < 3%, with quantification limits < 1µM.

161 *Determination of Rate Constants*

162 Second order rate constants for the reaction of •OH with the selected contaminants (k_{OH})
163 were measured in systems based on the photolysis of hydrogen peroxide by competition kinetics
164 with *p*-chlorobenzoic acid (pCBA) as a competitor (k_{OH} =5.0 × 10^9 $M^{-1}s^{-1}$) (Elovitz et al. 2000,
165 Huber et al. 2003, von Sonntag and von Gunten 2012). Second-order rate constants for the
166 reaction of fast-reacting compounds with ozone (k_{O3}) were determined by competition kinetics
167 with cinnamic acid (k_{O3}=3.8 × 10^5 $M^{-1}s^{-1}$ for pH 7 – 8 (Leitzke et al. 2001)). Competition kinetics
168 experiments were performed in duplicate by plotting ln ([C]/[C]$_0$) ([C]: concentration of target
169 compound) versus ln([competitor]/[competitor]$_0$), which allows a calculation of the unknown
170 rate constant from the slope of the straight line of such a graph (Elovitz et al. 2000, Elovitz and
171 von Gunten 1999, Huber et al. 2003, von Sonntag and von Gunten 2012). In the case of
172 compounds reacting slowly with ozone, k_{O3} values were determined in excess of either ozone or
173 the target compound at pH 2 under pseudo-first order conditions (Hoigné and Bader 1983a, von
174 Sonntag and von Gunten 2012). The excess of ozone or target compound, respectively, was ≥20.
175 The ozone concentration during the experiments was measured by the indigo method (Bader and
176 Hoigné 1981).

177 *Bioassays*

178 The Ames (*Salmonella*/microsome mutagenicity assay) test is a widely accepted bacterial
179 reverse mutation assay. It is used worldwide as a screening assay, and has been recognized for its

ease of use, rapidness, broad applicability, high sensitivity and reproducibility. The correlation between mutagenicity and carcinogenicity has been widely evaluated because carcinogenicity is of utmost human health concern and can result from mutagenicity. Original data in the 1970's showed a high correlation between carcinogenicity and mutagenicity detected by the Ames test (90% from 174 chemicals tested, including almost all of the known human carcinogens of that time (McCann et al. 1975)). A later study showed lower specificity for rodent carcinogens (58%), and for the non-carcinogens the specificity was 73% (Ashby et al. 1989). Several carcinogens are non-mutagenic; therefore, the Ames assay may underestimate carcinogenicity due to the inability to measure the specific mode of carcinogenicity (Ashby et al. 1989). A disadvantage of the Ames test is that prokaryotic cells differ from eukaryotic cells with respect to uptake and metabolism. To test the metabolites of the chemicals, a crude preparation of enzymes is obtained from the homogenized liver of rats (previously pre-treated with a substance for enzyme induction). This preparation is known as the S9 fraction and with added enzyme cofactors is called S9-mix. Addition of the S9-mix allows for the metabolization of AOP transformation products to mimic further transformation in a living organism. The presence and absence of the S9-mix indicates the metabolized and non-metabolized forms of the compound in question, respectively. The Ames test was performed as described in ISO 11350 (ISO 2012). Two mutations contained within bacteria were used in the Ames II assay: TAMix, which is comprised of a mixture of six Salmonella strains (measuring base pair substitution), and TA98 (measuring frameshift mutations). Positive controls were used as the reference compounds (see equation 1). 4-nitro-o-phenylenediamine (4-NODP) was used as a positive control for frameshift mutations (TA98), and 2-nitrofuran (2-NF) for base pair substitutions (TAMix). 2-

aminoanthracene (2-AA) was used as the positive control for both frameshift and base pair mutations in the presence of the S9-mix. Additional details are given in SI, Text S2.

The recombinant yeast estrogen screen (YES) test was used to detect and quantify the estrogenicity in the treated samples (Routledge and Sumpter 1996). Under well controlled and proper experimental conditions the yeast-based YES assay is a sensitive and reproducible test across laboratories for the screening of compounds interacting with the estrogen receptor (Dhooge et al. 2006). It was reported that false negative rates were 22% and false positive 7% on the estrogenic agonisms (for 105 chemicals tested (Kolle et al. 2010)). The suitability of this assay for pre-screening of water bodies was evaluated (Brix et al. 2010) and no significant difference was found between two laboratories. A low coefficient of variation (<9%) was reported also for positively identified compounds for 3 laboratories in a previous study (Dhooge et al. 2006). The YES test was performed as described by Rutishauser et al. (Rutishauser et al. 2004) (for details see SI, Text S2).

Toxic Equivalent Concept (TEQ)

The mutagenicity/estrogenicity in the samples was analyzed based on the toxic equivalent (TEQ) concept (Escher et al. 2008), which was used in previous studies (Dodd et al. 2009, Lee et al. 2008, Mestankova et al. 2011). This approach is suitable for quantification of the biological activity of mixtures and allows an evaluation of the development of these effects due to degradation of target compounds. The biological activity of samples taken during degradation, containing the known parent compounds and unknown degradation products, can be directly compared with the extent of the parent compound degradation.

TEQ (or more specifically MEQ and EEQ for mutagenicity and estrogenicity, respectively) is calculated by dividing the equivalent concentration required to reach 50% of the

maximum effect (EC_{50}) of the reference compound (17β-estradiol for EEQ and for MEQ positive controls of the test) by the EC_{50} of each sample (Eq. 1).

$$MEQ\ or\ EEQ = \frac{EC50\ (reference\ compound)}{EC50\ (sample)} \quad (1)$$

A positive control for the bioassay is used as the reference compound. If the parent compound presents mutagenicity or estrogenicity, the MEQ or EEQ, respectively of each sample can be normalized to its initial value (MEQ_0 or EEQ_0) as a measure of the development of the specific activity during degradation of the parent compound (Eq. 2).

$$\frac{MEQ}{MEQ_0}\ or\ \frac{EEQ}{EEQ_0} = \frac{EC50_0\ (sample)}{EC50\ (sample)} \quad (2)$$

If this ratio is plotted against the extent of degradation (C/C_0), in an ideal case the decrease of the parent compounds results in the corresponding stoichiometric decrease of the specific activity of the mixture of the transformation products. In this case, the degradation products do not show a significant biological response and these data are on a straight line with a slope of -1. If transformation products are causing a significant biological effect, a positive deviation from the "ideal case" is expected (points above the line with a slope of -1).

RESULTS AND DISCUSSION

Kinetic Data

Table 1 shows the selected compounds from the CCL3 with their measured and previously published second order rate constants for the reactions with ozone and ˙OH.

Table 1. Rate constants for the reactions of the selected compounds from the CCL3 with ozone and ˙OH. References to values from literature are given in brackets (superscripts), chemicals with rate constants in bold were studied for biological effects.

	k_{O3} ($M^{-1}s^{-1}$)	k_{OH} ($M^{-1}s^{-1}$)
1. Halogenated compounds		
1,2,3 - Trichloropropane	0.87 ± 0.03	$< 5 \times 10^8$
2. Olefines		
Dimethipin	**$(1.2 \pm 0.1) \times 10^5$**	**$(6.7 \pm 0.4) \times 10^9$**
3. Nitro compounds		
Nitrobenzene	**0.09 (Hoigné and Bader 1983a)**	**$3.9\text{-}4.2 \times 10^9$ (Buxton et al. 1988, García Einschlag et al. 2003)**
Nitroglycerin	0.04 ± 0.01	$< 5 \times 10^8$
4. Ethers, alcohols, and phenols		
1,4-Dioxane	0.3 (Eibenberger 1980a, Thomas 1965)	**$2.5\text{-}3.1 \times 10^9$ (Hoigné and Bader 1983b)**
Oxirane, methyl	0.09 ± 0.02	**$(5.7 \pm 0.4) \times 10^9$**
Methyl-tert-butyl ether (MTBE)	0.14 (Acero et al. 2001, Eibenberger 1980b)	**1.0×10^9 (Acero et al. 2001)**
Cumene hydroperoxide	**$(2.0 \pm 0.67) \times 10^5$**	**$(2.8 \pm 0.2) \times 10^9$**
5. Nitrogen containing compounds		
4,4'-Methylenedianiline	**$> 9 \times 10^7$ (Hoigné and Bader 1983a)**	**$> 5 \times 10^9$(estimated)**
Aniline	**0.9×10^8 (Hoigné and Bader 1983b)**	**$0.9\text{-}1.7 \times 10^{10}$ (Christensen 1972, DeLaat et al. 1996, Qin et al. 1985, Solar et al. 1986)**
Quinoline	**51 (Wang et al. 2004)**	**7×10^9 (Wang et al. 2004)**
6. Organophosphorous compounds		
Acephate	0.23 ± 0.01	**$(7.1 \pm 0.2) \times 10^9$**
Dicrotophos	0.03 ± 0.01	**$(5.5 \pm 0.2) \times 10^9$**
Methamidophos	0.02 ± 0.01	**$(8.2 \pm 0.4) \times 10^9$**
Fenamiphos	0.65 ± 0.01	**$(6.2 \pm 0.2) \times 10^9$**
7. Amides		
Metolachlor	**3 (DeLaat et al. 1996)**	**6.1×10^9 (DeLaat et al. 1996)**
Metolachlor ethanesulfonic acid (ESA)	**5.0 ± 0.4**	**7.1×10^9 (DeLaat et al. 1996)**
Metolachlor oxanilic acid (OA)	**7.0 ± 0.1**	**5.6×10^9 (DeLaat et al. 1996)**
N-methyl-2-pyrrolidone	**2 (Muruganandham et al. 2007)**	**4.9×10^9 (Gligorovski et al. 2009)**

Molinate	**500** (Chen et al. 2008)	**$1.3\text{-}6.9 \times 10^9$** (Reifferscheid and Heil , Shemer et al. 2006)
8. *N*-nitroso compounds		
N-nitrosodimethylamine (NDMA)	**0.05** (Lee et al. 2007)	**4.3×10^8** (Mezyk et al. 2004)
N–nitrosodiethylamine (NDEA)	**0.08** (Mestankova et al. 2014)	**7×10^8** (Mezyk et al. 2008)
N-nitrosodi-*n*-propylamine (NDPA)	**0.04** (Mestankova et al. 2014)	**2.3×10^9** (Landsman et al. 2007)
N-nitrosopyrrolidine (NPYR)	**0.33** (Mestankova et al. 2014)	**1.75×10^9** (Landsman et al. 2007)
N-nitrosodiphenylamine (NDPhA)	**8.5** (Mestankova et al. 2014)	**6.5×10^9** (Mestankova et al. 2014)

Second order rate constants for reactions with $^{\bullet}OH$ and ozone that were not available in the literature were experimentally determined. Of the 25 compounds in this study, two (1,2,3-trichloropropane and nitroglycerin) had second order rate constants for the reaction with $^{\bullet}OH < 5\times10^8$ $M^{-1}s^{-1}$, a benchmark for which an effective abatement of a target compound is not feasible by AOPs, unless the compound can undergo efficient direct photolysis (e.g., NDMA). Most organic contaminants react with $^{\bullet}OH$ with second order rate constants $\geq10^9$ $M^{-1}s^{-1}$. A benchmark for rate constants of the reaction of a target compound with ozone was set to > 10 $M^{-1}s^{-1}$, below which the reaction with ozone does not contribute significantly to the abatement of the parent compound. Direct UV photolysis was not relevant for most compounds evaluated for treatment with some exceptions, including nitrobenzene, dimethipin, cumene hydroperoxide, aniline, fenamiphos, and *N*-nitrosamines (Table 2), which were tested for formation of mutagenic and estrogenic compounds by direct photolysis.

Mutagenic and Estrogenic Activity of Parent Compounds

Based on the kinetic study, the 23 parent compounds that were determined to be treatable by AOPs (Table 1, $^{\bullet}OH$ rate constant $\geq 5\times10^{8}$ $M^{-1}s^{-1}$) were tested for parent compound mutagenicity and estrogenicity. In addition, 1,2,3-trichloropropane was evaluated based on its current importance to US EPA.

Most parent compounds tested yielded no responses in the Ames II or the YES assay, i.e. neither mutagenicity nor estrogenicity. Among the few compounds causing mutagenicity in the Ames II test were cumene hydroperoxide, (group 4, in the TAMix strain, without the addition of the S9-mix) and group 6 chemicals (Parker et al. in prep.). Only one parent compound was found to be estrogenic in the YES assay: 4,4'-methylenedianiline (MDA) (group 5) with an EC_{50} value of 1.1×10^{-4} M. In agreement with our findings, it was previously reported that MDA has a weak positive response with an EC_{50} of 1.9×10^{-4} M (using the recombinant yeast assay with 5 days of incubation) (Hamblen et al. 2003). MDA, which is structurally related to bisphenol A, shows low estrogen binding affinity (Blair et al. 2000). The responses of all parent compounds evaluated under the Ames and YES assays are provided in Table S3 (SI).

Development of Mutagenic Activity during AOPs

The development of the mutagenic activity following degradation by $^{\bullet}OH$, ozone, and direct photolysis of each compound is given in Table 2. In most cases no increase occurred but for some compounds an increase in biological activity from transformation products was observed (e.g., the case of quinoline, aniline, and several *N*-nitrosamine compounds). In the following sections, mutagenicity development during degradation of individual classes of compounds is discussed in more detail.

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Table 2. Evaluation of the mutagenic activity by the TA98 and TAMix Ames bioassays in absence (-S9) and presence (+S9) of the S9-mix after treatment of selected CCL3 compounds with ˙OH, ozone or UV light (direct photolysis, λ = 254 nm).

	oxidation by ˙OH radical				oxidation by ozone				direct photolysis			
strains:	TA98		TAMix		TA98		TAMix		TA98		TAMix	
metabolic activation:	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
1. Halogenated compounds												
1,2,3-trichloropropane	−⊠→	−⊠→	+⊠→	−⊠→	−⊠→	−⊠→	+⊠→	−⊠→	−⊠→	−⊠→	+⊠→	−⊠→
2. Olefines												
Dimethipin	− → −	− → −	− → −	− → −	− → −	− → −	− → −	− → −	− → −	− → −	− → −	− → −
3. Nitro compounds												
Nitrobenzene	- ↗ +	− → −	− → −	− → −	− → −	− → −	− → −	− → −	- ↗ +	− → −	− → −	− → −
4. Ethers, Alcohols, and Phenols												
1,4-Dioxane	− → −	− → −	− → −	− → −	−⊠→	-⊠→	− ⊠→	-⊠→	− ⊠→	-⊠→	− ⊠→	-⊠→
Oxirane, methyl	− → −	− → −	− → −	− → −	−⊠→	-⊠→	− ⊠→	-⊠→	− ⊠→	-⊠→	− ⊠→	-⊠→
Methyl-tert-butyl ether	− → −	− → −	− → −	− → −	−⊠→	-⊠→	− ⊠→	-⊠→	− ⊠→	-⊠→	− ⊠→	-⊠→
Cumene hydroperoxide	− → −	− → −	+ ↘ -	− → −	− → −	− → −	+ ↘ -	− → −	− → −	− → −	+ ↘ -	− → −
5. Nitrogen-containing compounds												
Aniline	− → −	− → −	− → −	− → −	− → −	− → −	− → −	− → −	− → −	- ↗ +	− → −	− → −
4,4'-Methylenedianiline	− → −	− → −	− → −	− → −	− → −	− → −	− → −	− → −	**n.a.**	**n.a.**	**n.a.**	**n.a.**
Quinoline	- ↗ +	- ↗ +	− → −	− → −	- ↗ +	- ↗ +	- ↗ +	- ↗ +	**n.a.**	**n.a.**	**n.a.**	**n.a.**
6. Organophosphorous compounds												
Acephate	+↘ -[?]	− → −	− → −	− → −	−⊠→	-⊠→	− ⊠→	-⊠→	−⊠→	-⊠→	− ⊠→	-⊠→
Dicrotophos	− → −	− → −	+↘ -[?]	− → −	−⊠→	-⊠→	− ⊠→	-⊠→	−⊠→	-⊠→	− ⊠→	-⊠→
Methamidophos	− → −	+ ↗ +	− → −	− → −	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →
Fenamiphos	− → −	− → −	+ ↘ -	− → −	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	− → −	− → −	+ ↘ -	− → −
7. Amides												
Metolachlor	− → −	− → −	− → −	− → −	−⊠→	-⊠→	− ⊠→	-⊠→	− → −	− → −	− → −	− → −

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Metolachlor ethanesulfonic acid	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
Metolachlor oxanilic acid	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
2-methyl-2-pyrrolidone	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	–⊠→	-⊠→	– ⊠→	-⊠→
Molinate	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –
8. *N*-nitroso compounds (Mestankova et al. 2014)												
N-nitrosodimethylamine	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
N-nitrosodiethylamine	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
N-nitrosodi-*n*-propylamine	– → –	– → –	- ↗ +	- ↗ +	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
N-nitrosopyrrolidine	– → –	– → –	- ↗ +	- ↗ +	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
N-nitrosodiphenylamine	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	- ↗ +	- ↗ +	– → –	– → –

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– → –	Parent compound activity was negative, and after treatment the sample was tested negative.
–⊠→	Insufficient degradation was achieved for testing.
+⊠→	Parent compound activity was positive, but insufficient degradation was achieved for testing.
+ ↘ -	Parent compound activity was positive, and after treatment the activity decreased proportionally to the parent compound.
+↘ -$^{?}$	Parent compound displayed a weak activity, and the activity decreased with treatment, but was too weak to be conclusively determined.
+ ↗ +	Parent compound activity was positive, and after treatment the activity increased.
- ↗ +	Parent compound activity was negative, and after treatment the activity increased.

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Group 2 - Olefines

The only olefin tested from the CCL3 list was dimethipin. Dimethipin showed no parent compound response for either of the Ames assay strains tested (TAMix, TA98). Dimethipin is amenable to oxidation by $^{\bullet}OH$ and by ozone, and also direct photolysis. When subjected to either of these processes, dimethipin showed no formation of mutagenic activity.

Group 3 - Nitro compounds

Direct photodegradation of nitrobenzene (NB) (Tables 1 and S1 (SI)) leads to formation of significant mutagenic activity in the strain TA98 in the absence of the S9-mix, as shown in Figure 2. The mutagenicity was quantified as MEQ-2-NF (for explanation see Materials and Methods). Further photolytic degradation of NB leads to the removal of the detected mutagenicity. Also during oxidation of NB by $^{\bullet}OH$ (via the UV/H_2O_2 process) a weak mutagenicity formation was observed. However, the observed formation of the mutagenic activity during degradation of aromatic nitro compounds should be interpreted cautiously due to documented false positives tests of these compounds in the Ames test (*McCann and Ames 1976; Maragos et al. 1993; Kirkland et al. 2007*).

Direct photolysis and oxidation of NB by $^{\bullet}OH$ leads to a formation of 2-nitrophenol, 3-nitrophenol, 4-nitrophenol, phenol, nitrohydroquinone, nitrocatechols and catechol (Beltran et al. 1998 , Chen et al. 2005, Chen et al. 2006, Matthews and Sangster 1967). NB itself is non-mutagenic in the strains TA98 and TA100 in the absence and presence of S9, only the substituted NBs were observed to be mutagenic (Assmann et al. 1997). 4-Nitrophenol was reported to be mutagenic in the TA98 in the absence of S9 (Yang et al. 2003) in contrast to earlier studies (Shimizu and Yano 1986). Mutagenicity was also reported for phenol, an additional possible transformation product of NB (Kamber et al. 2009, Kirkland et al. 2011).

Figure 2 here

Group 4 - Ethers, alcohols, and phenols

In group 4 (Tables 1 and S1 (SI)) only cumene hydroperoxide exerted mutagenicity. Its degradation by photolysis, oxidation by ozone and $^{\bullet}OH$ led to the removal of its mutagenic activity. The degradation of cumene hydroperoxide by all three treatment methods together with the mutagenic equivalents (MEQ) is shown in Figure 3.

Figure 3 here

The MEQ results show that the mutagenic activity of the parent compound decreases at or below the line for stoichiometric loss of mutagenicity (slope = -1). This means that mutagenicity is lost proportionally to the degradation of the parent compound. During degradation of other compounds from this group, no significant mutagenicity was revealed.

Group 5 - Nitrogen-containing compounds

None of the parent compounds of group 5 (Tables 1 and S1 (SI)) were mutagenic in the Ames tests (TA98 and TAMix). However, degradation of aniline and quinoline led to the formation of mutagenic compounds, which were detectable by the Ames test (TA98 and TAMix) (Table 2). The direct photolytic transformation products of aniline induced mutagenicity in the strain TA98 in presence of S9 (Figure 4).

Figure 4 here

Based on a previous study, the photolysis of aniline leads to the formation of hydrazobenzene, 4-aminodiphenylamine, 2-aminodiphenylamine, benzidine and azobenzene (Zechner et al. 1976). Benzidine has been recognized as a human carcinogen by the US EPA and

the IARC and it was positively tested for mutagenicity in the strain TA98 in the presence of the S9-mix (Chung et al. 2000, Sinsheimer et al. 1992). Azobenzene is also considered to be a probable human carcinogen (B2) and was tested positively in TA100 in the presence of the S9-mix. Hydrazobenzene showed the same results in TA100 in presence of the S9-mix. (Zeiger 1987). However, in the present study no significant mutagenicity was observed in the TAMix strains with and without S9, which are able to detect the same type of mutations as TA100.

During oxidation of quinoline (Q) with ozone, formation of mutagenicity was detected in both strains (in absence and presence of the S9-mix) during oxidation by ozone, but only in the TA98 strain during oxidation by $^{\bullet}$OH (Table 2). In a prior study, quinoline itself exhibited a positive response for base-pair substitution in TA100 in the presence of the S9-mix (Plumlee and Reinhard 2007). However, in the present study, the mutagenic potency of the parent compound Q was not detected in any strain used in this study, perhaps because the experimental conditions of the Ames test was not sensitive enough to detect the mutagenicity of quinoline.

Several possible transformation products of Q were shown to be mutagenic *in vitro*. Mutagenic activity of 5-hydroxyquinoline and 8-hydroxyquinoline (5-OH-Q and 8-OH-Q) (more details on the formation of hydroxyquinolines are given in the section on estrogenicity below) was detected in the TAMix (in presence of the S9-mix). It was previously demonstrated that 5-OH-Q and 8-OH-Q showed mutagenicity in TA98 and TA100 in presence of the S9-mix (Sideropoulos and Specht 1984, Smith et al. 1997, Willems et al. 1992). Also some other derivatives of quinoline were mutagenic in the TA100 in presence of the S9-mix (Willems et al. 1992).

Group 6 - Organophosphorous compounds

The results from the study of the mutagenic activity of organophosphorous compounds are described in detail elsewhere (Parker et al. in prep.). Briefly, a degradation of the

organophosphorous insecticide methamidophos by $^{\bullet}OH$ led to the formation of mutagenic activity in the TA98 strain in presence of the S9-mix. Fenamiphos and dicrotophos showed positive activity in the TAMix strain in absence of the S9-mix, and $^{\bullet}OH$ degradation (UV/H_2O_2) was effective at removing this mutagenic signal. Acephate showed a weak response in the TA98 strain in absence of the S9-mix, and oxidation by $^{\bullet}OH$ (UV/H_2O_2) was effective for the removal of this activity.

Group 7 - Amides

No mutagenicity formation was observed during degradation of chemicals from group 7.

Group 8 – *N*-nitroso compounds

The results of this class of compounds have been published in another study (Mestankova et al. 2014); here only a brief summary will be provided. The UV photolysis of the non-mutagenic NDPhA leads to the formation of mutagens detectable in the TA98 strain (with and without the S9-mix). Oxidation of both *N*-nitrosodi-*n*-propylamine and *N*-nitrosopyrrolidine by $^{\bullet}OH$ revealed the formation of direct mutagens detectable in the TAMix strains (Zeiger 1987).

Development of Estrogenic Activity

An overview of all the results from estrogenicity testing is given in Table S4 (SI). 23 parent compounds were tested in the YES assay and only 4,4'-methylendianiline (MDA) showed estrogenic activity in the YES test. The development of the estrogenic activity of MDA as EEQ/EEQ_0 was measured during its degradation with ozone and $^{\bullet}OH$ (Figure 5).

Figure 5 here

Both processes (ozone and $^{\bullet}OH$) lead to a decrease of the overall estrogenic activity, following a slight initial increase due to $^{\bullet}OH$. The EEQ decreased stoichiometrically with the

384 decrease of the concentration of MDA during oxidation as indicated by the values being close to
385 the line with a slope of −1.

386 From the 23 target compounds tested, quinoline (Q) is the only one resulting in a
387 formation of estrogenicity detected by YES test during its oxidation by •OH. The formation of
388 estrogenic activity was confirmed under two different UV/H_2O_2 experimental set-ups: (1) a
389 medium-pressure mercury vapor lamp (including a band-pass filter in the wavelength range of
390 308-410 nm) in a solution containing 5 mM (170 mg/L) H_2O_2 and (2) a low pressure mercury
391 vapor lamp (emitting primarily at 254 nm) with 1 mM (34 mg/L) H_2O_2 (Figure 6).

392 **Figure 6 here**

393 The formation of the estrogenic activity was also confirmed for a lower Q concentration
394 of 1 µM (~0.13 mg/L) (Figure S6), which is closer to environmentally relevant conditions. The
395 oxidation of 100 µM (~13 mg/L) Q leads to a maximum estrogenic activity of approximately $2 \times$
396 10^{-10} M EEQ. Given that the potency of Q relative to E2 is $3x10^{-4}$, a concentration of 0.24 mg/L
397 would therefore be needed to lead to an estrogenicity comparable to 1 ng/L 17β-estradiol. The
398 levels of estrogenicity measured were well below the effect concentrations relevant to humans
399 (Brand et al., 2013). The oxidation of Q by ozone did not result in any estrogenic activity, nor
400 did the direct photolysis of Q with a low-pressure lamp.

401 The degradation of Q by •OH was previously studied in detail (Nicolaescu et al. 2005a, b,
402 Zhang et al. 2003). Hydroxyl radical attack occurs preferentially on the electron-rich benzene
403 ring by an OH addition reaction, leading to a hydroxylation of Q in positions 5-7. Further
404 oxidation of these products can lead to the formation of quinolone-5,8-dione. The pyridine ring is
405 less susceptible to an •OH attack, but it still occurs, as the products of its hydroxylation are also
406 detected in systems where Q is transformed by •OH. While one of the main degradation products,

8-hydroxyquinoline, was reported to not exhibit estrogenic activity by the recombinant yeast assay with 3 days exposure (Miller et al. 2001), together with other hydroxylated quinolines (6- and 7-OH-Q) it exhibited a clear positive response for estrogenic activity by the YES 5-day assay (Schultz and Sinks 2002). All hydroxylated quinolines (with a hydroxyl group in positions 2-8) were evaluated by the YES test in the present study. The EC_{50} values for the estrogenic hydroxylated quinolines determined in this study (3 days exposure YES test) are presented in Table 3 together with literature data of the YES test with 5-days exposure. Hydroxylated quinolines with a hydroxyl group on the pyridine ring did not exhibit an estrogenic response.

Table 3. EC_{50} values of estrogenic hydroxylated quinolines in the YES test compared with previously published data, in which the compounds were tested with the recombinant yeast assay with longer incubation times (Schultz and Sinks 2002) (5 days, instead of 3).

	This study (3-d EC_{50}, M) (95% confidence interval)	5-d EC_{50} (M) (Schultz and Sinks 2002)
6-OH-Q	**8.0×10^{-4}** ($6.5\text{-}9.7 \times 10^{-4}$)	3.4×10^{-4}
7-OH-Q	**1.4×10^{-3}** ($1.1\text{-}1.6 \times 10^{-3}$)	6.9×10^{-4}
8-OH-Q	**6.6×10^{-8}** ($3.1 \times 10^{-8}\text{-}1.46 \times 10^{-7}$)	3.4×10^{-7}

8-OH-Q is the most potent of the estrogenic compounds among the hydroxyquinolines, however, no *in vivo* data on its estrogenicity are available. A relatively high toxicity, i.e. impairment of viability, of 8-OH-Q towards yeast cells probably causes the ambiguous results obtained for its estrogenicity testing (note also the large 95 % confidence interval of EC_{50}).

The absence of estrogenicity in samples taken during oxidation by ozone can be explained by the formation of different transformation products. The reactions of Q with ozone (relatively slow) take place at the benzene ring leading to the cleavage of 5,6 or 7,8 bonds

(Andreozzi et al. 1992) and the formation of quinolinic acid, which can be further oxidized to a *N*-oxide derivative. A minor ozone attack was observed on the pyridine ring through formation of an *N*-oxide.

CONCLUSIONS

Advanced oxidation processes are able to degrade the selected compounds from the CCL3. Based on a thorough kinetic assessment of the 25 CCL3 compounds studied, 23 were found to be amenable to hydroxyl radical-based treatment, with second-order $^{\bullet}OH$ rate constants in the range of $3–8\times10^{9}$ $M^{-1}s^{-1}$. All compounds tested in the groups containing olefines, ethers, alcohols and phenols, amides, and *N*-nitroso compounds were degradable by $^{\bullet}OH$.

Ozonation, photolysis and AOPs are relevant not only for the abatement of the parent compound, but also for the removal of biological activity as illustrated by the mutagenic activity removal of cumene hydroperoxide or the estrogenic activity removal of MDA. 6 of the 23 evaluated compounds exhibited transformation products causing mutagenic or estrogenic activity detectable by *in vitro* assays. Mutagenic activity (via $^{\bullet}OH$, ozone, or direct photolysis) occurred in 9 treatment cases with the most frequent detected in the TA98 strain (frameshift mutation). The formed mutagens were mostly direct mutagens, meaning they do not need metabolic activation by the S9 enzymes to become mutagenic. Estrogenic activity (via $^{\bullet}OH$, ozone, or direct photolysis) was generated only from the oxidation of quinoline by $^{\bullet}OH$. However, when examined at environmentally relevant concentrations, quinoline oxidation produced only low estrogenic activity, probably not relevant in an overall risk assessment.

Based on the results from this study, the possibility of generating biological effects as indicated in the Ames II and YES assays during advanced oxidation processes, while rare, cannot be completely ruled out. If ozone- or UV-based AOPs are implemented for drinking water

451 treatment from water resources with significant anthropogenic contamination, the use of
452 bioassays to assess potential risks associated with AOPs, similar to the current study should be
453 considered.
454

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Figure Captions for WR 11746

Figure 1. Experimental procedure for testing of the selected CCL3 compounds and their transformation products (TPs): (i) Assessment of the susceptibility of the target compounds to oxidation by $^{\bullet}OH$, (ii) effect removal during oxidative transformation of parent compound, (iii) test for toxic transformation products with alternative modes of action. The main criteria for the feasibility of an oxidative process was based on the second order rate constant for the reaction with $^{\bullet}OH$. Typically, compounds with low $^{\bullet}OH$ rate constants do not react with ozone. Photolysis was also a selection criteria but except for a few compounds (see below), it is typically overruled by $^{\bullet}OH$ radical reactions in AOPs.

Figure 2. Mutagenic equivalents MEQ-2-NF (2-NF: 2-nitrofuran) during degradation of nitrobenzene by UV photolysis in the TA98 strain without the S9-mix. For explanation of MEQ-2-NF see Materials and Methods section.

Figure 3. Development of relative mutagenicity during cumene hydroperoxide abatement by ozone, direct photolysis and $^{\bullet}OH$ in the TAMix strains without the addition of the S9-mix.

Figure 4. Development of mutagenicity (expressed as mutagenic equivalents MEQ-AA (AA: 2-aminoanthracene) during transformation of aniline by direct photolysis in the TA98 strain in presence of the S9-mix. For explanation of MEQ-AA see Materials and Methods section.

Figure 5. Development of EEQ/EEQ_0 during degradation of 4,4'-methylendianiline (MDA, 100 µM, pH 7) by ozone and $^{\bullet}OH$. EEQ measured by the YES assay.

Figure 6. Estrogenic activity (in EEQ) during transformation of quinoline (100 µM) by $^{\bullet}OH$: (a) medium-pressure lamp (filtered to emit between 308-410 nm) in a photoreactor with 5 mM H_2O_2; (b) low-pressure lamp with 1 mM H_2O_2.

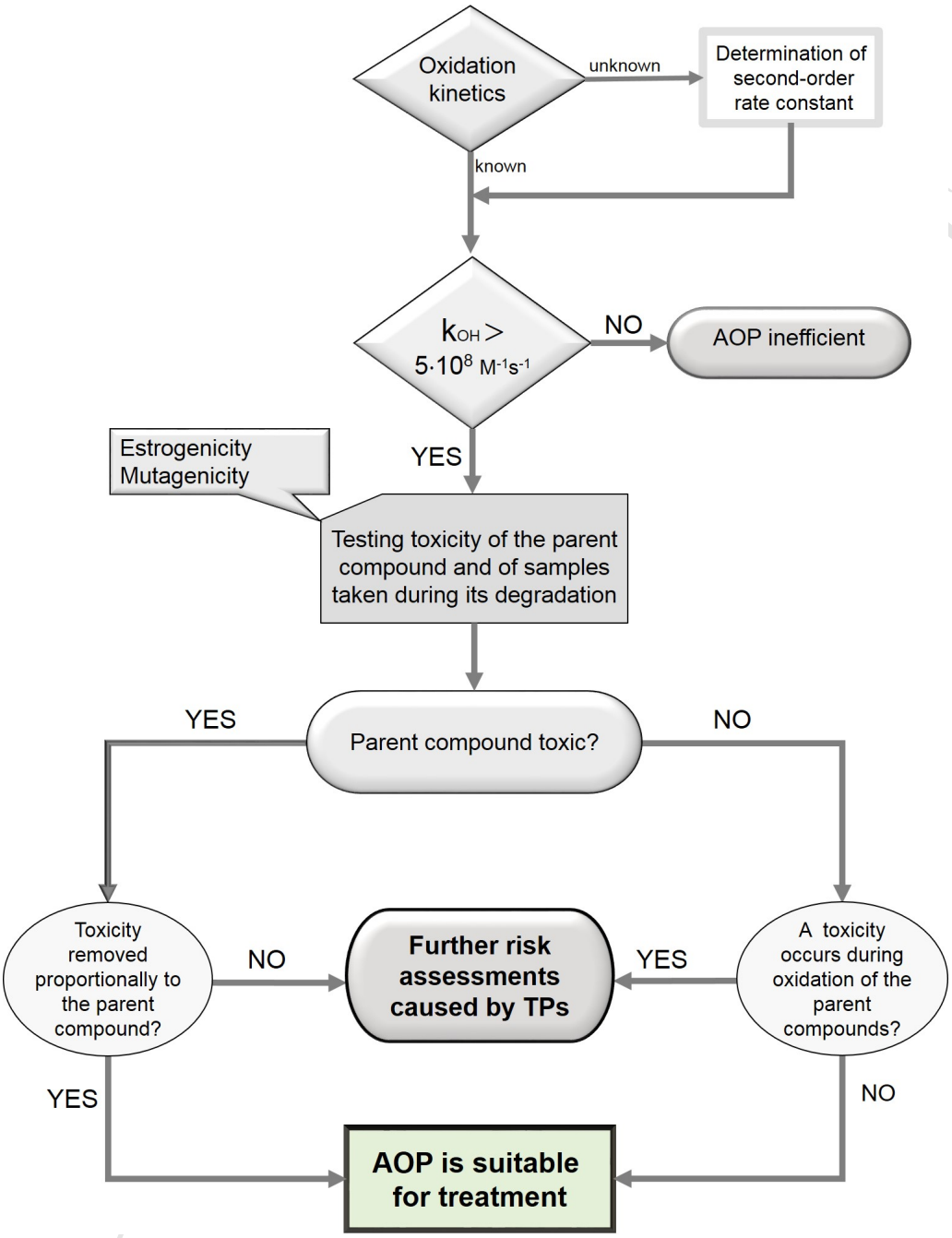


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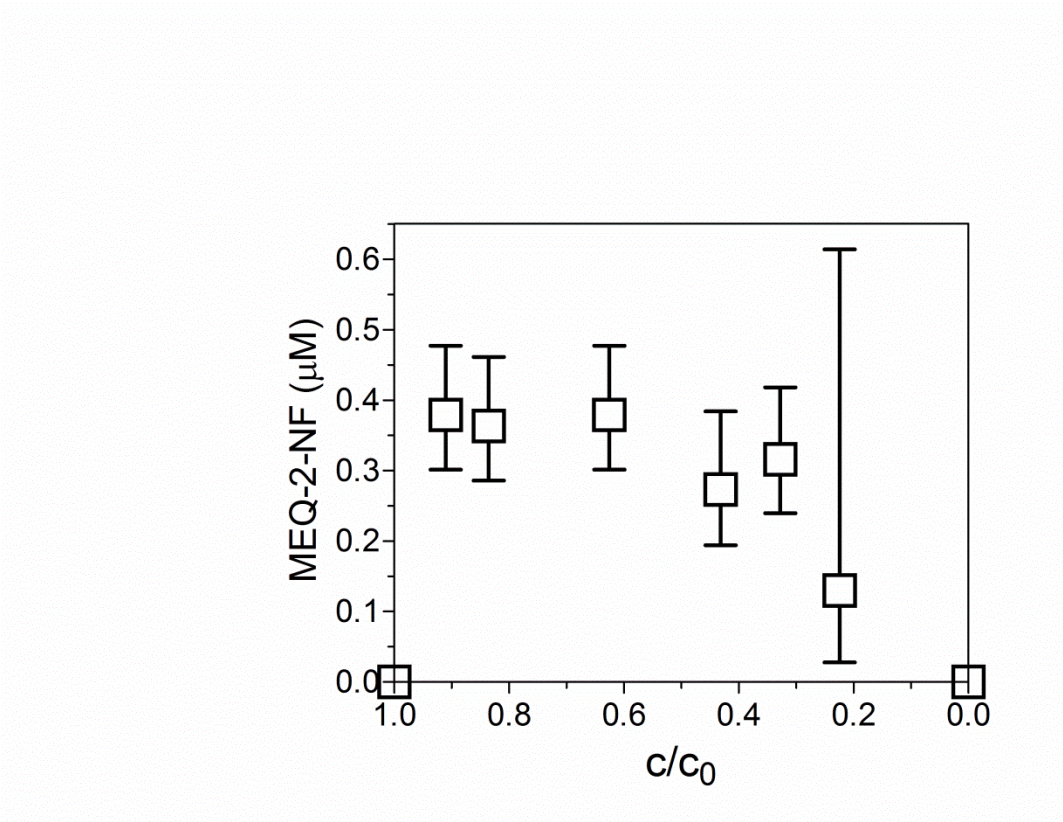


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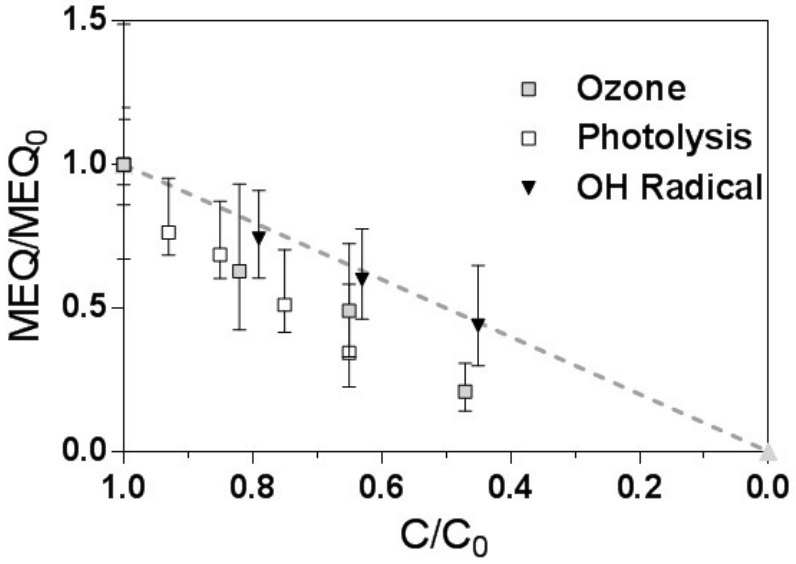


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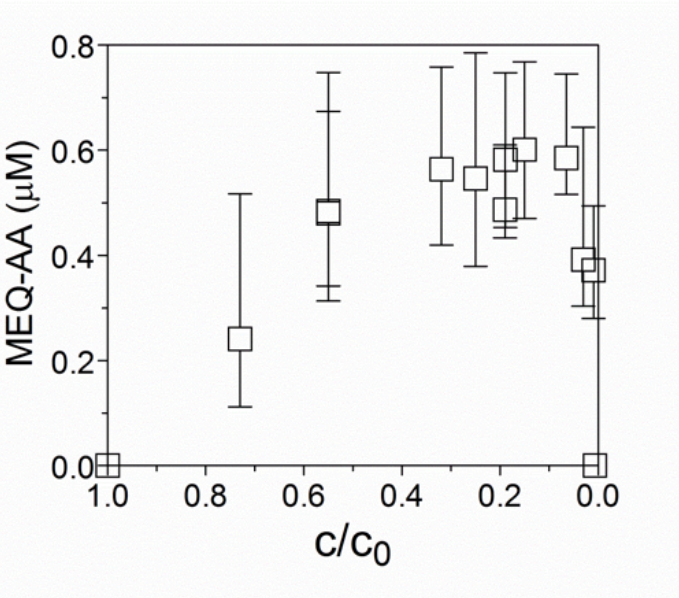


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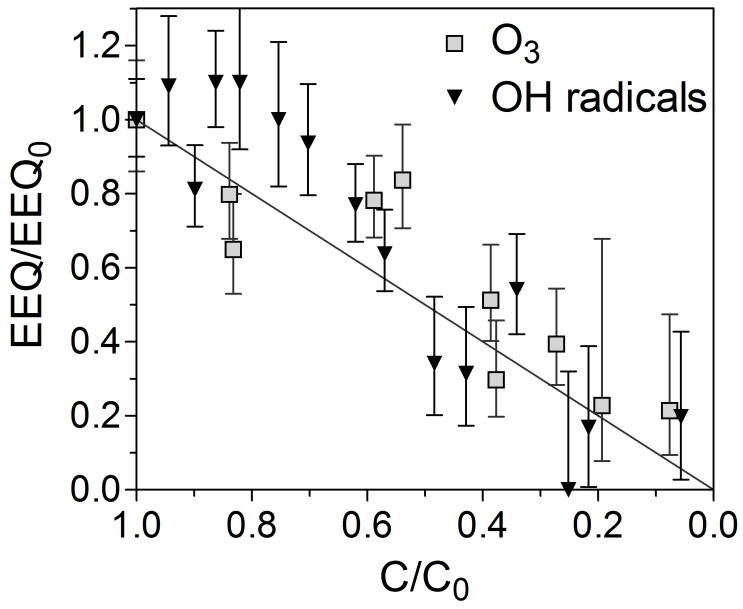


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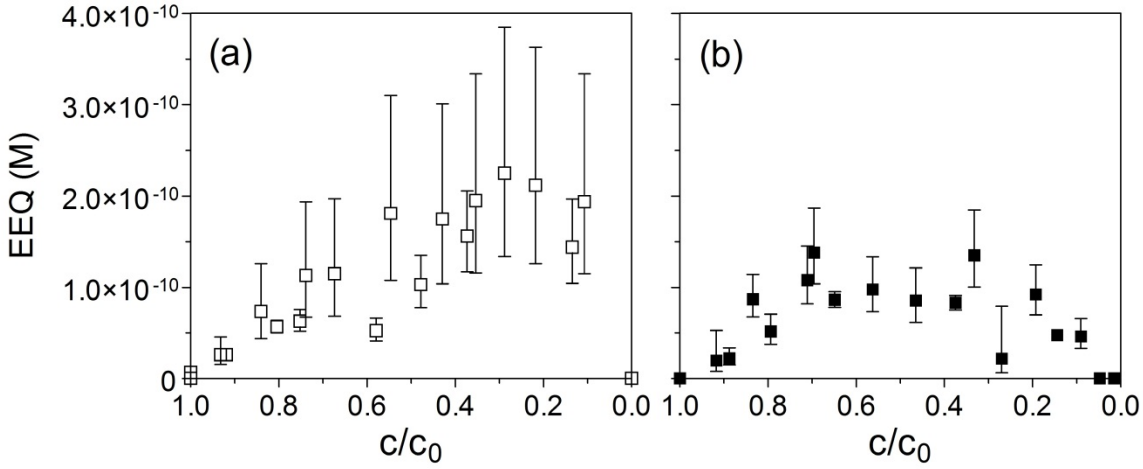


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Highlights

For "Transformation of Contaminant Candidate List (CCL3) compounds during advanced oxidation processes: toxicity assessment"

1. Ozone and UV-based advanced oxidation processes were studied
2. 25 Chemicals on the US EPA Contaminant Candidate List 3 (CCL3) were evaluated
3. Transformation products were evaluated for mutagenicity and estrogenicity
4. Most treatment cases resulted in a loss of biological activity upon oxidation
5. Increases in biological activity post-treatment was found for a number of compounds

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