Assessing Advanced Oxidation Transformation Products

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This thesis entitled

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has been approved for the Department of Civil, Environmental, and Architectural Engineering

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

Abstract

Assessing Advanced Oxidation Transformation Products

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Thesis directed by Professor Karl G. Linden.

Advanced water treatment technologies are being evaluated and implemented for treating organic micropollutants in water. Included among several viable technologies is a collection of advanced oxidation processes. Of primary interest in water are UV- and ozone-based oxidation systems. UV-hydrogen peroxide and ozone-hydrogen peroxide are advanced oxidation technologies appropriate for treating water contaminated with trace levels of organic chemicals. Unlike water treatment processes such as activated carbon and air stripping which transfer chemicals from one physical phase to another, these technologies are capable of destroying chemicals, and transforming them to degradation products. Although this destruction of contaminants is generally beneficial, the formation of byproducts (from the water matrix) or transformation products (from the micropollutants) that retain harmful biological activity is a possibility.

The objectives of this thesis were designed in order to assess the possible formation of toxic byproducts following oxidative treatment. A parallel analytical and toxicological potential analysis utilizing both analytical and biological tools following the oxidative degradation of several chemical contaminants found on the United States Environmental Protection Agency's Candidate Contaminant List 3 was performed, including following the changes in genotoxicity (mutagenicity), estrogenicity, and neurotoxicity while the contaminants were undergoing oxidative

treatment. Mutagenicity dominated with regard to formation of toxicity post-oxidation in the contaminants tested. However, most chemical contaminants were amenable to advanced oxidation treatment, and no formation of toxicity was observed.

The synergistic use of biological filtration following advanced oxidation was explored, as providing an additional barrier to treatment could prove useful for the removal of various transformation products. Both biological sand and biologically activated carbon were studied with respect to the removal of genotoxic chemical byproducts. Assimilable organic carbon removal is an additional driver for implementing biological filtration downstream of an oxidation processes. The formation of assimilable organic carbon with regards to UV-based oxidation processes was explored. The UV-based process was found to cause no increase in assimilable organic carbon concentration when undergoing both low and medium pressure UV photolysis.

A policy review was conducted that included an exploration into group contaminant regulation, using the USEPA Contaminant Candidate List 3 as a framework

Group contaminant regulations by both structure (biological mode of action) and the contaminants intended use or purpose was performed. A lack of correlation existed between the health and occurrence data generated within each grouping parameter. An alternative approach to aid in emerging contaminant regulation was provided that included an effect-directed analysis that could act as an early warning system for water utilities with respect to unintended constituents or chemical byproducts.

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Table of Contents

List of Figures

List of Tables

1. Introduction and Scope

Chapter 1 of this dissertation provides the motivation for this research, a general testing strategy, and the research objectives that were studied. A literature review focusing on advanced oxidation and bioassay analysis is provided as Chapter 2. Chapters 3 and 4 are to be submitted as individual publications that include background, methods, and results pertinent to each publication. Chapter 5 includes additional work on Objectives 4 and 5. Chapter 6 is meant to be submitted as an individual publication, regarding drinking water policy. Conclusions, applications, and recommendations for future work are presented as Chapter 7.

1.1. Motivation and Impact

Advanced water treatment technologies are being evaluated and implemented for treating organic trace organic contaminants, micropollutants, in water. Included among several viable technologies is a collection of advanced oxidation processes (AOPs). Of primary interest in water are UV- and ozone-based oxidation systems. UV-hydrogen peroxide (H_2O_2) and ozone-H_2O_2 are advanced oxidation technologies appropriate for treating water contaminated with trace levels of organic chemicals. Unlike water treatment processes such as activated carbon and air stripping which transfer chemicals from one physical phase to another, oxidation technologies are capable of destroying chemicals, and transforming them to degradation products. Although this destruction of contaminants is generally beneficial, the formation of byproducts (from the water matrix) or transformation products (from the micropollutants) that retain harmful biological activity is a possibility.

The concerns of biological and chemical degradation products led to the regulation of metabolites of pesticides in the drinking water directive of the European Union in 1998 (EU 1998).

AOPs were primarily used for treatment of atrazine in France in the 1990s but it has been shown that atrazine was not mineralized but transformed into mainly deethyl- and deisopropylatrazine (Acero, Stemmler et al. 2000). In the late 1990s, this triggered a ban of AOPs for drinking water treatment in France. Since then research has been performed in the field of oxidation of micropollutants with emphasis on kinetics and product formation, more recently resesarch also with regard to toxicological effects. The main oxidants in the AOPs UV/H_2O_2 and O_3/H_2O_2 are OH radicals and ozone. Generally, oxidative degradation of micropollutants with these oxidants leads to oxygen-rich compounds, which are typically more hydrophilic than their parent compounds. Therefore, their ability to penetrate through cell membranes decreases, which typically leads to a loss in the baseline toxicity (Escher, Baumgartner et al. 2008). In general, oxidative transformation products are less toxic than their parent compounds, unless a compound with a specific toxicity is formed. In some recent studies that were conducted, it has been shown that the primary attack of estrogenic compounds with oxidants such as OH radicals, ozone, chlorine, bromine, chlorine dioxide and ferrate leads to a stoichiometric loss of the estrogenic activity tested with the Yeast Estrogen Screen Assay (Huber, Ternes et al. 2004; Rosenfeldt, Chen et al. 2007; Lee, Escher et al. 2008). Even though the samples were only tested for one endpoint, namely estrogenicity, additional effects are highly unlikely, because the concentrations of estrogenic compounds are typically very low in wastewater treatment plant effluents and drinking water (low ng/L range).

Based on these and other kinetic studies on antibiotics and other chemical contaminants it can be concluded, that the transformation of all investigated compounds by ozone and OH radicals is very efficient and a viable process for water treatment. However, for typical oxidant doses in water and wastewater treatment, mineralization of micropollutants to carbon dioxide, water and mineral

acids will not occur, and intermediate chemical species will remain that may or may not retain toxicological properties.

Chemicals of current interest in the United States are those on the Contaminant Candidate List 3 (CCL 3) as they are being explored for possible regulation by the United States Environmental Protection Agency (USEPA) in the future. The use of AOPs for mitigating these chemicals was assessed in relation to the formation of transformation products to fully understand the implications of utilizing oxidation technologies in the treatment of water.

1.2. Research Objectives

The major themes of this study included testing the degradability of CCL3 contaminants by AOPs, the toxicity of the degradation products post-oxidation, the removal of toxic byproducts by biofiltration, the formation and mitigation of assimilable organic carbon (AOC) from AOPs, and the policy implications of transformation products as depicted in Figure 1.1.

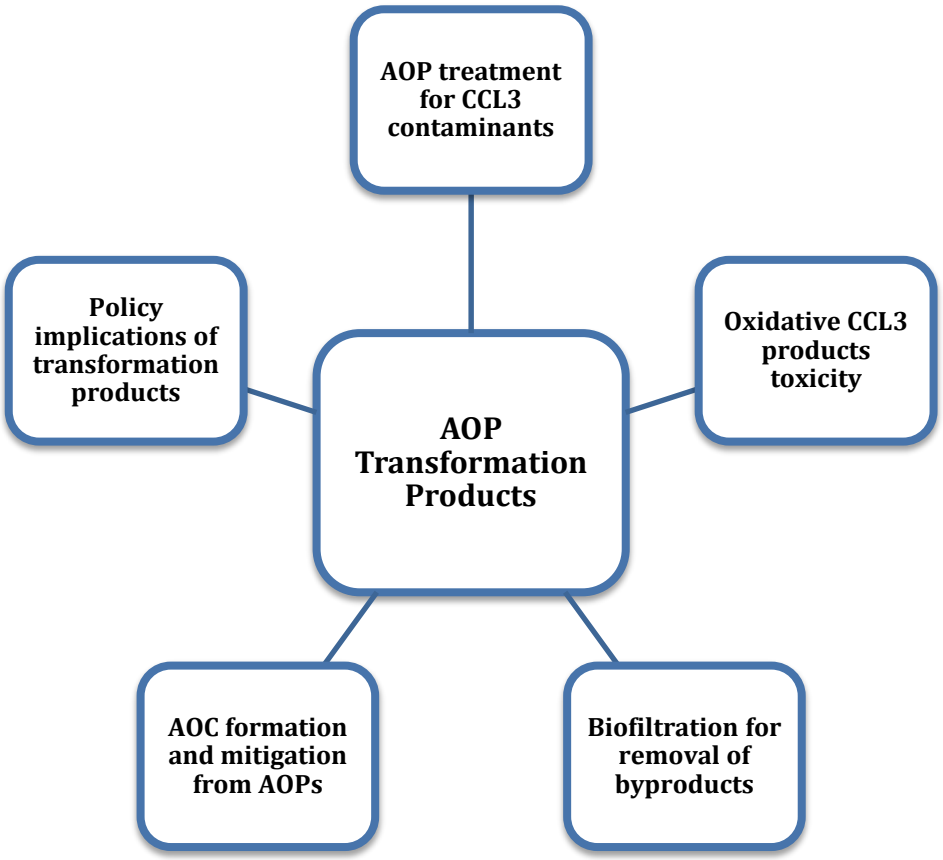

Figure 1.1 Major topics explored in this study as they relate to CCL3 transformation products post AOP treatment

1.2.1. Defined Research Objectives

Defined research objectives of this dissertation can be found numbered 1-5 below, with their corresponding chapter referring to where they are reported.

4

1. Assess the treatability of CCL3 contaminants selected by AOP. (Chapters 3 and 4)
2. Assess the toxicity (by defined endpoints) of CCL3 contaminants before and after degradation by AOP. (Chapters 3 and 4)
3. Investigate the use of biological filtration for the removal of toxic/unknown byproducts following AOP. (Chapter 5)

4. Investigate the formation and mitigation of AOC following AOP treatment. (Chapter 5)
5. Explore the policy implications of using transformation processes (i.e. advanced oxidation) for the treatment of CCL3 contaminants. (Chapter 6)

1.3. Hypothesis

The defined research objectives above led to the formation of the following hypothesis in order to provide a technical framework for this dissertation:

1. Assess the treatability of CCL3 contaminants selected by AOP. (Chapters 3 and 4)
    a. Most, if not all, CCL3 contaminants selected will be amenable to hydroxyl radical degradation, and therefore treatable by AOP.
    b. Most CCL3 contaminants selected will not be amenable to destruction by molecular ozone or UV photolysis alone.
2. Assess the toxicity (by defined endpoints) of CCL3 contaminants before and after degradation by AOP. (Chapters 3 and 4)
    a. A small percentage, likely less than 30%, of CCL3 compounds tested will result in byproducts that exhibit more toxic potential than the parent compound after undergoing transformation by AOP.
    b. More mutagenic activity is likely to be observed with the CCL3 compounds selected than estrogenic activity.
3. Investigate the use of biological filtration for the removal of toxic/unknown byproducts following AOP. (Chapter 5)
    a. Biological sand filtration will be less effective than biological granular activated carbon for the removal of toxic oxidation products following AOP.

4. Investigate the formation and mitigation of AOC following AOP treatment. (Chapter 5)
    a. AOC formation is likely to occur post ozone-AOP treatment, but not as likely post UV-AOP treatment.
5. Explore the policy implications of using transformation processes (i.e. advanced oxidation) for the treatment of CCL3 contaminants. (Chapter 6)
    a. Grouping contaminants by structural properties, or modes of action within the human body will provide further guidance for regulations.
    b. A more holistic matrices effect approach to emerging contaminant regulation will be challenging to implement, but will provide a more comprehensive barrier for distributed water suppliers.

1.4. General Approach

The overall research goal of this dissertation was to explore and evaluate the use of UV and ozone-based AOPs for mitigating CCL3 contaminants. To achieve the previously defined research objectives, the CCL3 was divided amongst two research institutions, with ten CCL3 contaminants being selected for study in this dissertation research. These contaminants are included in the literature review presented in Chapter 2. CCL3 contaminants were examined for both UV-and ozone-based AOP processes for changes in toxicity following oxidation.

A full degradation pathway was not developed for each contaminant, due to time and material constraints, however an effect-directed analysis (EDA) approach was utilized. EDA allows a response to be measured and quantified using bioanalytical tools without the need for a full byproduct analysis. In the cases where toxicological activity of the parent compound holds or is increased despite achieving the targeted level of parent compound treatment, an attempt was

made to identify the degradation products and pathways to understand the specific oxidation process. With this knowledge, better aid can be applied in refining up and downstream processes to better remove these toxicologically relevant constituents. The EDA approach is shown graphically as Figure 1.2.

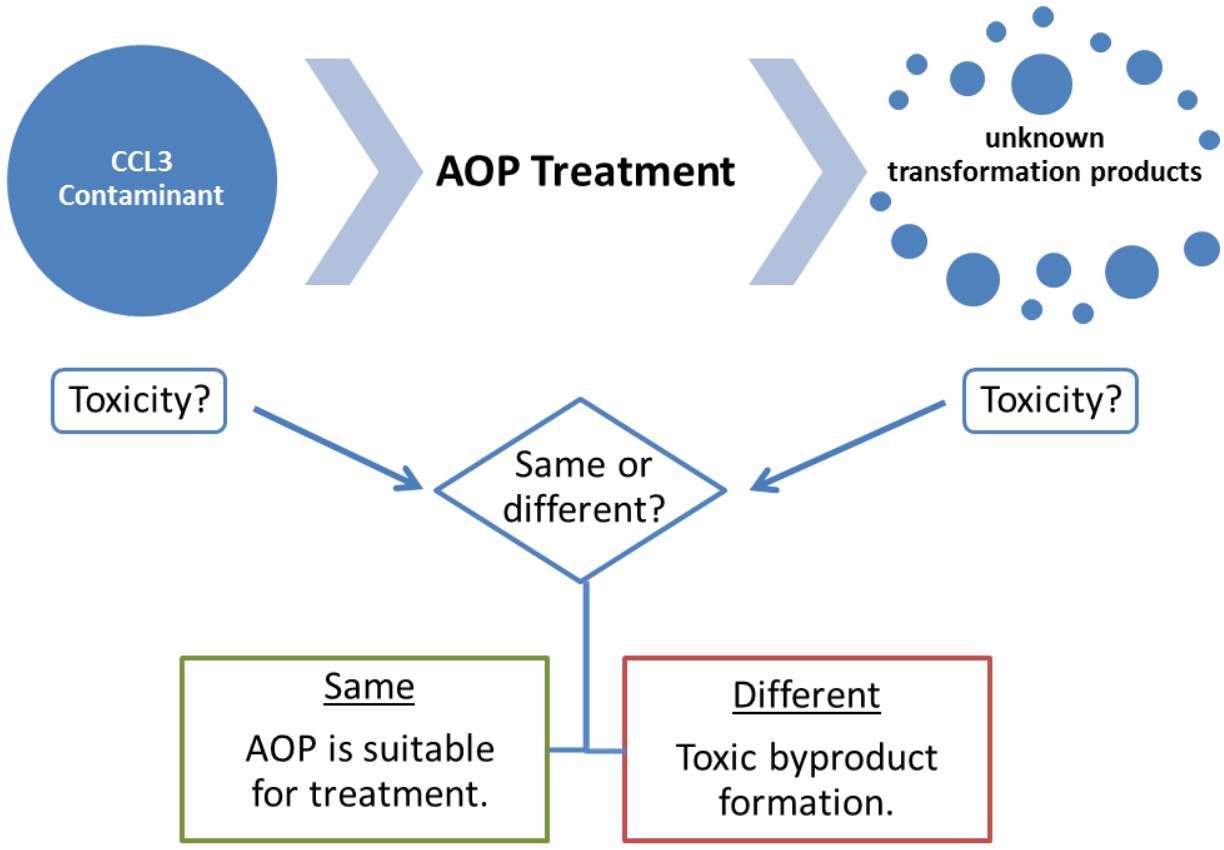

Figure 1.2 General approach for CCL3 treatment with advanced oxidation and the treatment of CCL3 contaminant

EDA was also taken in the investigation of biofiltration following advanced oxidation systems, by utilizing a toxicologically relevant compound, after undergoing oxidation. In addition to the use of biofiltration for the removal of toxic oxidation processes, the formation of AOC following UV-AOP processes was explored using bench-scale methods.

Following the theme of toxicologically relevant transformation processes, regulations surrounding emerging contaminants, specifically CCL3, were explored with regards to chemical grouping and EDA as a tool for regulators.

2. Literature Review and Approach

2.1. Advanced oxidation processes

In 1987, Glaze et al. first established the concept of advanced oxidation processes (AOPs) (Glaze, Kang et al. 1987). AOPs were defined as the oxidation process, which generate hydroxyl radicals in sufficient quantity to affect in water treatment. A wide variety of AOPs have been studied and implemented for many years, including ozonation, UV/ozone, UV/hydrogen peroxide, ozone/hydrogen peroxide, and UV/photocatalysis. Most commonly, a combination of strong oxidants, catalysts, and irradiation (e.g. UV) are used to produce oxidants, such as UV coupled with chlorine (Watts, Rosenfeldt et al. 2007). A list of typical AOPs, both chemical and non-photochemical, developed by Huang et al. can be seen in Table 2.1 (Hauang, Dong et al. 1993).

Table 2.1 Typical AOP systems, including photochemical and non-photochemical AOP systems (Hauang, Dong et al. 1993)
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AOPs have many advantages including high rates of pollutant oxidation, as a result of the production of fast-reacting, non-selective hydroxyl radicals (•OH), which are known to be one of the strongest oxidants in water (Hoigne, Bader et al. 1985). •OH radicals have the capability to oxidize most organic compounds to water, carbon dioxide, and mineral salts (Ollis, Pelizzetti et al. 1991). Bischof et al. showed that molecules of atrazine, desethylatrazine, and simazine can be mineralized to carbon dioxide in the UV/H_2O_2 process (Bischof, Hofl et al. 1996). AOPs are

also advantageous because of their ability to function in a variety of natural water matrices (Andreozzi, Caprio et al. 1999), (Comninellis, Kapalka et al. 2008), (Czech and Nazimek 2006). Common disadvantages of AOPs are high treatment costs, storage of reactive chemicals (e.g. ozone, hydrogen peroxide, etc.), and high-energy sources (e.g. UV lamps) (Kochany and Bolton 1992).

2.1.1. UV/H_2O_2

Drinking water disinfection using UV irradiation is already in practice; however, photolysis alone does not lead to the destruction of as many organic contaminants as an advanced oxidation process. Adding hydrogen peroxide (H_2O_2) in combination with UV irradiation at a wavelength of 254 nm (λ = 254 nm) results in the cleavage of the O-O bond (Equation 2.1), and generates hydroxyl radicals (•OH) (Legrini, Oliveros et al. 1993). A high concentration of hydrogen peroxide is needed to produce large amounts of hydroxyl radicals due to the low molar extinction coefficient (ε = 19.6 $M^{-1}s^{-1}$) at λ = 254 nm (Andreozzi, Caprio et al. 1999). However, there is a limit to hydrogen peroxide addition, as it can act as a scavenger of hydroxyl radicals (Equation 2.2) when the concentration is too high (Glaze, Kang et al. 1987). Therefore, an important component of UV/H_2O_2 system operation is the determination of an optimal hydrogen peroxide concentration, based on the water quality parameters, and the targeted level of treatment.

$$H_2O_2 + hv \rightarrow \bullet OH + \bullet OH$$

Equation 2.1

$$\bullet OH + H_2O_2 \rightarrow HO_2 \bullet$$

Equation 2.2

The combination of UV/H_2O_2 has been proven effective in the treatment of a variety of water treatment contaminants, such as herbicides and pharmaceuticals (Keen, Baik et al. 2012),

(Linden, Rosenfeldt et al. 2007), (Liu, Wu et al. 2007). This process has also been shown effective for the destruction of chlorophenols and other chlorinated compounds (Hirvonen, Tuhkanen et al. 1996; Trapido, Hirvonen et al. 1997; Nicole, De Laat et al. March 1991). An advantage of using the UV/H_2O_2 process is the potential for two processes to take place simultaneously for contaminant destruction, including (1) photolysis of contaminants and (2) hydroxyl radical reactions (Rosenfeldt and Linden 2004). For UV irradiation to effectively react with hydroxyl radical a water with very high UV transmittance is required. This often requires pre-filtration steps in order to lower the turbidity to an acceptable level prior to UV/H_2O_2 treatment.

Additionally, downstream hydrogen peroxide quenching is required in most applications, especially when a chlorine residual is required (e.g. drinking water distribution). There are several options for hydrogen peroxide quenching including chemical and non-chemical options. Chemical options can include free chlorine, bovine catalase, sulfite, and thiosulfate for laboratory analysis of samples (Keen, Dotson et al. 2013), but in practice large amounts of chemicals may not be practical for quenching hydrogen peroxide residual (e.g. bovine catalase). Non-chemical options can include granular activated carbon (GAC) filtration, which was found to be highly effective at quenching hydrogen peroxide and can provide the benefit of filtration post-AOP processes (Linden, Dotson et al. 2012).

2.1.2. **Ozone**

AOP treatment using ozone has been in practice for some time, and is the most commonly applied technology compared to other AOPs. Applications have included disinfection, odor control, and color removal (Rice, Evison et al. 1981; Rice, Robson et al. 1981). Ozone as a treatment technology for micropollutants works by two mechanisms, (1) direct oxidation by

ozone, and (2) by indirect oxidation by hydroxyl radicals. The first process, direct oxidation, is characterized by its selectivity of contaminants and low reaction rates (Hoigné and Bader 1983). The second process, indirect oxidation, provides the same advantages to treatment as any AOP producing hydroxyl radicals.

The first step of process 1 works by the decomposition of ozone by hydroxide ions (Equation 2.3). This forms a hydroperoxyl radical, which is in an equilibrium state (Equation 2.4). The superoxide anion radical and ozone react to form an ozonide anion radical (Equation 2.5), which then decomposes into oxygen and hydroxyl radical (Equation 2.7) (Von Sonntag and von Gunten 2012). In theory, two hydroxyl radicals are produced for every 3 ozone molecules in the system (Equation 2.8).

$$O_3 + OH^- \rightarrow O_2 \bullet + HO_2 \bullet$$ **Equation 2.3**

$$HO_2 \bullet \rightarrow O_2 \bullet^- + H^+$$ **Equation 2.4**

$$O_3 + O_2 \bullet^- \rightarrow O_3 \bullet^- + O_2$$ **Equation 2.5**

$$O_3 \bullet^- + H^+ \rightarrow HO_3 \bullet$$ **Equation 2.6**

$$HO_3 \bullet \rightarrow \bullet OH + O_2$$ **Equation 2.7**

$$3O_3 + OH^- + H^+ \rightarrow 2OH \bullet + 4O_2$$ **Equation 2.8**

Limitations for the implementation of ozone include influent water quality parameters, as natural water constituents, such as DOM, that have an ozone-demand (Park, Hwang et al. 2001), and therefore upstream treatment is required upstream. An additional limitation is the formation of bromate, which is a regulated contaminant (Vongunten, Hoigne et al. 1994; Boorman, Dellarco et al. 1999). The high energy cost associated with ozone is a down-side, as well as the potential danger of on-site generation and hazardous chemical storage.

11

2.1.3. **Ozone/H_2O_2**

Ozone coupled with hydrogen peroxide (O_3/H_2O_2), the "peroxone process", was first presented in 1982 by Staehelin and Hoigne (Staehelin and Hoigne 1982). The decomposition cycle of ozone with hydrogen peroxide produces hydroxyl radicals, by following Equations 2.9 through 2.10 (Gottshchalk, Libra et al. 2000), (Hoigné 1982).

$$\boldsymbol{H_2O_2 \rightarrow HO_2^- + H^+} \qquad \textbf{Equation 2.9}$$

$$\boldsymbol{HO_2^- + O_3 \rightarrow HO_2 \bullet + O_3 \bullet^-} \qquad \textbf{Equation 2.10}$$

The pathway described above continues, and two ozone molecules produce two •OH radicals, as described by Equation 2.11.

$$\boldsymbol{2O_3 + H_2O_2 \rightarrow 2 \bullet OH + 3O_2} \qquad \textbf{Equation 2.11}$$

Studies have shown added advantages to the peroxone process, compared to that of ozone alone, such as with the degradation of atrazine in river water (Paillard, Brunet et al. 1988). Performance of ozone/H_2O_2 systems is dependent upon several factors including introduction point of hydrogen peroxide, alkalinity, and other radical/ozone scavengers. This system is also dependent upon the ozone dose and contact time (Duguet, Brodard et al. 1985).

Kinetic modeling of the peroxone process was completed using PCE as a model compound (Glaze and Kang 1989; Glaze and Kang 1989). Further modeling practices have taken place for O_3/H_2O_2 systems, with inputs for hydrogen peroxide concentration, ozone concentration, and contact time (Glaze, Lay et al. 1995). Advantages of the O_3/H_2O_2 combination are that it has a high yield of hydroxyl radicals, easy adaptation using existing ozone facilities, and it typically has the lowest process cost based on •OH radical oxidation. Disadvantages are similar to that of

the other AOP processes including high energy use, hazardous chemical storage, and the need to quench residual hydrogen peroxide.

2.1.4. **Water Quality Effects**

Scavenging species are those compounds or constituents in a water matrix that result in unintended treatment, thereby decreasing the efficiency of the advanced oxidation process for the intended target of treatment. Hydroxyl radical scavengers include dissolved organic matter (DOM), alkalinity (pH dependent – carbonate/bicarbonate), and other unknown constituents. Several values of the radical scavenging rate of DOM (k_{DOM}) have been reported. Zepp et al. reported a k_{DOM} value of $2.6x10^4$ $M^{-1}s^{-1}$ and others have reported values ranging from $1\text{-}5x10^4$ $M^{-1}s^{-1}$ (Hoigne and Bader 1979; Haag and Hoigne 1985; Zepp, Hoigne et al. 1987; Westerhoff, Song et al. 1998). The reaction of hydroxyl radical has been reported with both carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) as $4.2x10^8$ and $1.5x10^7$ $M^{-1}s^{-1}$, respectively (Chen and Hoffman 1973). The level of hydroxyl radical scavenging is concentration dependent upon the species, and often on the pH of the water (i.e. the carbonate buffer system).

Kinetic models have been developed in order to account for the radical scavengers and "unknowns" in natural water systems. Elovitz and von Gunten developed a kinetic model that measures the hydroxyl radical/ozone ratios in natural waters, the R_{ct} concept (Elovitz, Shemer et al. 2008). A kinetic model for UV/H_2O_2 hydroxyl radical production was developed by Linden and Rosenfeldt, and also works in natural water systems (Rosenfeldt and Linden 2007) while using a hydroxyl radical probe.

13

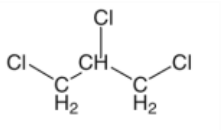
2.2. Contaminant Candidate List selection

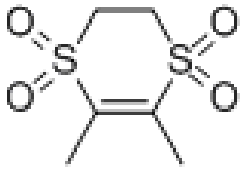
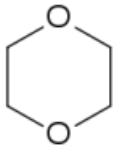
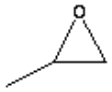
The dominant mechanism of degradation for advanced oxidation processes is the action of hydroxyl radicals. Therefore, the main selection criterion for this study was the likelihood that the contaminant would be degraded by hydroxyl radical. Contaminants were selected based on a fast reaction rate with hydroxyl radicals (•OH), partition coefficient of chemical into the water phase (k_{OW}), health and occurrence data, and analytical capabilities. An extensive literature review was performed to identify published hydroxyl radical reaction rates (k_{OH}) and known octanol-water partition coefficients (k_{OW}) for these compounds. Hydroxyl radical and ozone reaction rates for compounds that were not available were determined, and presented in Chapters 3 and 4. Contaminants with low •OH reaction rates ($k_{OH} < 5 \times 10^8$ $M^{-1}s^{-1}$) were generally dismissed, as they would not be appropriate candidates for AOP treatment. It is likely that the contaminant would be out-competed by natural constituents in the water matrix, such as natural organic matter or alkalinity, or would be outcompeted by another organic contaminant with a higher hydroxyl radical rate constant. The $logK_{OW}$ values were determined based on a literature review of the individual compounds, and when published values were not found, modeling software was utilized. A compound with a high $logK_{OW}$ indicates that it is more favorable to be in a solid-phase and will likely be removed by coagulation and/or filtration prior to an oxidation process, which would typically be found downstream of conventional treatment in a water treatment facility.

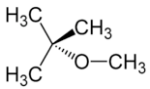
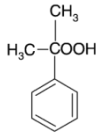
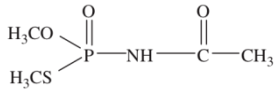
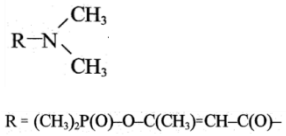
Contaminant groups were evaluated based on similar structural properties, along with bioassay screening for similar toxicity endpoints. Table 2.2 displays the compounds that were selected specifically for study in the scope of this dissertation.

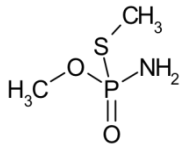
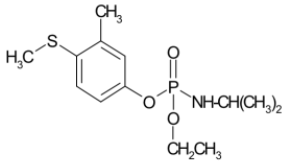
14

Table 2.2 Selected CCL 3 contaminants including known oxidation rate constant data, octanol-water partition coefficients, USEPA defined use, and justification for selection for this thesis study.

	CAS	k_{O3}, k_{OH} ($M^{-1}s^{-1}$)	Reference for Kinetic Data	$\log K_{OW}$ @ 25°C	Analytical Method	Use	Selection Justification
Group 1. Halogenated Compounds							
1,2,3-Trichloropropane 	96-18-4	--	--	1.79	EPA 624	It is an industrial chemical used in paint manufacture.	This compound is a leading emerging contaminant, with no information available as to how the compound degrades via AOP, and was therefore be studied.
Group 2. Olefines							
Dimethipin	55290-64-7	$k_{O3} \approx > 10^5$ $k_{OH} \approx > 5.0 \times 10^9$	--	-0.17	EPA 525.3	It is used as a herbicide and plant growth regulator.	Low K_{OW} (highly water soluble), relevant kinetic rates, and high occurrence data.

							
Group 4. Ethers, Alcohols, phenols, aldehydes and other oxygen containing compounds							
1,4-Dioxane 	123-91-1	$k_{O3} = 3.0 \times 10^{-1}$ $k_{OH} = 3.1 \times 10^{9}$ $k_{OH} = 2.5 \times 10^{9}$	(Thomas 1965; Eibenberger 1980; Hoigné and Bader 1983)	-0.27	CDC 1602	It is used as a solvent or solvent stabilizer in the manufacture and processing of paper, cotton, textile products, automotive coolant, cosmetics and shampoos.	High occurrence data, high water solubility, and interesting biodegradation byproduct investigation (Stefan and Bolton 1998).
Oxirane, methyl 	75-56-9	--	--	0.27	CDC 1612	It is an industrial chemical used in the production of other substances.	No published kinetic data is available, low K_{OW} and unknown byproducts are of interest.
Methyl-tert-butyl ether	1634-04-4	$k_{O3} = 0.14$ $k_{OH} = 1.9 \times 10^{9}$	(Acero, Haderlein	1.24	CDC 252080	It is used as an octane booster in gasoline, in the	High occurrence data and unknown

			et al. 2001)			manufacture of isobutene and as an extraction solvent.	oxidation byproducts expected.
Cumene hydroperoxide 	80-15-9	--	--	2.17	EPA 8260	It is used as an industrial chemical and is used in the production of other substances.	High occurrence data, no known kinetic data.
Group 6. Organophosphorous compounds							
Acephate 	30560-19-1	k_{OH} = 7.1 x 10^9	--	-0.85	EPA 538	It is used as an insecticide	Low K_{OW} – high water solubility, high occurrence data, and no published kinetic data.
Dicrotophos 	141-66-2	k_{OH} = 5.5 x 10^9	--	0.50	EPA 538	It is used as an insecticide	Low K_{OW} – high water solubility, high occurrence data, and no published kinetic data.
Methamidophos	10265-92-6	k_{OH} = 8.2 x 10^9	--	-0.80	EPA 538	It is used as an insecticide.	Low K_{OW} – High water solubility, no published kinetic data, and

						transformation product of acephate	high occurrence data.
Fenamiphos 	22224-92-6	k_{OH} = 6.2 x 10^9	--	3.25	EPA 538	It is used as an insecticide	Although it has a lower water solubility, there is no published kinetic data, this compound has high occurrence data, and toxicologically relevant oxidation products are expected(Nordmeyer and Dickson 1990).

2.3. Bioassay Evaluation of Biological Activity

Selection of specific bioassays for CCL3 contaminants to be studied (Table 2.2) was evaluated and included in this literature review. Bioassay analysis in Chapters 3 and 4 followed the testing strategy presented in Figure 2.1. After assessing oxidation kinetics, compounds were subjected to bioassay screening. First, a bioassay relevant for the specific toxicity of the parent compound was used. The selection of the assay was based on previously published literature. This initial testing evaluated the impact of the studied degradation process on the elimination of the specific toxicity. We compared the extent of the degradation directly with the changes in the specific toxicity during degradation. To quantify the evolution of toxicity during the degradation of contaminants, the toxic equivalent concept (TEQ) is widely applied to express the toxicity of environmental samples, and has been previously demonstrated (Escher 2008). If the parent compound dominates the overall toxicity, the disappearance of the parent compound is directly proportional to the decrease in toxicity of the mixture and no further action involving the identification of the transformation products is warranted. In contrast, a deviation from the parent compound degradation proportionality indicates the formation of toxic transformation products, and an effect-directed analysis could be applied to identify the toxic compounds (Biran, Yagur-Kroll et al.).

In addition to tracking the parent compound's toxicity, all contaminants, regardless of their specific toxicity were subjected to a suite of screening assays, listed in bold in Figure 2.1. Therefore, if the specific toxicity of the byproduct(s) differs from that of the parent compound, the general screening assays (AMES II and YES) will indicate other forms of toxicity that may

be present. In this study, positive controls were used in order to determine the variability and sensitivity of each test run.

Figure 2.1 displays the steps taken during bioassay implementation for both parent compounds and samples post AOP treatment. After necessary oxidation kinetics were determined, all compounds underwent the same process. If the parent compound has a known specific toxicity, then it was evaluated in the first step. All compounds, regardless of available information on parent toxicity, underwent screening for genotoxicity and estrogenicity using the Ames II and YES assays. All compounds were subjected to both UV initiated and Ozone initiated indirect photolysis, and samples taken during oxidative degradation were evaluated by the same screening mechanisms used for the parent compound. Non-specific cytotoxicity was detected by the combination of Ames II and YES assays, however, these are not cyotoxic-specific assays meaning that the sensitivity of these assays was low. The toxicities (Cytotoxicity and Neurotoxicity) are examples of specific toxicities that were only tested for if the specific mode of toxicity was known, such as neurotoxicity for the organophosphorous pesticides (Group 6).

At various time points during the oxidation of the parent compound, samples were drawn for toxicity assays. The oxidation processes lead to the formation of a cascade of degradation products, where only a few of them have been identified. Therefore, it is not possible to do a prediction of toxicity based on the literature or proposed degradation pathways, and this type of work is then based extensively on original laboratory work.

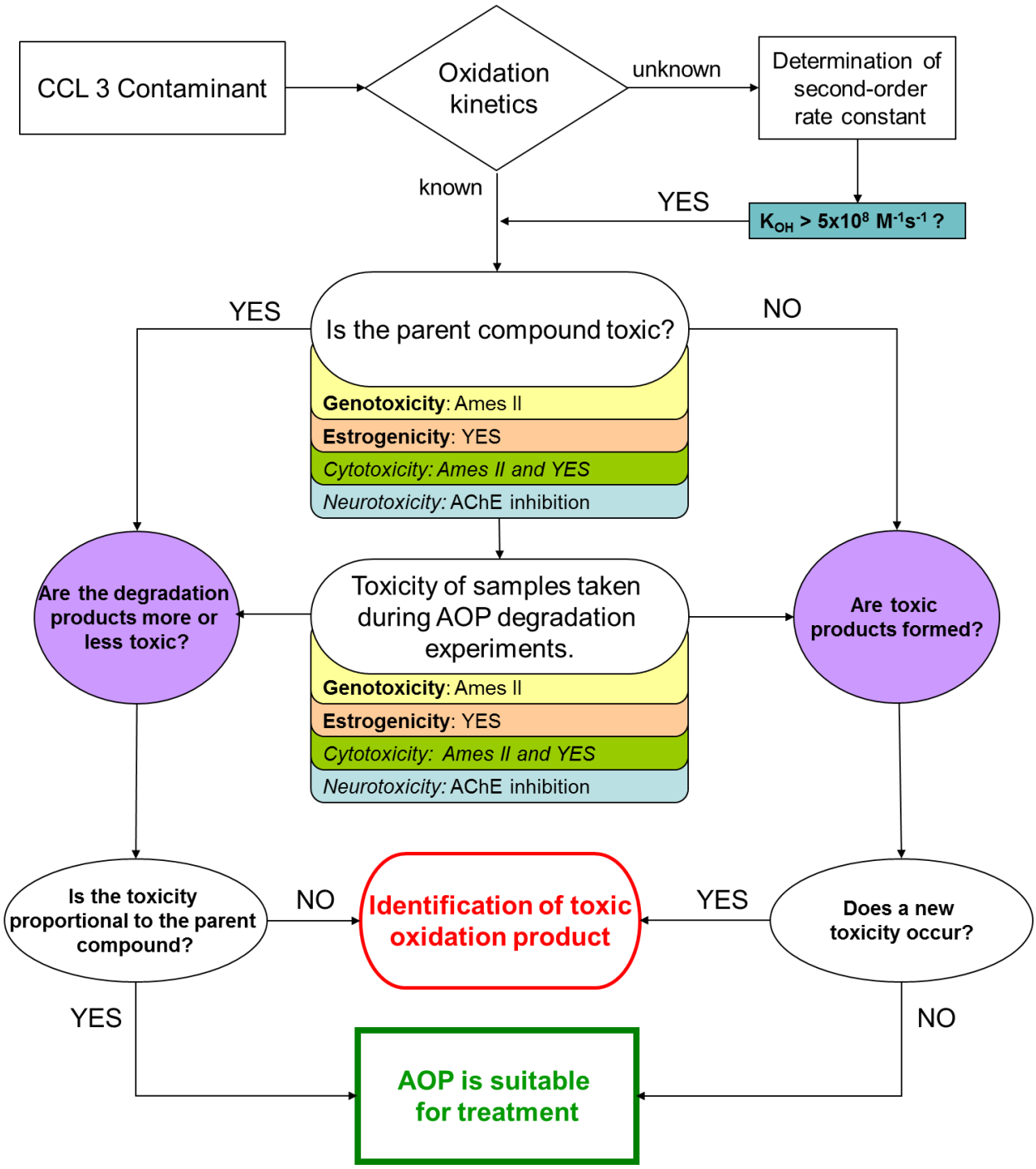


Figure 2.1 Flow chart for evaluating the oxidation testing conditions, bioassay selection, and byproduct identification for each CCL3 contaminant selected for investigation.

2.4. Toxic Equivalents Concept (TEQ)

The toxicity of the samples taken during degradation experiments will be assessed by *in vitro* established bioassays. The Toxic Equivalent Concept (TEQ) for the quantification of the evolution of toxicity has been described in previous studies (Dodd, Kohler et al. 2009; Mestankova, Escher et al. 2011). This concept can be further extrapolated to include additional terms such as Mutagenic Equivalent Concept (MEQ) or Estrogenic Equivalent Concept (EEQ). Quantification of each term is the same, but has as specific toxicity correlated to the bioassay being analyzed.

This approach allows for the direct comparison of the toxicity of known parent compounds and unknown degradation products with known kinetics. TEQ is calculated by dividing the equivalent concentration required to reach 50% of the maximum effect *in vivo* (EC50) of the reference compound (positive control) by the EC50 of each sample as given in Equation 2.1 below.

$$TEQ = \frac{EC50\,(\text{reference compound})}{EC50\,(\text{sample})} \qquad \textbf{Equation 2.12}$$

The ratio of the TEQ of the degraded compound sample to the initial value (parent compound) allows us to quantitatively evaluate changes in toxicity during the oxidative degradation of a compound, as seen in Equation 2.2.

$$\frac{TEQ}{TEQ_{t=0}} = \frac{EC50_{t=0}}{EC50} \qquad \textbf{Equation 2.13}$$

If the post-oxidation mixture's toxicity is dominated by the parent compound, the degradation of the parent compound will be directly proportional to the decrease in toxicity of the post-oxidation mixture as indicated by the straight line (slope = -1.0) on Figure 2.2.

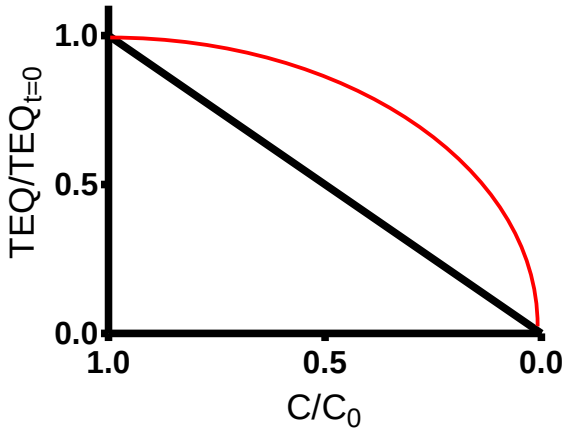


Figure 2.2 Ratio of TEQ of sample to TEQ of parent compound (black line = ideal; red line = non-ideal)

When the toxicity decreases linearly (black line in Figure 2.2), this indicates either less toxic degradation products than the parent compound or this indicates that any toxic compounds that are formed have a low enough concentration not to observe any effect. If any toxic compound is formed, a positive deviation from the "ideal case" is observed (e.g. the red line). If the toxicity trend of degradation products is "non-ideal", then this warrants further analytical degradation. It should be noted that the indication of toxicity decreasing is only indicative of the specific toxicity measured in the assays chosen. There could still exist a mode of toxicity formed post-oxidation treatment that would be measured in an alternative *in vitro* or *in vivo* assay.

2.4.1. Reference Compound Selection

Selection of a reference compound to be used for calculating TEQs depends on the measured endpoints of toxicity chosen. The most common choice for a reference compound is the positive control compound utilized in the standard test method or standard operating procedures (SOPs). For estrogenic activity, 17-β-estradiol (E2) is typically used, whereas for mutagenic activity the positive controls for bacterial strains to be used are reported in the literature (Murk 2002; OECD 2004). Reference compounds for each bioassay explored for project selection are discussed subsequently in this chapter.

2.5. Effect-Directed Analysis (EDA)

The effect-directed analysis (EDA) is an environmental toxicology tool that is based on a combination of fractionation procedures, bio-testing, and subsequent chemical analyses (Brack 2003; Hecker and Hollert 2009; Biran, Yagur-Kroll et al. 2010). EDA was applied when the formation of a toxic compound is detected during the degradation of the parent compound ("non-ideal" case). EDA is commonly used in order to detect toxic compounds in mixtures. The identification of the toxic degradation products is important to aid in the understanding of the mechanism and structural features leading to the formation of toxic products during AOPs.

Table 2.3 below containing the "Reported end points of the parent compound" displays published references for genotoxicity and estrogenicity data that can be compared to EDA of the selected compounds.

Following parent compound specific toxicity screening, when available, the next step includes assessing the genotoxicity and estrogenicity to ensure that the studied processes do not lead to the formation of a new toxicophore with modes of action of particular concern to human health. A toxicophore is a structural feature, responsible for the toxicity of the compounds (i.e. binding to a specific receptor). This general bioassay screening step allows for the identification of patterns leading to the formation of hazardous transformation products, with regard to parent compound structure and bioactivity. For estrogenic activity, the Yeast Estrogen Screen (YES) assay was utilized, and for genotoxicity the Ames II bioassay was used.

Bioassay analysis of samples taken during the oxidative degradation experiments (Chapters 3 and 4) identifies if a genotoxic or estrogenic compound is formed. When this is the case, the EDA procedure was used to try and localize the compound in the degradation mixtures, while also attempting to identify the responsible degradation product. The goal of utilizing bioassays in this project was not to study the mode of action of chemicals, but to evaluate the possibility of the formation of degradation products having a chosen mode of action. Some conclusions are provided in the subsequent chapters, but further studies are needed to quantify the potency of the formed compounds and their specific chemical mode of action.

2.6. Genotoxicity Bioassay Selection

There are three major end points of genetic damage associated with human disease: gene mutations (point mutations or frameshift mutations), clastogenicity (i.e. structural chromosome

aberrations), and aneuploidy (i.e. numerical chromosome aberrations). There exists no single assay that can cover all genotoxic endpoints. Several rapid tests *in vitro* with bacteria and mammalian cell culture have been designed and standardized. For the assessment of genotoxicity in this study, only gene mutations were studied using the Ames II bioassay. The Ames II bioassay has been validated for the assessment of risk posed by chemicals, in the OECD/EU test guideline (TG 471, and TG 487). A combination of micronucleus with the bacterial Ames test was reported to be sufficient for detection of all relevant carcinogens and genotoxins (Kirkland, Reeve et al. 2011). Kirkland et al. reported for the combination of gene mutations, clastogenicity, and aneuploidy, a detection of 90% of rodent carcinogens could be achieved (from 372 chemicals tested) (Kirkland, Aardema et al. 2005), and this was confirmed in a later study (Kirkland, Reeve et al. 2011). The European Environmental Mutagen Society (GUM) recommended that Stage-1 *in-vitro* genotoxicity testing should consist of the Micronucleus and Ames bioassays (Pfuhler, Albertini et al. 2007). The European Food Safety Authority recommended this strategy for genotoxicity testing applicable to its food and feed safety assessment (EFSA 2011). The papers of Kirkland et al. contain nearly 1,000 chemicals, including all of the types of compounds covered in this study. An extensive literature review in

Table 2.3 contains many references specific to the contaminants in this study involving specific modes of toxicity. While recognizing that there is no single bioassay that incorporates all genotoxic endpoints, an attempt was made to couple two bioassays, and due to validation constraints only the Ames II bioassay was selected. The information listed in Table 2.3 is displayed as it is still relevant for contaminant toxicity and testing of CCL3 chemicals.

2.6.1. **Ames II Bioassay**

The Ames (*Salmonella*/microsome mutagenicity assay) is a widely accepted bacterial reverse mutation assay. The test utilizes *Salmonella typhimurium* strains containing an engineered mutation, which renders bacteria unable to synthesize the essential amino acid histidine. In the absence of an external histidine source, proliferation of bacteria is impossible. A new mutation at or close to the site of the pre-existing mutation caused by a compound can restore the gene function (a reversion of the mutation), allowing cells to resume synthesis of histidine. Mutagenic compounds cause an increase in the number of revertant colonies relative to the background levels that are able to repair themselves. The number of revertant colonies formed indicates the relative level of genotoxic activity of the exposure mixture.

The Ames test is used world-wide as an initial screening assay, and has been recognized for its ease of use, rapidness, broad applicability, high sensitivity and reproducibility. Large data sets are available due to its importance in chemical assessment. The engineered strains have different mutations in various genes; each is responsive to mutagens that act via different mechanisms. This specificity of the various strains provides useful information on the types of mutations. Alternately, one strain is not sufficient to detect all gene mutagens, OECD guidelines 471, recommends the use of at least five strains. For water quality determination, the combination of *Salmonella typhimurium*, TA 98, and TA 100 or TA Mix is recommended (ISO 11350).

Initially, the number of revertant colonies on agar plates was counted, making the original version of this test laborious. The Ames fluctuation test decreased the scale of the procedure, and the evaluation of the revertants was determined by counting the number of wells containing a color shift (from purple to yellow) in the presence of a bromocresol pH indicator (pH shift caused by accumulation of catabolites). Further development of the assay resulted in the Ames II test, which is a test kit comprised of a mixture of six *Salmonella* strains (TA Mix) allowed in combination due to a low spontaneous reversion that measure base pair mutations, in combination with the *Salmonella* strain TA98, which is the strain responsive to frameshift mutations. The sensitivity of Ames II has been shown to be comparable to the traditional Ames test (Kamber, Fluckiger-Isler et al. 2009). In this research, the strains inherent to the Ames II test were utilized for the screening of genotoxicity. Additionally, highly specific strains sensitive to a specific mode of genotoxic action caused by alkylating agents of some studied compounds were used.

The correlation between mutagenicity and the Ames test has been widely reported. Original data 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 the time (McCann, Choi et al. 1975). The later work of Ashby et al. (1989) showed lower specificity for rodent carcinogens (58%), and for the non-carcinogens the specificity was 73% (Ashby, Tennant et al. 1989). Several of the negatively tested carcinogens are non-mutagenic, so the results were underestimated, due to the inability to measure the specific mode of carcinogenicity(Ashby, Tennant et al. 1989). Following the work of Kirkland et al., the Ames test is shown to be sensitive to the CCL3 compounds selected for this study (McDaniels, Reyes et al. 1990; Reifferscheid and Heil 1996; Yasunaga, Kiyonari et al. 2004).

A disadvantage of the Ames test is that prokaryotic cells differ from eukaryotic cells with respect to uptake and metabolism (metabolic activation is added), as well as chromosomal and DNA repair. This study is looking for toxicity relevant to humans, and the differences between prokaryotic and human (eukaryotic) cells can result in different uptakes (due to the presence of the eukaryotic cell wall). The differences will mainly be seen with metabolites, because prokaryotic cells are devoid of the enzymes involved in the activation of promutagens. So, if the metabolites of the tested compounds are responsible for the genotoxicity, and are not formed in sufficient concentrations, the test will give a false-negative result.

2.6.2. **umuC Bioassay**

The umuC bioassay was also considered as a screening assay for genotoxicity. This fast and simple method is ideal for a high throughput of samples, making it an ideal screening assay for a study design similar to this project. It is based on the induction of a SOS response of the repair system as a consequence of DNA damage. The strain of *Salmonella typhimurium* carries a fused gene umuC'-'lacZ. When the DNA-damage SOS-repair system is activated, lacZ, the gene for β-galactosidase activity, is also activated. The β-galactosidase activity is measured using a simple colorimetric assay. In comparison to the Ames test, the main advantage is that one single bacterial strain is necessary to detect various types of mutagens. However, the umuC is unable to provide any further information about specific genotoxin action, such as a frameshift or base-pair mutation. Both tests have been subjected to extensive validation, with the percentage of false-positive and false-negatives being comparable (McDaniels, Reyes et al. 1990; Reifferscheid and Heil 1996; Yasunaga, Kiyonari et al. 2004). After considering both tests, the added advantages

included with the Ames test (specific genotoxin action) make it more favorable for the detection of gene mutations. Therefore this assay was not used further in this study.

2.7. Estrogenic Activity

There are three general categories of estrogenic assays: estrogen receptor (ER) binding assays, which measures the binding affinity of the tested compound to the ER, cell proliferation assays (E-Screen) which measures an increase in cell numbers of an estrogen sensitive cell line (mammalian breast cancer) in the presence of an estrogenic compound, and reporter gene assays which are based on the ability of a compound to stimulate ER-dependent transcriptional activity (mammalian cell-based or yeast cell-based reporter gene). Three specific assays for the determination of estrogenic activity were explored for this research: Yeast Estrogen Screen (YES), E-Screen, and ER-CALUX assay.

2.7.1. Yeast Estrogen Screen (YES)

The YES assay is beneficial due to its simplicity to perform, rapid results, and relatively inexpensive cost compared to similar assays. The yeast cells contain a human estrogen receptor (hER) and plasmids carrying the reporter gene *lac-Z* encoding the enzyme β-galactosidase. In the presence of estrogenically active compounds, the synthesis of β-galactosidase is induced. The β-galactosidase activity is detected by a chromogenic substrate. The advantage of this test is the absence of complex interactions between the ER and other receptors and inherently low background levels. Disadvantages associated with this assay include: i) the yeast cell wall and transport mechanisms differ from mammalian cells, thereby potentially altering the activity of the tested compounds in comparison to mammalian cells (Routledge and Sumpter 1996; Legler 2002); ii) the assay has been reported to cause false negatives; and iii) the sensitivity is lower

compared to mammalian cell-based assays. The EC50 values (in tens of ng E2/L) are generally one order of magnitude higher than in the mammalian cell-based assays.

Following the outlined research approach (Figure 2.1), samples taken during oxidation degradation experiments (Chapters 3 and 4) contain relatively high concentrations of the parent compound and its degradation products, if any are formed. The lower sensitivity of the YES assay should therefore not limit detection of estrogenic compounds. However, another limitation of the test is that cytotoxic effects could potentially occur at higher concentrations causing interferences if the yeast cells are more sensitive to the cytotoxic effect rather than the estrogenic effect; the same cytotoxic effect limitation is also observed with mammalian cells. The YES assay is appropriate for estrogenic activity measurements of concentrated mixtures that can contain compounds interfering with growth and viability of cells. Both the low cost of and the ease of use are favorable for application of this test for the screening of numerous samples, and are possibly applicable to be implemented at moderately equipped water quality laboratories.

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 an estrogen receptor (Dhooge, Arijs et al. 2006). Kolle et al. (2010) reported false negative rates were 22%, and 7% false positive on the estrogenic agonisms (for 105 chemicals tested (Kolle, Kamp et al. 2010). The suitability of this assay for pre-screening of water bodies was evaluated (Brix, Noguerol et al. 2010) and no significant difference was found between two laboratories. A low coefficient of variation (< 9.0%) was reported for the positive identification of compounds within three laboratories in previous work (Dhooge, Arijs et al. 2006). The advantages of this assay over others lead to it be selected as the estrogenic activity screening assay for this research.

2.7.2. **E-Screen**

The E-Screen shows a higher sensitivity than the YES assay; it is based on estrogen-dependent cell proliferation of MCF-7 breast cancer cells. It is a relatively demanding assay, not only regarding the cultivation of the cells but also for the relatively long incubation period of 4 to 6 days. Growth and proliferative response of MCF-7 cells depend strongly on the strain and on culture conditions and procedure, which can lead to significant variability across laboratories (Escher 2008; Biran, Yagur-Kroll et al. 2010; Kirkland, Reeve et al. 2011). Another consideration is that non-estrogenic compounds can also stimulate cell proliferation in the E-Screen assay (Bryce, Bemis et al. 2007).

2.7.3. **ER-Calux**

Another option for estrogen screening assays is a gene-reporter cell line, such as the ER-CALUX assay. This mammalian-based reporter gene assay uses T47D human breast adenocarcinoma cells expressing endogenous estrogen receptor (ER) and stably transfected with an estrogen responsive luciferase reporter gene containing three ERs. In the presence of an estrogenic compound, the activation of ER leads to the expression of the luciferase gene, the luciferase activity is then measured by luminescence (after lysis of cells, in the presence of the substrate luciferin). ER-CALUX is a recently developed assay with detection limits and performance comparable to the E-Screen. It is highly specific to estrogenic compounds, as opposed to the E-Screen, because responses can be measured only upon interaction with the ER. This mammalian-based reporter gene assay can distinguish estrogen agonists and antagonists. ER-CALUX is currently more expensive than the E-Screen due to licensing fees, but the test is considerably more rapid to perform than the E-Screen. This test can be utilized as a challenge assay, in case of ambiguity in results obtained by the YES assay.

2.8. Neurotoxicity

In the grouping of contaminants, organophosphate pesticides were grouped according to structural properties, but with a specific bioassay, the Acetylcholinesterase, bioassay in consideration for testing of neurotoxicity. Neurotoxicity is not yet systematically tested in vitro. Because of the anatomical and physiological complexity of the nervous system, it is difficult to represent by the existing ***in vitro*** tests (Harry, Billingsley et al. 1998; Harry and Tiffany-Castiglioni 2005). Non-animal methods and alternative testing strategies for assessing neurotoxicity are currently being discussed (Bal-Price, Hogberg et al. 2010), but due to a lack of published literature, it would be difficult to systematically study neurotoxicity. The AChE inhibition assay is a well-known endpoint of neurotoxicity, and was described for structurally defined compounds (organophosphates, carbamates, tertiary and quaternary amines) (Enz and Floersheim 1996). Since we selected four organophosphorous compounds, we measured the specific mode of their action, by AChE inhibition during their degradation.

2.8.1. Acetylcholinesterase Inhibition (AChE) Bioassay

Organophosphate pesticides act by specifically binding to the enzyme acetylcholinesterase (AChE), blocking AChE and thereby inhibiting the hydrolysis of the neurotransmitter acetylcholine (ACh). The inhibition of AChE is the relevant end point for their neurotoxicity. The AChE in vitro assay was developed by Ellman et al. (Ellman, Courtney et al. 1961). The acetylcholinesterase enzyme hydrolyses the substrate to yield acetate and thiocholine, the free thiol groups are quantified by reagent dithio-bis(2-nitrobenzoic acid) (DTNB) giving a yellow color caused by 4-nitrothiolate production that can be measured colorimetrically using a plate reader. The specific wavelength intensity is proportional to the AChE activity, with the decrease of intensity being proportional to the level of inhibition.

2.9. Contaminant Bioassay Coupling from Literature

An extensive literature review was performed in order to confirm the screening assays and also to display a knowledge base of specific genotoxic and estrogenic bioassays being used for testing of CCL3 contaminants. For the organophosphorous insecticides, the literature reports that most organophosphorous pesticides (Pan, San Francisco et al.) are directly, or after biotransformation (oxons are formed), very potent acetylcholinesterase inhibitors (Chen, Sharpless et al. 2006; Bosgra, van Eijkeren et al. 2009). Some evidence suggests that organophosphates could also mediate genotoxic effects at low concentrations (Hreljac, Zajc et al. 2008; Kumar, Nagpure et al. 2010; Li, Li et al. 2010; Ukpebor, Llabjani et al. 2011). Phenolic compounds formed through the oxidation of organophosphorous compounds (Yamada, Terasaki et al. 2011) can also exhibit an estrogenic activity. This information was taken into account during the specific toxicity assay selection, by choosing a neurotoxicity assay.

Table 2.3 Literature review performed for several CCL3 contaminants selected previously in Table 2.2.

1. Halogenated Compounds	Genotoxicity	Estrogenicity
1,2,3-trichloropropane	-MN: human lymphocytes (Tafazoli and Kirsch-Volders 1996) +SCE: V79 (von der Hude, Scheutwinkel et al. 1987) + MN: AHH-1 and h2E1 cells (Doherty, Ellard et al. 1996) +Ames: TA153k and TA100 (+S9) (Ratpan and Plaumann 1988)	
4. Oxygen containing compounds	Genotoxicity	Estrogenicity
1,4-dioxane	This carcinogenic compound is a weak genotoxic compound tested in vitro (Morita and Hayashi 1998) its carcinogenic effect can be caused by a non-genotoxic mechanism (Kirkland, Aardema et al. 2005) - CA, MN, SCE: CHO cells (Morita and Hayashi 1998) - Ames: TA1535, TA1537, TA100 and TA98 (±S9) (Morita and Hayashi 1998)	
Oxirane, methyl-	+ Ames (Tennant, Margolin et al. 1987) + SCE: mouse lymphoma L5178Y cells (Tennant, Margolin et al. 1987) + CA: CHO cells (Tennant, Margolin et al. 1987)	
Methyl tert-butyl ether (MTBE)	- Ames : TA98, TA100, TA104, and TA1535 (±S9)(Kado, Kuzmicky et al. 1998) - Ames: TA98, TA100, YG1041, and YG1042 (±S9) (Vosahlikova, Cajthaml et al. 2006) + (?) Comet: peripheral blood lymphocytes (Chen, Hseu et al. 2008)	
Butylated hydroxyanisole	+ micronucleus: CHL/IU cells(Matsushima, Hayashi et al. 1999) +CA: CHO cells (Anderson and Phillips 1999)	+ YES (Miller, Wheals et al. 2001)
Cumene hydroperoxide	+ Ames II (Piegorsch, Simmons et al. 2000) + Ames: TA98 (+S9) (Yamaguchi and Tsutomu 1980) + Ames: TA102 and *E. coli* WP2uvrA2 (±S9) (Diehl, Willaby et al. 2000)	

	- Ames: TA98 and TA100 (±S9)(Diehl, Willaby et al. 2000) +(?) Comet, MN: mouse lymphoma L5178Y *tk*$^{+/-}$ cells (Brink, Richter et al. 2009)	
6. Organo-phosphorous compounds	Genotoxicity	Estrogenicity
Acephate	+ CA, MN, SCE: human peripheral lymphocytes (Özkan, Yüzbaşıoğlu et al. 2009) + CA, Comet: human peripheral lymphocytes (Das, Shaik et al. 2008) - CA, SCE, MN: mouse bone marrow cells (Carver, Bootman et al. 1985) - Ames: TA 98, TA100 and TA1535 (Hour, Chen et al. 1998)	- ER-CALUX (Kojima, Fukunaga et al. 2005)
Dicrotophos	+ SCE: CHO cells (Nishio and Uyeki 1981) +(?) Ames TA97 TA98 TA100 TA102 TA1535 (±S9) (Wu, Chye et al. 2010) + CA: CHO-K1 cells (Wu, Chye et al. 2010) + Comet: HepG2 cells (Wu, Chye et al. 2010)	
Disulfoton	- Ames: TA98, TA100 (±S9) (CEPA 2000) + Ames: TA1535 (±S9) (CEPA 2000) + SCE: CHO cells (CEPA 2000)	
Methamidophos	+ Ames: TA 98 and 100 (±S9) (Karabay and Oguz 2005)	- ER-CALUX (Kojima, Fukunaga et al. 2005)
Fenamiphos	- Ames: TA100 and TA98 (±S9) (Sui, Kawakami et al. 2009) - SCE: Chinese hamster V79 cells (Chen, Sirianni et al. 1982)	

Abbreviations: CA: chromosomal aberration

SCE: sister chromatid exchange

MN: micronuclei formation

(+S9), (-S9) in the presence, in the absence of metabolic activation

(?) weak answer or conflicting results

3. Effect-Directed Analysis for the Treatment of Organophosphorous Insecticides in Drinking Water using Advanced Oxidation

3.1. Abstract

Advanced oxidation processes are utilized for their ability to treat emerging contaminants with the fast reacting and non-selective hydroxyl radical. Organophosphorous insecticides are a common drinking water contaminant, with 12 different compounds in this class being found on the US EPAs most recent Candidate Contaminant List (CCL3). The use of advanced oxidation processes for the treatment of organophosphorous insecticides was explored in this study, by coupling biological and analytical tools to follow degradation. Four insecticides were explored for advanced oxidation treatment: acephate, dicrotophos, fenamiphos, and methamidophos. All four compounds were fast reacting with hydroxyl radical, all reacting at or greater than $5.5x10^9$ $M^{-1}s^{-1}$. Three major endpoints of toxicity were studied: estrogenicity, genotoxicity (mutagenicity), and neurotoxicity. Oxidized products of methamidophos exhibited in an increase in mutagenicity, which subsequently decreased with time, possibly due to the hydrolysis of the daughter products formed. This work provides insight into appropriate treatment technologies for these organophosphorous contaminants that not only evaluate parent compound removal, but also the toxicity of the oxidative products.

3.2. Introduction

Emerging contaminants in the United States that are being explored for regulation are placed onto the US EPAs Candidate Contaminant List (CCL). On the most recent CCL list published by the EPA (CCL3), there are twelve organophosphorous insecticides (USEPA 1996). Organophosphorous insecticides are widely used in the United States, and are prevalent in the

influent drinking water supply (USEPA 2009). These insecticides act by specifically binding to the enzyme acetylcholinesterase (AChE), blocking AChE and thereby inhibiting the hydrolysis of the neurotransmitter acetylcholine (ACh). ACh inhibition is not specific to insects, where the delivery of the insecticide was meant for, but can also occur if ingested by humans. Several studies have previously reported the neurological effects of organophosphorous insecticide ingestion (Wadia, Sadagopa.C et al. 1974; Gromov and Rozengart 1976; Bradwell 1994).

New technologies are being implemented for control of contaminants of emerging concern (CECs), like organophosphorous insecticides, in water treatment. AOPs have proven to be an effective technology for micropollutant control due to their ability to destroy contaminants, and not just remove them by a phase transfer mechanism like sorption or air stripping (Huber, Canonica et al. 2003; Huber, Ternes et al. 2004; Lee, Escher et al. 2008). Several types of advanced oxidation treatment including O_3/H_2O_2, UV/H_2O_2, UV/O_3, $UV/O_3/H_2O_2$, UV/catalyst/H_2O_2 (including the "photo-Fenton" process with Fe(III) complexes as a catalyst), VUV/H_2O (vacuum UV photolysis of water) and UV/TiO_2, are available (Braun and Oliveros 1997) and might be considered for applications to drinking water purification. UV/H_2O_2 and O_3/H_2O_2 were explored in this study for use in generating hydroxyl radicals for organophosphorous destruction.

When a water is treated with an AOP, those contaminants with sufficiently high hydroxyl radical rate constants (typically greater than $5x10^8\ M^{-1}s^{-1}$) will be transformed into a daughter-product(s). These products may or may not retain the parent compound toxicity or could develop a new form of toxicity. The toxicity of the chemical products subsequent to a transformation based treatment process can be assessed using a technique known as effect-direct analysis (EDA). In an unknown water matrix with unknown daughter products, the toxic equivalents

concept (TEQ) can be used to quantify toxicity. The toxic equivalents concept expands to specific measurements of potential toxicity, such as the mutagenic equivalents concept (MEQ), measuring mutagenic potential and neurotoxic equivalents concept (NEQ), measuring potential neurotoxicity by acetylcholinesterase inhibition. TEQ approach saves time and money on analytical techniques, and allows for a more comprehensive review of contaminant treatment by evaluating the products formed. The objectives of this research included (1) a determination of the AOP treatability of four organophosphorous insecticides (acephate, dicrotophos, fenamiphos, and methamidophos) and (2) an assessment of toxicity for the transformation products formed from the AOP treatment.

3.3. Methods

3.3.1. Chemical Identification

Compounds were analytical grade, (Sigma-Aldrich, MO, USA), and identified using an Agilent HPLC ion trap mass spectrometer (MS) with an Agilent Eclipse XDB-C_8 column, following US EPA standard method 538. At time 0, a gradient of 70% water (laboratory grade with 1% formic acid) and 30% acetonitrile (analytical grade) were run, and at time 5 minutes, a gradient of 30% water and 70% acetonitrile was used, with a final gradient shift at a time of 7 minutes of 100% acetonitrile.

3.3.2. Experimental Setup

UV/H_2O_2 based experiments employed a quasi-collimated beam, with four low-pressure (LPUV) lamps (ozone free, 15 watt, #G15T8) housed above a 4 inch aperture equipped with a manual shutter. Each lamp emitted radiation principally at 254 nm, and incident irradiance was measured at a wavelength of 254 nm with a calibrated radiometer (International Light Inc.,

Model 1700/SED 240/W). The UV fluence (i.e. dose) was calculated from the incident irradiance measurement, exposure time, depth of sample and water quality characteristics (Bolton and Linden 2003). Contaminants were dosed into MilliQ water at a concentration of 1,000 µg/L, hydrogen peroxide was added at a concentration of 5 mg/L, aqueous sample solutions were irradiated at a temperature of 25.0 ± 0.5 °C, and a buffered pH of 7.2 ± 0.1.

Experiments involving the ozonation of samples were performed by preparing solutions containing 10 to 1,000 µg/L of the studied compound and were buffered to pH 7 and 0.74 mg/L of t-butanol (a hydroxyl radical scavenger) was added with variable volumes of ozone stock solution to obtain the desired percentages of degradation based solely on ozonation. An ozone stock solution, approximately 60 mg/L, was produced by sparging O_3-containing oxygen through Milli-Q water that was cooled in a jacketed beaker. The solutions were stirred in borosilicate glass vials (screw-top) at room temperature, and ozone was added by ozone-demand free pipet tips. Ozone concentration in the concentrated stock solution was measured by direct measurement at a wavelength of 258 nm in a diluted solution of ozone stock and MilliQ water. Hydrogen peroxide was added at a concentration of 5 mg/L to all contaminant solutions directly before ozone was added to the solutions, and the solutions were quenched with a bovine catalase solution prior to analysis, within 4 hours of quenching.

3.3.3. **Determination of Rate Constants**

Apparent second-order rate constants for the reaction of ozone and hydroxyl radicals with the selected contaminants were determined by competition kinetics. The hydroxyl radical rate constant (k_{OH}) for the contaminants was measured with the addition of *p*-chlorobenzoic acid (pCBA) as a competitor with a known k_{OH} value of 5×10^9 $M^{-1}s^{-1}$ (Elovitz, Gunten et al. 2000). For each experiment, competition kinetics were measured twice, and the slope was determined

from the plots of $\ln([C]/[C]_0)$ as a function of $\ln([competitor]/[competitor]_0)$, where C was the concentration of the contaminant and the rate constant was calculated from the slope following equations presented in previous publications (Shemer, Sharpless et al. 2006; Elovitz, Shemer et al. 2008). Reaction constants with ozone (k_{O3}) were determined by direct measurement of the compound reacting with ozone, by following the degradation of ozone over time and analytically following the decay of the parent compound over time (Hoigné and Bader 1983).

3.3.4. **Yeast Estrogen Screen (YES bioassay)**

The yeast estrogen screen (YES) bioassay was used to test and quantify the estrogenicity of AOP treated organophosphorous insecticides (Routledge and Sumpter 1996). The test is based on a recombinant strain of the yeast *Saccharomyces cerevisiae*, which contains a reporter gene for the human estrogen receptor hER and expression plasmids carrying the reporter gene *lac-Z* encoding the enzyme β-galactosidase. β-galactosidase enzyme releases chlorophenol red from the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG). A color change was measured photometrically, using a Biotek Epoch plate reader (VT, USA).

3.3.5. **Ames Mutagenicity Assay (Ames II)**

The Ames II test (*Salmonella*/microsome mutagenicity assay) uses *Salmonella typhimurium* strains with engineered mutations, which render the bacteria unable to synthesize histidine. The lack of histidine does not allow the bacteria the ability to reproduce without an external histidine source. A gene mutation, caused by an external source, can restore the gene function (reverse mutation) and allows the bacteria to produce histidine. TA98, measuring frameshift mutation, and TAMix, comprised of a mixture of six strains and measuring point mutations were used in this study, following ISO 11350 (Acero, Haderlein et al. 2001). The test was considered valid if the percentage of revertant wells in the negative controls was lower than 10% (from 48 wells),

and the positive controls caused ≥ 80% revertants in the positive wells. Bacteria strains (TA98, TAMix) and S9 rat liver enzyme were obtained from Xenometrix (location). S9 rat liver enzyme was added alongside of the chemical before the initial incubation, in the presence of a histidine-rich indicator in order to mimic liver function activity, inducing metabolism of compounds.

3.3.6. **Acetylcholinesterase Inhibition Assay (AChE assay)**

The AChE inhibition assay measures the kinetic inhibition of the enzyme acetylcholinesterase. The level of neurotoxicity of the mixtures is proportional to the time-based inhibition of the production of acetylcholinesterase. Experiments were performed in a 96 well plate, according to a well referenced standard method (Ellman, Courtney et al. 1961) by Ellman et al. Lyophilized Electric Eel enzyme (AChE 0.26U/mg) was used, and enzyme activity was assessed to ensure validity. Solutions of paraoxon and parathion were used as positive controls for the experiments, with concentrations ranging from $1.82x10^{-6}$ to $4x10^{-4}$ M used in a 96 well plate. Positive controls were made in ethanol, and pipetted into a 96 well plate, and the ethanol was allowed to evaporate. Samples were then redissolved in 50 μL of 0.05 M phosphate buffer. Five μL of N-bromosuccinimide (NBS), (Sigma-Aldrich, 99%) was used to provide bromination of the contaminants in question, as an alternative type of oxidation (other than hydroxyl radicals) that could lead to a toxic byproduct (Lee, Kim et al. 2002). After bromination, the samples were thoroughly mixed on a shaker table for 2 minutes, and 5 μL of ascorbic acid (Sigma-Aldrich, 99%) was added to all wells, and the plates were shaken again for 1 minute. Samples that were not exposed to bromination were treated identically, but without the addition of NBS. A 40 μL aliquot of acetylcholinesterase enzyme solution was added into every well and placed on the shaker table for thorough mixing for 10 minutes. After this time, 40 μL of acetylthiocholine solution (Sigma-Aldrich, 98%) and dithiotonitrobenzoic acid solution (DTNB) (Sigma-Aldrich,

99%) were added to each well, plates were mixed for 1 minute on a shaker table and were then immediately read on a plate reader (Biotek Epoch, VT, USA) for absorbance at 420nm for 10 minutes each at 30 second intervals.

3.3.7. Toxic Equivalents Concept (TEQ)

To quantify the evolution of toxicity during the degradation of contaminants, the toxic equivalent concept (TEQ) is widely applied to express the toxicity of environmental samples, and has been previously demonstrated (Escher 2008). When applied directly to mutagenic or neurotoxic activity, the quantification can be referred to as the mutagenic equivalents concept or MEQ or neurotoxic equivalents concept (NEQ). If the parent compound dominates the overall toxicity, the disappearance of the parent compound is directly proportional to the decrease in toxicity of the mixture and no further action involving the identification of the transformation products is required. In contrast, a deviation from the parent compound degradation proportionality indicates the formation of toxic transformation products, and an EDA was then applied to identify the toxic compounds (Biran, Yagur-Kroll et al.).

3.4. Results and Discussion

3.4.1. Kinetic Data

Table 3.1 contains the reaction rate constants for all four insecticides, with all four minimally reacting with ozone. All four compounds studied exhibited a second-order OH radical rate constant of greater than $5x10^{8}\ M^{-1}s^{-1}$, indicating that they were all expected to be significantly degraded in a water matrix treated with an AOP.

Table 3.1 Organophosphorous compound k_{OH} and k_{O3} rate constants.

	k_{O3} ($M^{-1}s^{-1}$)	k_{OH} ($M^{-1}s^{-1}$)
Acephate	0.023 ± 0.001	7.1×10^9
Dicrotophos	0.019 ± 0.004	5.5×10^9
Methamidophos	0.031 ± 0.002	8.2×10^9
Fenamiphos	0.017 ± 0.001	6.2×10^9

3.4.2. **Estrogenic Activity**

Figure 3.1shows the estrogenic activity observed while using the YES assay during the OH radical-based degradation of the four insecticides. All insecticides showed a negative response (i.e., no estrogenic activity) in comparison to the positive control, for estrogenic activity. While no estrogenic activity was noted with the YES assay for the parent compound and none developed during degradation into transformation products, estrogenic activity has been observed previously for several organophosphorous insecticides, using alternative estrogenic assays (Raun Andersen, Vinggaard et al. 2002).

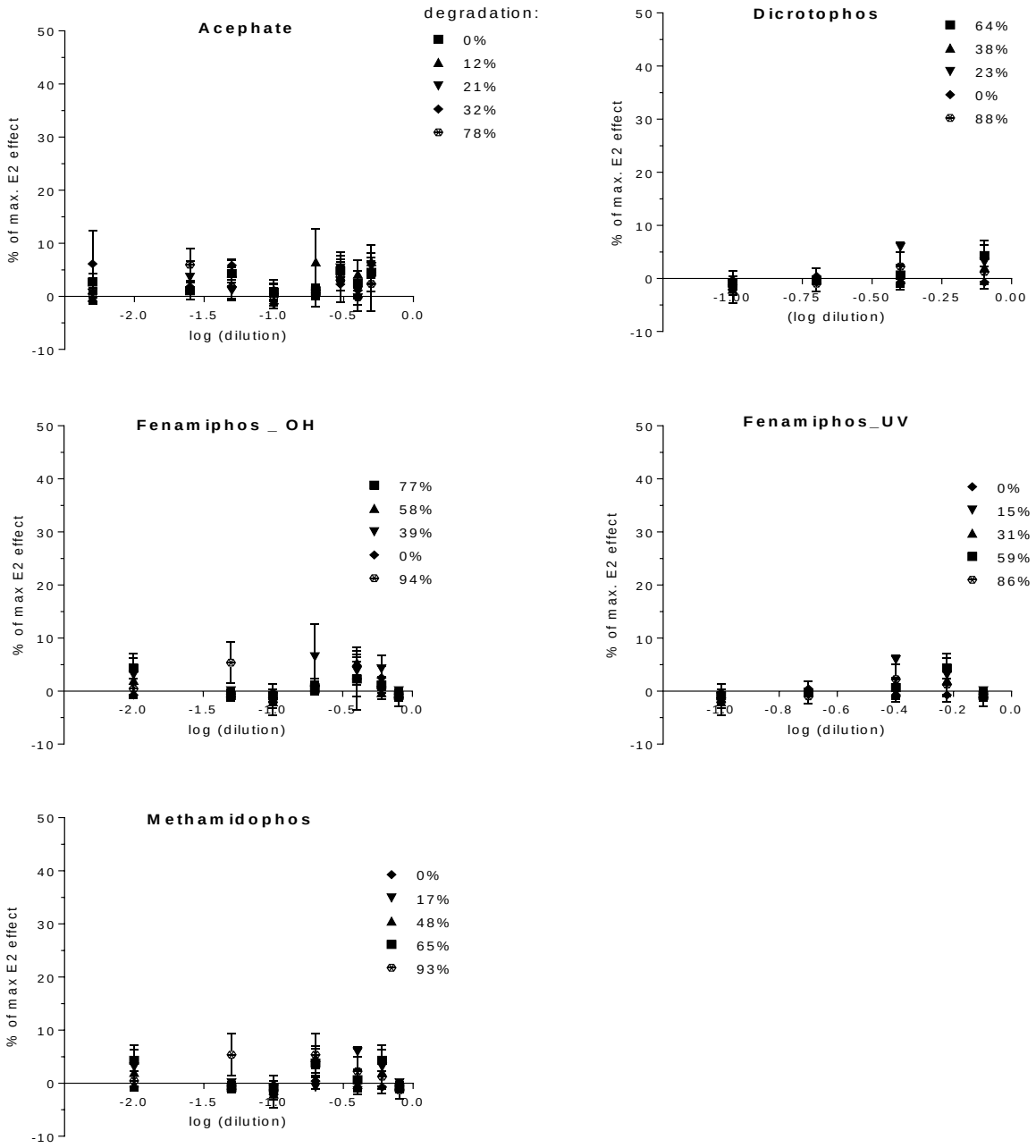


Figure 3.1 Estrogenic activity measured in triplicate of recombinant strains of yeast *Saccharomyces cerevisiae* during degradation of insecticides by hydroxyl radical, and Low Pressure UV (Fenamiphos only).

3.4.3. **Mutagenic Activity**

Table 3.2 presents a summary of the data describing the mutagenic activity of all four insecticides. All four compounds showed at least one positive response to the various strains used in the AMES assay, with and without metabolic activation. Since the compounds were not fast reacting with molecular ozone, the degradation of the compounds by ozone alone was not followed using toxicity assays. Acephate showed a low-positive initial response in the TA98 strain, without the addition of metabolic activation by S9 rat liver enzyme, and this response decreased with increasing AOP treatment. Dicrotophos and fenamiphos caused positive responses in the TAMix combination strains without the addition of metabolic activation in the parent compound form, and the response decreased with increasing hydroxyl radical treatment. Methamidophos showed a parent compound response with metabolic activation in the TA98 strain, and showed an increase in this response with the addition of hydroxyl radicals during AOP treatment.

Table 3.2 Mutagenicity results of acephate, dicrotophos, methamidophos, and fenamiphos using the AMES assay with and without S9 rat liver enzyme, following degradation by hydroxyl radicals.

strains:	TA98		TAMix	
metabolic activation:	-S9	+S9	-S9	+S9
Acephate	+ ↘ -?	– → –	– → –	– → –
Dicrotophos	– → –	– → –	+ ↘ -?	– → –
Methamidophos	– → –	+ ↗ +	– → –	– → –
Fenamiphos	– → –	– → –	+ ↘ -	– → –

– → Parent compound activity was negative, and after treatment, sample was negative.

+ ↘ - Parent compound activity was positive, and after treatment, activity decreased proportional to parent compound.

+ ↘ -? Parent compound displayed weak activity, and activity decreased with treatment, but was too weak to conclusively determine.

+ □ Parent compound activity was positive, and after treatment, activity increased.

Methamidophos mutagenic activity (TA 98, indicating a frameshift mutation in the presence of S9) first decreases with hydroxyl radical degradation, and then increases with increasing advanced oxidation treatment (Figure 3.2). In order to quantify the oxidation products and parent compound response of methamidophos degradation by hydroxyl radicals, the MEQ was employed, as illustrated in Figure 3.3. The red line in Figure 3.3 indicates an ideal toxicity signal degradation response if it followed the parent compound decay exactly. If the toxicity decreases at or below that of the parent compound, it indicates that no toxic oxidation products are formed from AOP treatment. Methamidophos AOP treatment led to a toxicity increase above the line of the parent compound, indicating an increase in byproduct formation toxicity that was greater than that of the parent compound, having an MEQ/MEQ_0 ratio of >1.

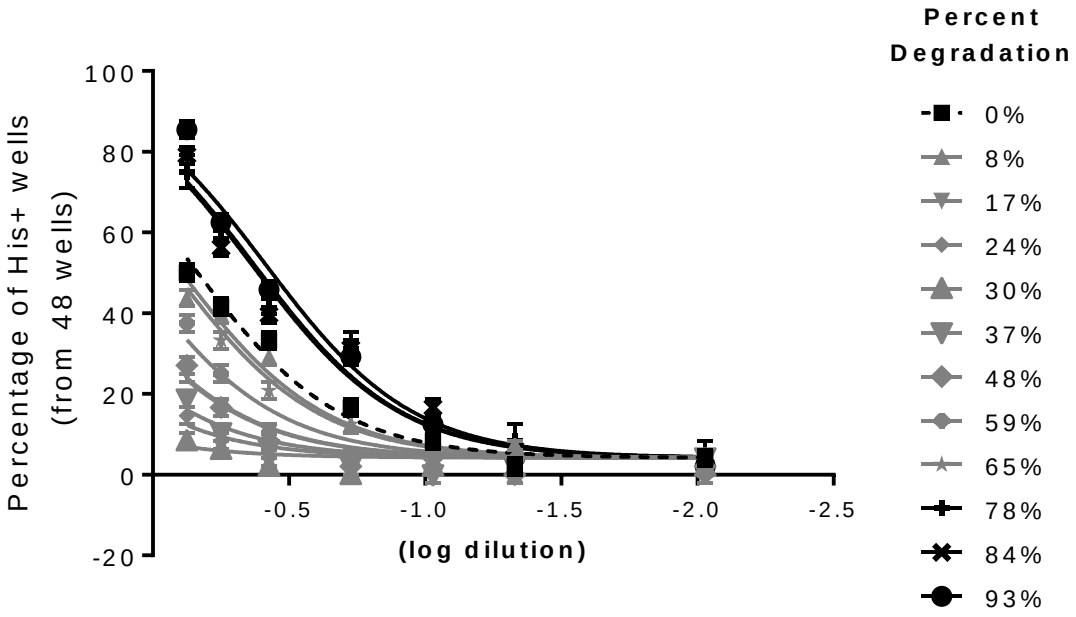


Figure 3.2 Mutagenicity measured in triplicate by AMES bioassay results of Methamidophos with only active strain TA98 + S9 enzyme. Dashed line indicate parent compound response (0% degradation), and solid dark lines indicate increased toxicity signal due to byproduct formation (78, 84, and 93% degradation).

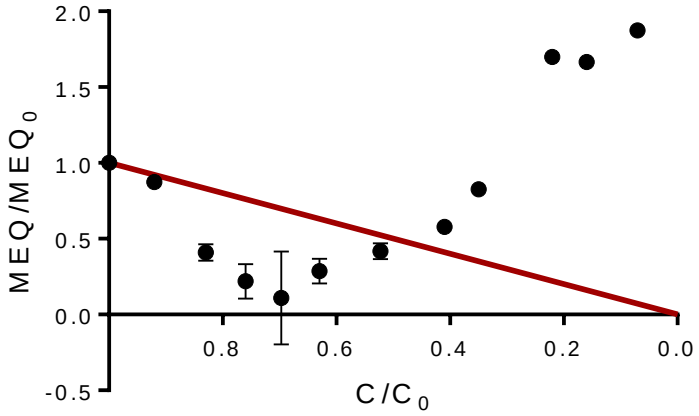


Figure 3.3 Normalized mutagenic equivalents of Methamidophos measured in triplicate under degradation by hydroxyl radical in TA98 strain in the presence of S9; the solid line indicates an ideal response with toxicity correlating to the decrease in parent compound concentration and toxicity.

Identification of the daughter products responsible for this increase in toxic response in the AMES assay was not successful using various solid and liquid-phase extraction and gas and liquid chromatography analytical identification methods. However, the hydrolysis of AOP treated methamidophos after 24 hours in the water matrices, resulted in a steady reversal of the toxic response (Figure 3.4).

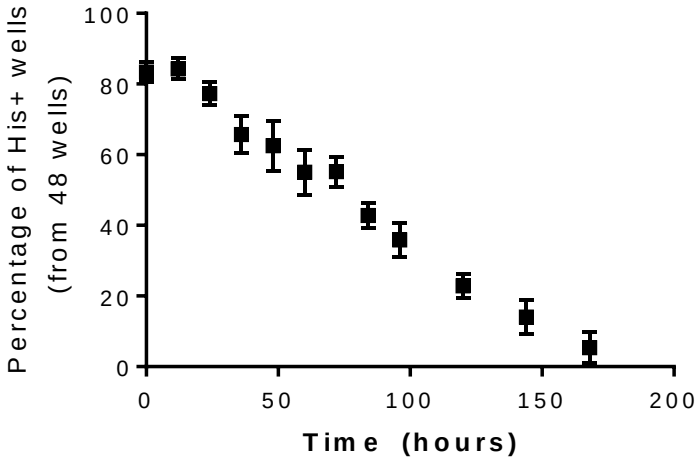


Figure 3.4 Mutagenicity measured in duplicate by AMES bioassay results of methamidophos treated with hydroxyl radicals to 90% degradation in active strain TA98 + S9 enzyme over time in the laboratory water matrices, showing a decrease in toxicity over time due to further byproduct degradation.

3.4.4. **Nuerotoxic Activity**

Not all of the organophosphorous compounds tested reacted strongly enough with the AChE assay such that their neurotoxicity activity can be detected. **Error! Reference source not found.** hows the neurotoxic equivalents, as measured by AChE inhibition, before and after AOP treatment with and without bromination of the oxidation products using NBS.

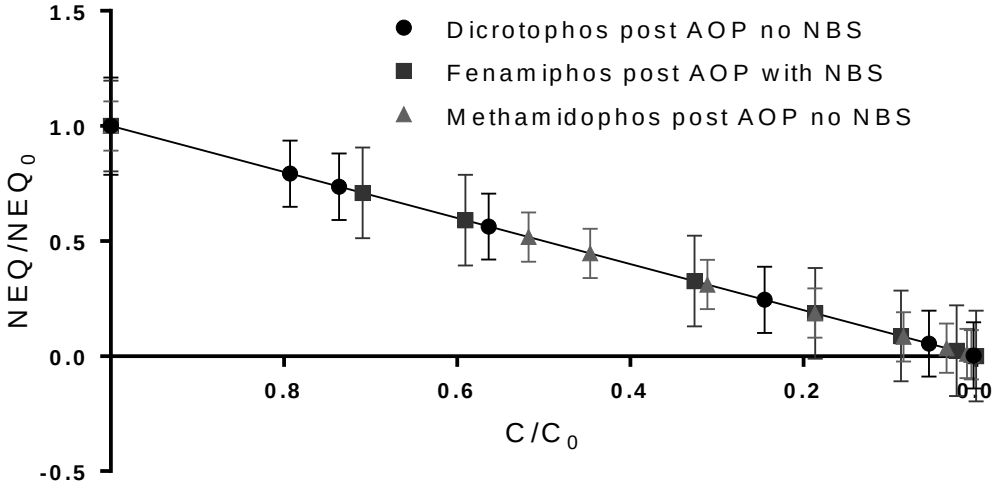


Figure 3.5 Acetylcholinesterase inhibition measured in triplicate, expressed as neurotoxic equivalents (NEQ), of the dicrotophos, fenamiphos, and methamidophos with and without NBS in the AChE assay, with the solid line indicating ideal degradation (slope = -1).

NEQ was applied to the compounds responsive to the AChE assay: dicrotophos, fenamiphos, and methamidophos. Dicrotophos and methamidophos both showed a positive response in the assay without the additional bromination, and the neurotoxicity decreased proportionally to that of the parent compound while undergoing UV-AOP treatment. Fenamiphos has previously been shown to exhibit a positive response in the AChE assay (Worek, Thiermann et al. 2004), which is consistent with these results. The response decreased with increasing oxidative treatment by UV/H2O2 AOP, proportional to that of the parent compound. Acephate was not responsive in our AChE assay both before and after AOP treatment, with and without the addition of NBS.

All organophosphorous insecticides that exhibited a positive parent compound response showed that UV-AOP was effective for decreasing neurotoxicity potential as judged by AChE activity. No new toxicity compared to that of the parent compound was detected in the AChE

assay following UV-AOP treatment. With regard to the neurotoxicity indicator AChE inhibition, UV-AOP is an effective treatment for removal of the organophosphorous insecticides studied.

3.5. Conclusions

AOPs are an effective treatment technology for organophosphorous insecticides, with all four in this study reacting quickly with hydroxyl radicals. None of the contaminants examined or their decay products showed any estrogenic activity pre or post AOP treatment using the YES. All compounds showed an active genotoxic (mutagenic) response in the AMES II assay, and only methamidophos degradation by UV-AOP resulted in an increase in mutagenicity. The initial treatment of methamidophos showed an increase in mutagenic activity in parallel with the parent compound transformation during AOP treatment. However, the mutagenic response slowly decreased with time, likely due to the further transformation of these daughter products. The daughter products of methamidophos were not able to be identified, however, it is plausible that they could potentially be DNA intercalating agents, based on previous research indicating a frameshift mutation response in the AMES test (Ames, Bartsch et al. 1972). Acetylcholinesterase inhibition was evident in three of the four organophosphate parent compounds but decreased in parallel with the parent compound decay upon increasing advanced oxidation treatment.

4. Applicability of AOPs for removal of CCL3 contaminants

*This paper was written jointly in collaboration with EAWAG, and is not the sole work of Austa M. Parker.

4.1. Abstract

Emerging contaminants in the United States that are being explored for potential regulation are those found on the Contaminant Candidate List (CCL). Advanced oxidation processes (AOPs) are being implemented for the treatment and control of these emerging contaminants. Transformation products from these oxidation processes are generally unknown and difficult to characterize, but could potentially be more than or equally as toxic as the parent compound. An effect-direct analysis (EDA) approach was taken to explore the toxicity of transformation products before and after advanced oxidation. Generally, advanced oxidation was found to be an appropriate treatment technology for the emerging contaminants tested.

4.2. Introduction and Background

The presence of micropollutants in water resources is a current issue. Advanced oxidation processes (AOPs) are efficient for the majority of micropollutants. In this paper we are showing results on the screening of selected contaminants from the USEPA CCL3 list undergoing oxidation for toxic potential relevant for human health, including estrogenic and mutagenic activity.

4.2.1. USEPA Candidate Contaminant List (CCL)

Chemicals on the US EPA's Contaminant Candidate List (CCL) 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). The CCL process starts with a broad list of chemicals and then narrows the list following a screening process (USEPA 2009). This process ends in a regulatory determination with a set of contaminants, made public, which the USEPA could potentially regulate. The most recent CCL is the CCL-3, which contains 104 chemical and 12 microbial contaminants (USEPA 2009).

4.2.2. **Advanced Oxidation Processes**

Water treatment processes for treatment of chemical contaminants may employ either physical removal or destructive technologies. Physical removal technologies such as membranes, adsorption, and filtration move contaminants out of the bulk water phase for concentration and elimination. An alternative to physical removal processes are destructive or transformation processes, which transform the contaminants from its parent compound form, altering its chemical structure.

AOPs are a category of transformation processes that work by producing the fast-reacting non-selective hydroxyl radical that reacts with a wide array of micropollutants. There are several types of AOPs, but two that are commonly used include the combination of ozone/H_2O_2 and UV/H_2O_2. Ozone and UV based AOPs have both been utilized to provide treatment of regulated and emerging contaminants at full-scale facilities.

Destruction of contaminants is generally beneficial; however, the possibility of the transformation products retaining or forming harmful biological activity is a fundamental issue. Concern surrounding chemical degradation products led to the regulation of degradation products of pesticides in the drinking water directive of the European Union in 1998 (EU 1998). It is therefore important to study the degradation products of contaminants treated with transformation technologies.

AOPs generally lead to the removal of biological activity from micropollutants, as shown previously for end-points such as estrogenicity, antibacterial activity and toxicity towards algae (Lee, Escher et al. 2008; Dodd, Kohler et al. 2009; Mestankova, Escher et al. 2011). However, in a few cases during the oxidation of parent compounds transformation products with completely different modes of toxic potential were formed. Formation of *N*-nitrosodimethylamine (NDMA), a disinfection byproduct, was detected during ozonation of its precursors, such as the bromide-catalyzed oxidation of dimethylsulfamide, a metabolite of the fungicide tolylfluamide (Schmidt and Brauch 2008; von Gunten, Salhi et al. 2010) or ozonation of dyes with dimethylaminogroups. . It is therefore important to study the oxidation of various classes of contaminants for the formation of potentially toxic oxidation products.

4.2.3. Contaminant Selection and Rationale

The 104 chemical contaminants from the USEPA CCL3 were distilled down to 25 specific compounds to evaluate in this study. The list of chemicals was grouped into classes based on their structure and biological mode of action, and were reviewed for toxicological relevance of byproducts and likelihood of oxidation and a few chemicals were selected from each class of compounds chosen for study. Halogenated compounds were not selected for study based on slow reaction rates with ozone and •OH radicals (von Gunten 2003). Olefines and nitro compounds were both selected for study based on their potential to be fast reacting with hydroxyl radical and their potential for toxic byproduct formation (Onstad 2005). The classification of oxygen containing compounds were chosen based on previous research showing similar compounds with interesting degradation products and fast-reactions with hydroxyl radicals (Schmidt 2008). Nitrogen containing compounds and organophosphorous compounds were both expected to react quickly

with hydroxyl radical, and both include degradation products that potentially lead to relevant byproducts (Nordmeyer and Dickson 1990). The amides, nitroso compounds, and aliphatic and aromatic compounds were all candidates for AOP treatment, and several compounds tested positive for genotoxic and estrogenic activity previously (Osano, Admiraal et al. 2002; von Gunten 2003; Hammes, Salhi et al. 2006; Ramseier and von Gunten 2009). Metallorganics were the last category identified, and although they are expected to react quickly with hydroxyl radical, the probability of the contaminants being found downstream at a water treatment facility are low, and were therefore not selected. Further information supporting selection of compound classes can be found presented in Table 4.6.

Figure 4.1 outlines the experimental process for this study, with the research goals being (1) determining whether or not the CCL3 compounds selected are able to be transformed by AOPs (2) determine whether or not the byproducts from these compounds are more toxic than their parent compounds.

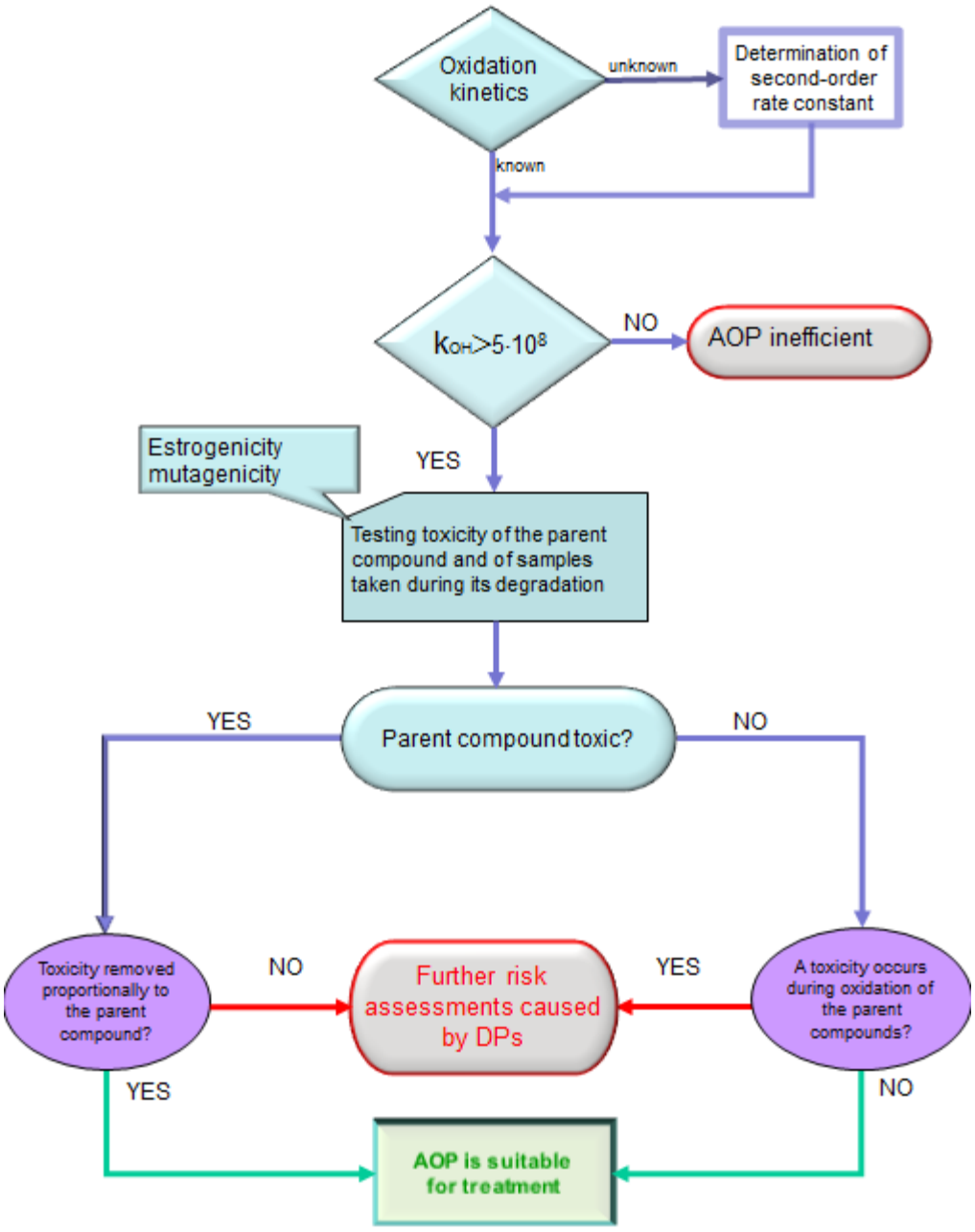


Figure 4.1 Experimental design overview of advanced oxidation process, including testing for unknown byproducts.

After the necessary oxidation kinetics were determined, all of the compounds underwent toxicity testing at various time points during degradation. The bioassay screening included measurements of estrogenic and mutagenic activity by the YES and AMES II bioassays.

4.3. Materials and Methods

4.3.1. Chemicals

All chemicals used as the parent compounds were standards of analytical grade of ≥ 99% purity (suppliers and purity indicated in Table 4.7 in SI). Chemicals used for preparation of media or reaction mixtures were ≥ 95% purity.

4.3.2. Hydroxyl Radical and Ozone setup

Hydroxyl radicals for the study were generated using two different setups. At Eawag (E), oxidation by hydroxyl radicals was based on the photolysis of hydrogen peroxide and carried out in glass-stoppered quartz tubes (internal diameter: 15 mm; external diameter: 18mm) using a temperature controlled merry-go-round photoreactor (MGRR) model 125 (DEMA Hans Mangels GmbH, Bornheim-Roisdorf, Germany), which was equipped with a Heraeus Noblelight model TQ718 medium-pressure mercury lamp that was operated at a power output of 500 Watts. The lamp was placed in a cooling jacket containing a UVW-55 glass filter (DEMA), which provided a band-pass filter in the wavelength range of 308 to 410 nm. Direct photolysis was inhibited due to the wavelengths filtered below 308 nm, leaving the only degradation of the compounds present due to hydroxyl radicals generated. The solutions contained 100 μM of the parent compound (or lower when limited by its solubility) and 5 mM H_2O_2 (buffered at pH = 8 by phosphate buffer). At each time point in degradation, samples were quenched with bovine catalase prior to quantifying the parent compounds by HPLC.

At CU Boulder (B), hydroxyl radicals were generated using low pressure UV coupled with hydrogen peroxide (LPUV/H_2O_2). Experiments were performed using a quasi-collimated low pressure beam, with four LPUV lamps (ozone free, 15 watt, #G15T8) being housed above a 4 inch aperture equipped with a manual shutter. Incident irradiance was measured at a wavelength of 254 nm with a radiometer (International Light Inc., Model 1700/SED 240/W). The UV fluence (i.e. dose) was calculated from the incident irradiance measurement, sample depth, and water quality characteristics (Bolton and Linden 2003).

Contaminants were dosed into aqueous sample solutions at a concentration of 1000 µg/L, hydrogen peroxide was added at a concentration of 5 mg/L, aqueous sample solutions were irradiated at a temperature of 25.0 ± 0.5 °C, and a buffered pH of 7.2 ± 0.1. Following exposure, samples were dosed with a bovine catalase solution to quench residual hydrogen peroxide, and the degradation of contaminants was followed by either HPLC-MS or GC-MS (see supporting information for analytical methods Table SI 2).

Experiments involving ozonation of samples were performed in the same manner by both research institutions (E&B). Solutions containing 100 µM of the studied compound were buffered to pH 7 and 10 mM t-butanol (a hydroxyl radical scavenger) was added with variable volumes of ozone stock solution to obtain the desired percentages of degradation. An ozone stock solution, usually approaching 60 mg/L, was produced by sparging O_3-containing oxygen through Milli-Q water that was cooled in an ice bath, or a jacketed beaker. Concentration of the stock solution was determined by direct UV at 258 nm. The solutions were stirred in borosilicate glass vials (screw-top) at room temperature, and ozone was added by either injecting the ozone stock solution via a gas-tight glass syringe or using ozone-demand free pipet tips. Initial ozone concentrations were typically targeted at 0.5, 1, 2.5, 5, and 10 mg/L. Ozone concentration in the

reaction mixture was measured using the indigo method (Bader and Hoigné 1981). Hydrogen peroxide was added at a concentration of 5 mg/L to all contaminant solutions directly before ozone was added to the solutions, and the solutions were quenched with a bovine catalase solution prior to analysis.

4.3.3. Determination of Rate Constants

Apparent second-order rate constants for the reaction of the contaminants with ozone and hydroxyl radicals were determined by competition kinetics. The hydroxyl radical rate constant (k_{OH}) for the contaminants was measured with the addition of *p*-chlorobenzoic acid (pCBA) as a competitor with a known k_{OH} value of 5×10^9 $M^{-1}s^{-1}$ (Elovitz, Gunten et al. 2000). For fast-reacting compounds, reaction rates with ozone (k_{O3}) were determined by competition kinetics with cinnamic acid, with a well-developed k_{O3} value of 3.8×10^5 $M^{-1}s^{-1}$ for pH 7 to 8 (Leitzke, Reisz et al. 2001) . In the case of slow-reacting compounds with ozone, the k_{O3} value was measured following the decay at pH 3 where the ozone decay is stabilized thus increasing the ozone exposure. Reactions were run in the presence of excess substrate in order to fulfill pseudo-first order conditions. The initial ozone concentration was lower than 50 μM and the concentrations of the studied compounds were at least 20 times higher than the initial ozone concentration. Ozone reaction rates were determined by following the decay of ozone and the decay of the compound, measuring ozone by utilizing the indigo method (Bader and Hoigné 1981). For each experiment, competition kinetics were measured in duplicate, and the slope was determined from the plots of ln $([C]/[C]_0)$ versus ln$([competitor]/[competitor]_0)$ and the rate constant was calculated from the slope following equations presented in previous publications (Elovitz and von Gunten 1999; Elovitz, Gunten et al. 2000).

4.3.4. **Yeast Estrogen Screen (YES)**

The yeast estrogenic screen (YES) was used to quantify the estrogenicity in samples (Routledge and Sumpter 1996). It is based on a recombinant strain of the yeast *Saccharomyces cerevisiae*, which contains a gene for the human estrogen receptor (hER) and expression plasmids carrying the reporter gene *lac-Z* encoding the enzyme β-galactosidase. Estrogenically active compounds that bind to the hER induce the expression of the *lac*-Z gene followed by the synthesis of the enzyme β-galactosidase. This enzyme releases chlorophenol red from the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG). The color change is measured photometrically and allows for the quantification of the estrogenic potential of the chemicals in the samples. The test is valid unless the measured OD620 of the negative controls is higher than 0.1, and the effective concentration at 50% (EC50) values for the positive control ethinyl estradiol (E2) are not higher than 0.5nM. A positive estrogenic response occurs when the sample's OD is at least twice the OD of the negative control, and a dose-response relationship exists.

4.3.5. **Ames Mutagenicity Assay (Ames II)**

The Ames II (*Salmonella*/microsome mutagenicity assay) uses strains of *Salmonella typhimurium* that have been genetically modified with a mutation, making the bacteria unable to synthesize the essential amino acid, histidine, making proliferation of the bacteria impossible without an external histidine source. A mutation at the site of the pre-existing mutation or nearby can restore the gene function (a reversion of the mutation) and allow cells to synthesize histidine. Mutagenic compounds cause an increase in the number of revertant colonies relative to the background levels. The quantification of the revertants is determined by counting the number of wells shifted from purple to yellow in color in the presence of bromocresol pH indicator. Two

mutations contained within bacteria were used in the Ames II assay, TA Mix, which comprised a mixture of six *Salmonella* strains (measuring point mutations) and TA98 (measuring frameshift mutations). The Ames II test was performed as described in ISO 11350 [21]. The test was considered valid only if the percentage of revertant wells in the negative controls was lower than 10% (from 48 wells) and the positive controls in concentrations from the ISO 11350 gave a percentage of revertants ≥ 80%. The strains of Ames II and S9 (induced by Aroclor) were obtained from Xenometrix (KS, USA). In order to ensure the sensitivity of the assay to the metabolization of byproducts, some batches of S9 were added in larger quantities than in the protocol of ISO 11350.

4.3.6. **Mutagenic Equivalents Concept (MEQ)**

To quantify the evolution of toxicity during the degradation of contaminants, the toxic equivalent concept (TEQ) is widely applied to express the toxicity of environmental samples, and has been previously demonstrated (Escher 2008). When applied directly to mutagenic activity, the quantification can be referred to as the mutagenic equivalents concept or MEQ. If the parent compound dominates the overall toxicity, the disappearance of the parent compound is directly proportional to the decrease in toxicity of the mixture and no further action involving the identification of the transformation products was required. In contrast, a deviation from the parent compound degradation proportionality indicates the formation of toxic transformation products, and an effect-directed analysis was then applied to identify the toxic compounds (Biran, Yagur-Kroll et al.).

4.4. Results and Discussion

4.4.1. Kinetic Data

Table 4.1 shows the CCL3 contaminants tested in this study, and their corresponding ozone and hydroxyl radical degradation rate constants. Values of each compound are included, with references to rate constants that were published previously in literature.

Table 4.1 Hydroxyl radical and ozone rate constants – footnote for method used to determine the fundamental parameter and bold text referring to chemicals and processes studied for toxicity.

	k_{O3} ($M^{-1}s^{-1}$)	k_{OH} ($M^{-1}s^{-1}$)
1. Halogenated Compounds[B]		
1,2,3 - Trichloropropane	0.87 ± 0.03	< 5 x 10^8
2. Olefines[B]		
Dimethipin	**1.2 x 10^5**	**6.7 x 10^9**
3. Nitro compounds[E]		
Nitrobenzene	0.09 (Hoigné and Bader 1983; Buxton, Greenstock et al. 1988; García Einschlag, Carlos et al. 2003)	**3.9-4.2 x 10^9 (Hoigné and Bader 1983; Buxton, Greenstock et al. 1988; García Einschlag, Carlos et al. 2003)**
Nitroglycerin	0.043 ± 0.008	< 5 x 10^8
4. Ethers, alcohols, and phenols[B]		
1,4-Dioxane	0.3 (Thomas 1965; Eibenberger 1980; Hoigné and Bader 1983)	**2.5-3.1 x 10^9 (Thomas 1965; Eibenberger 1980; Hoigné and Bader 1983)**
Oxirane, methyl	327	**5.7 x 10^9**
Methyl-tert-butyl ether (MTBE)	0.14 (Acero, Haderlein et al. 2001)	**1.0 x 10^9 (Acero, Haderlein et al. 2001)**
Cumene hydroperoxide	**2.0 x 10^5**	**2.8 x 10^9**
5. Nitrogen containing[E]		
4,4'-Methylenedianiline	**> 9 x 10^7 (Hoigné and Bader 1983)**	**> 5 x 10^9**
Aniline	**1 x 10^8 (Christensen 1972; Hoigné and Bader 1983; Solar, Solar et al. 1986)**	**0.9-1.7 x 10^{10}**
Quinoline	**51 (Wang, Huang et al. 2004)**	**0.7-1.0 x 10^{10}**
6. Organophosphorous compounds[B]		
Acephate	0.023 ± 0.001	**7.1 x 10^9**
Dicrotophos	0.019 ± 0.004	**5.5 x 10^9**
Methamidophos	0.031 ± 0.002	**8.2 x 10^9**
Fenamiphos	0.017 ± 0.001	**6.2 x 10^9**
7. Amides[E]		
Metalachlor	2.9 ± 0.1	**6.1 x 10^9**
Metalachlor ethanesulfonic acid (ESA)	5.0 ± 0.4	**7.1 x 10^9**
Metalachlor oxanilic acid (OA)	7.0 ± 0.05	**5.6 x 10^9**
N-methyl-2-pyrrolidone	1.8 ± 0.1	**3.1 x 10^9**

Molinate	**500** **(Shemer, Sharpless et al. 2006; Chen, Wu et al. 2008)**	**1.3-6.9 x 10^9**
8. Nitroso compounds[E]		
N-nitroso-diethylamine (NDEA)	0.077 ± 0.001	**7.0 x 10^8**
N-nitroso-dimethylamine (NDMA)	0.021 ± 0.002	**4.5 x 10^8**
N-nitroso-di-n-propylamine	0.043 ± 0.008	**2.3 x 10^9**
N-nitroso-diphenylamine	**8.5**	**6.5 x 10^9**
N-nitroso-pyrrolidine (NPYR)	5.0 ± 0.4	**7.1 x 10^9**

[B] Compounds were studied by the US Team (CU-Boulder)
[E] Compounds were studied by the Swiss Team (EAWAG)

Hydroxyl radical and ozone degradation rate constants that were missing from literature were experimentally determined. Of the 25 compounds in this study, two (1,2,3-trichloropropane and nitroglycerin) contained hydroxyl radical rate constants that were lower than the benchmark set at $5x10^8$ $M^{-1}s^{-1}$. This benchmark was chosen to represent a conservative reaction rate that contaminants must react more quickly than to be considered relevant for AOP treatment, as most organic contaminants react at a magnitude of 10^9 $M^{-1}s^{-1}$.

4.4.2. Mutagenicity and Estrogenicity

Table 4.2 presents a shortened list of compounds that contained parent compounds that tested positive for mutagenic or estrogenic activity. A full list of results of all compounds can be found as Table SI 3. All compounds, regardless of parent compound toxicity were tested for toxic byproduct formation after oxidative treatment. Compounds that could be degraded by hydroxyl radical, ozone alone, or photolysis treatment were tested. 1,2,3-trichlorpropane tested positive in parent compound form for mutagenic activity without metabolic activity (-S9), but had a very low rate constant with hydroxyl radical so was not included in additional screening.

Cumene hydroperoxide, as well as all organophosphorous compounds tested showed a positive response in the AMES test, as previously published (Parker, Spangler et al. 2014). Only 4,4'-methylenedianiline tested positive for estrogenic activity in the parent compound form.

Table 4.2 Contaminants studied that showed either Mutagenic or Estrogenic activity.

	Mutagenic Activity				Estrogenic Activity
	TA98		TAMix		
	-S9	+S9	-S9	+S9	
1. Halogenated Compounds					
1,2,3-Trichloropropane	-	-	+	-	-
4. Ethers, Alcohols, and Phenols					
Cumene hydroperoxide	-	-	+	-	-
5. Nitrogen containing compounds: Anilines, amines, hydrazine, heteroaromatic compounds, isocynate					
4,4'-methylenedianiline	-	-	-	-	+
6. Organo-phosphorous compounds					
Acephate	+?	-	-	-	-
Dicrotophos	-	-	+?	-	-
Methamidophos	-	+	-	-	-
Fenamiphos	-	-	+	-	-

\+ Positive activity observed.
\- Negative or no activity observed.
+? Weak activity observed.

4.4.3. Evolution of Mutagenic Activity

The evolution of mutagenic activity following degradation by hydroxyl radical, ozone, and direct photolysis (in some cases) of each compound can be found in Table 4.3 With arrows indicating treatment, red responses indicating an increase in toxicity, and green responses indicating a decrease in mutagenic activity. Most treatment cases did not cause an increase in toxic byproduct formation, however some did and were further explored, as discussed.

Table 4.3 Evaluation of mutagenic activity by AMES bioassay following treatment of CCL contaminants Degradation results of AMES bioassay

	oxidation by OH radical				reaction with ozone				direct photolysis			
strains:	TA98		TAMix		TA98		TAMix		TA98		TAMix	
metabolic activation:	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
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: Anilines, amines, hydrazine, heteroaromatic compounds, isocynate												
Aniline	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	- ↗ +	– → –	– → –
4,4'-methylenedianiline	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→
Quinoline	- ↗ +	- ↗ +	– → –	– → –	- ↗ +	- ↗ +	- ↗ +	- ↗ +	–⊠→	-⊠→	– ⊠→	-⊠→
6. Organo-phosphorous compounds												
Acephate	+↘ -?	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	–⊠→	-⊠→	– ⊠→	-⊠→
Dicrotophos	– → –	– → –	+↘ -?	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	–⊠→	-⊠→	– ⊠→	-⊠→
Methamidophos	– → –	+ ↗ +	– → –	– → –	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →
Fenamiphos	– → –	– → –	+ ↘ -	– → –	- ⊠ →	- ⊠ →	- ⊠ →	- ⊠ →	– → –	– → –	+ ↘ -	– → –
7. Amides												

Metolachlor	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
Metolachlor ethanesulfonic acid	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
Metolachlor oxanilic acid	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
2-methyl-2-pyrrolidone	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	–⊠→	-⊠→	– ⊠→	-⊠→
Molinate	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –
8. Nitroso compounds												
N-nitrosodimethylamine	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
N-nitrosodiethylamine	– → –	– → –	– → –	– → –	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
N-nitrosodi-n-propylamine	– → –	– → –	- ↗ +	- ↗ +	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
N-nitrosopyrrolidine	– → –	– → –	- ↗ +	- ↗ +	–⊠→	-⊠→	– ⊠→	-⊠→	– → –	– → –	– → –	– → –
N-nitrosodiphenylamine	– → –	– → –	– → –	– → –	– → –	– → –	– → –	– → –	- ↗ +	- ↗ +	– → –	– → –

– → – Parent compound activity was negative, and after treatment sample was negative.

–⊠→ Insufficient degradation was achieved through treatment for testing.

+ ↘ - Parent compound activity was positive, and after treatment activity decreased proportional to parent compound.

+↘ -? Parent compound displayed weak activity, and activity decreased with treatment, but was too weak to conclusively determine.

+ ↗ + Parent compound activity was positive, and after treatment activity increased.

- ↗ + Parent compound activity was negative, and after treatment activity increased.

4.4.4. **Olefines**

Only dimethipin in the olefines group was tested for mutagenicity, dimethipin. Dimethipin showed no parent compound response for either of the AMES assay strains tested. Dimethipin is amendable to oxidation by hydroxyl radical, degradation by ozone, and also direct photolysis treatment. When subjected to all three of these treatment processes, dimethipin showed no formation of mutagenic activity. The raw AMES data results showing negative response in all strains and metabolic activation scenarios with hydroxyl radical, ozone, and photolysis treatment can be seen as Figure 4.2 through Figure 4.4.

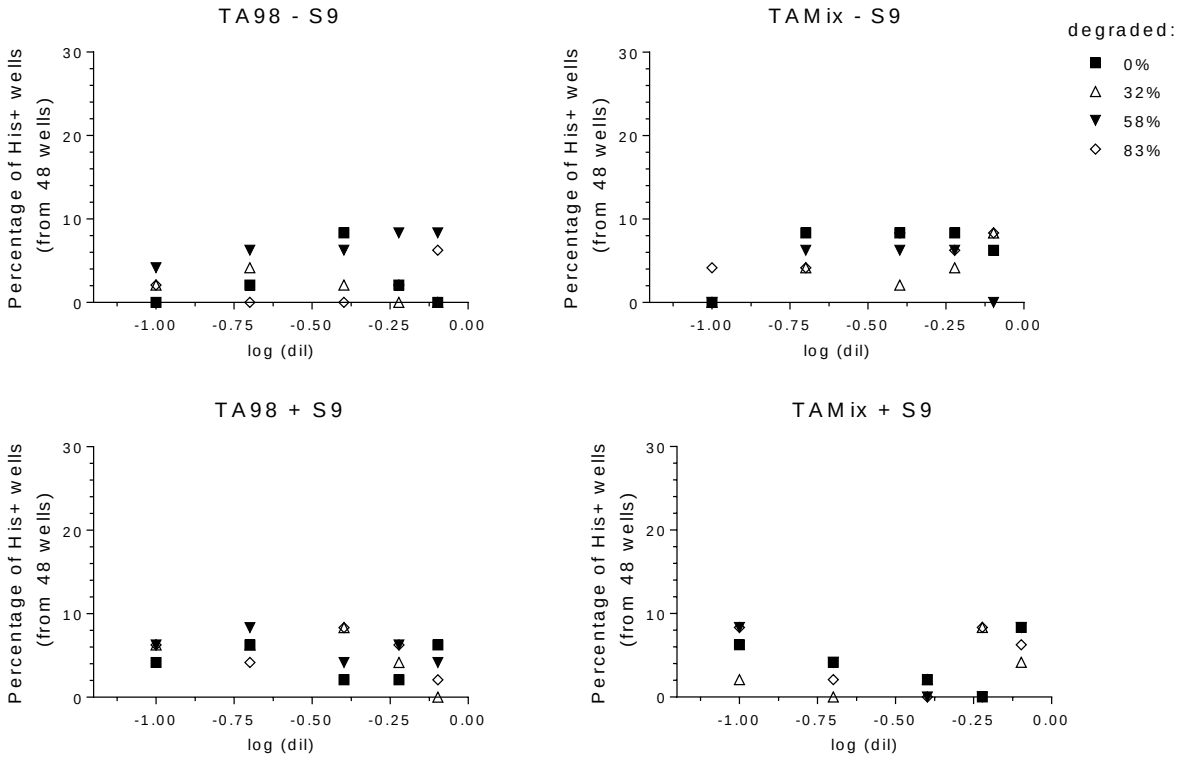


Figure 4.2 Mutagenicity in strains of Ames II during degradation of dimethipin by hydroxyl radical.

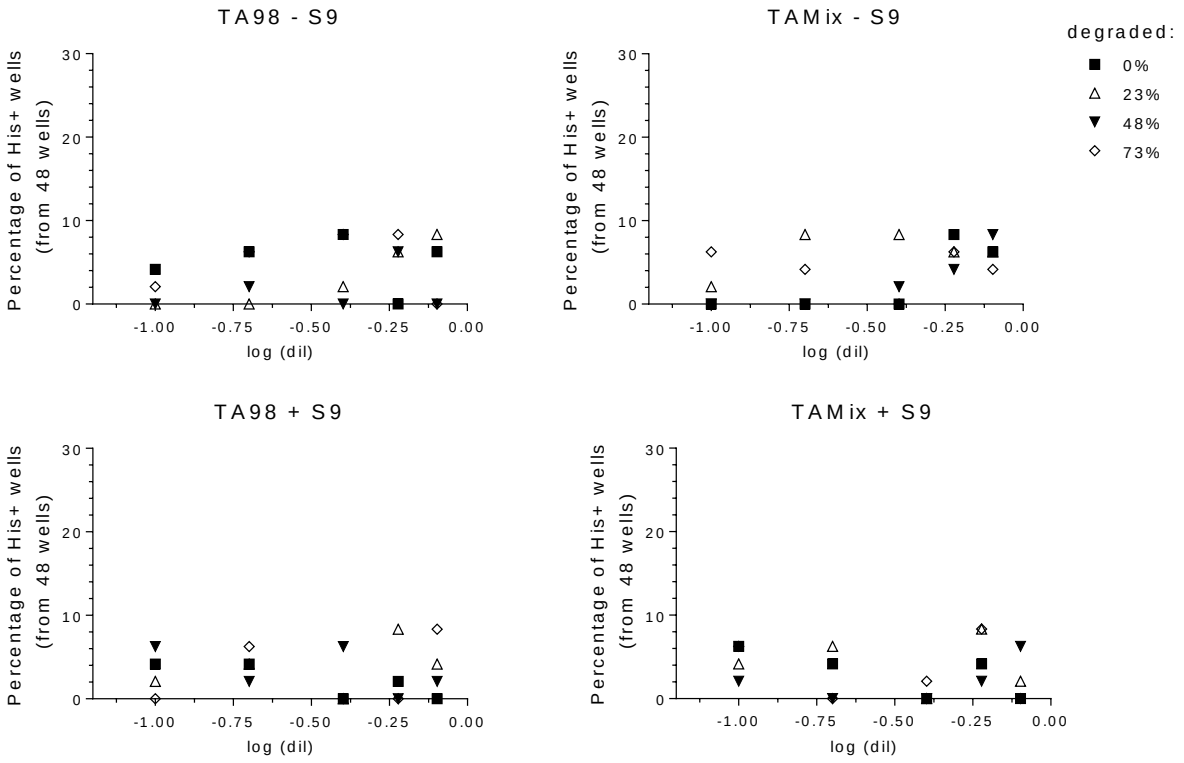


Figure 4.3 Mutagenicity in strains of Ames II during degradation of dimethipin by ozone.

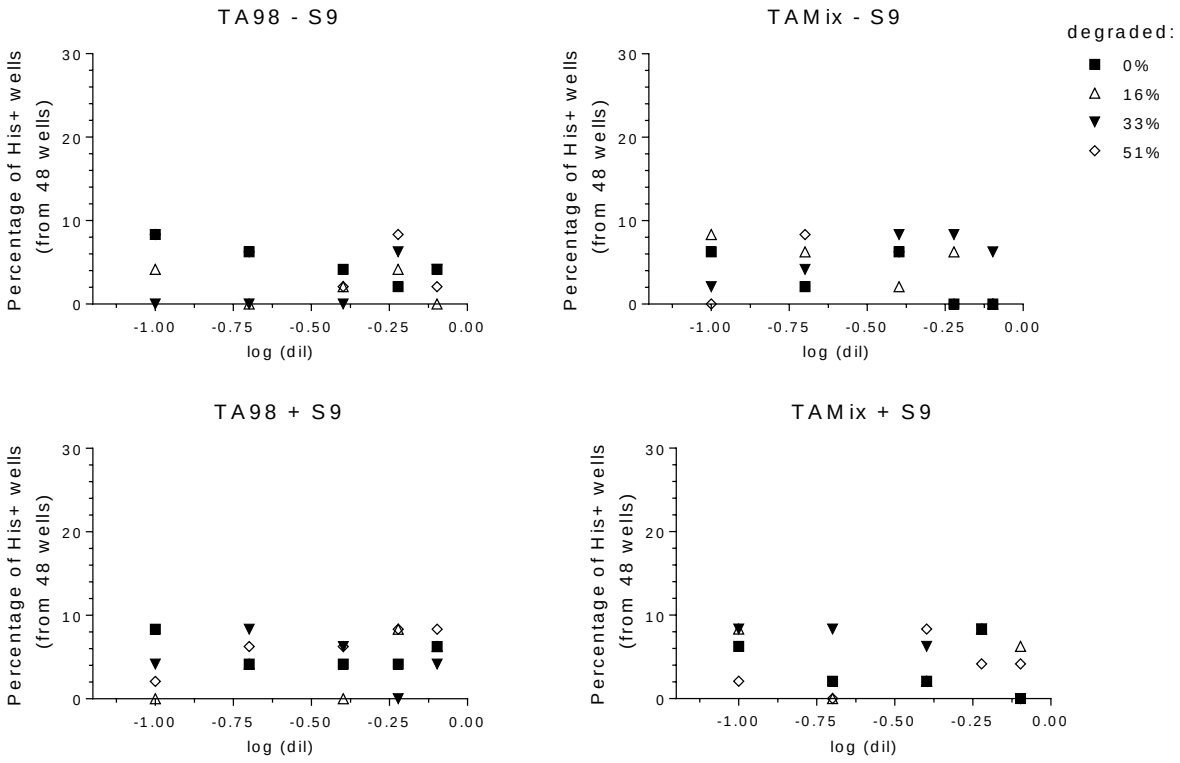


Figure 4.4 Mutagenicity in strains of Ames II during degradation of dimethipin by direct photolysis.

4.4.5. **Nitro Compounds**

Direct photodegradation of nitrobenzene in the nitro compounds group leads to formation of significant mutagenic activity in the strain TA98 in the absence of S9. The mutagenicity was quantified as MEQ-2-NF. Also during oxidation of NB by hydroxyl radical (in system H_2O_2 + hν) a weak mutagenicity formation was observed, as shown by Figure 4.5.

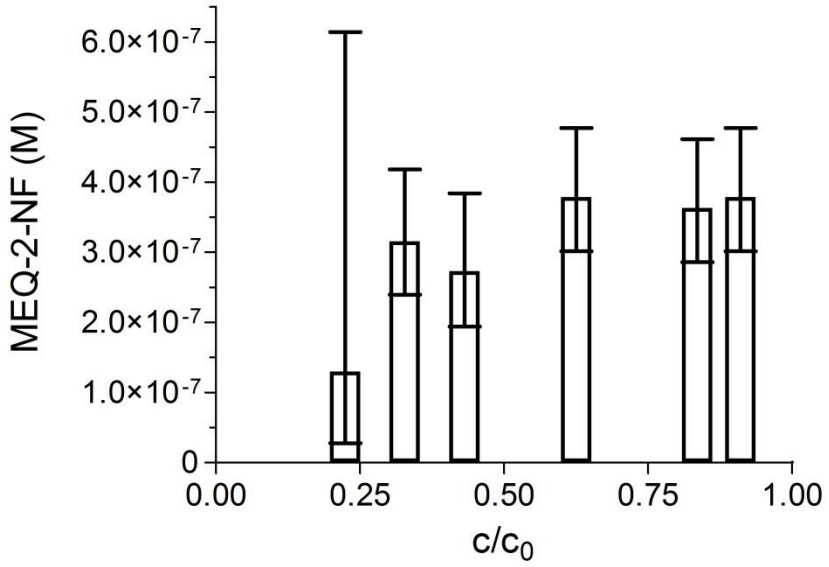


Figure 4.5 Mutagenic equivalents of nitrobenzene as a function of decreasing parent compound concentration while undergoing degradation by hydroxyl radical (E).

Degradation of nitrobenzene (NB) leads to formation of o-nitrophenol, m-nitrophenol, p-nitrophenol, phenol, nitrohydroquinone, nitrocatechol and catechol. NB itself is non-mutagenic in strains TA98 and TA100 in the absence and presence of S9, only the substituted NBs tested as mutagenic (Aßmann, Emmrich et al. 1997). P-nitrophenol was reported to be mutagenic in the TA98 in the absence of S9 by Yang, Xu et al. (2003) (Yang, Xu et al. 2003) contrary to previous studies by Shimizu and Yano (1986) (Shimizu and Yano 1986). Mutagenicity results were also reported for phenol previously (Kamber, Fluckiger-Isler et al. 2009; Kirkland, Reeve et al. 2011).

4.4.6. Ethers, Alcohols, and Phenols

Three of the four compounds tested in the ethers, alcohols, and phenols group 4 showed no parent compound toxicity and no degradation product toxicity in the AMES bioassay. Cumene hydroperoxide showed parent compound toxicity. No increase in toxic byproduct formation was observed after hydroxyl radical, ozone, and low-pressure direct photolysis treatment. The

degradation of cumene hydroperoxide by all three treatment methods following the mutagenicity, and quantified mutagenic equivalents (MEQ), can be seen as Figure 4.6.

The MEQ results show that the mutagenic activity of the parent compound decreases at or below the ideal red line (slope = -1), and therefore no increase in mutagenic activity was observed with engineered treatment.

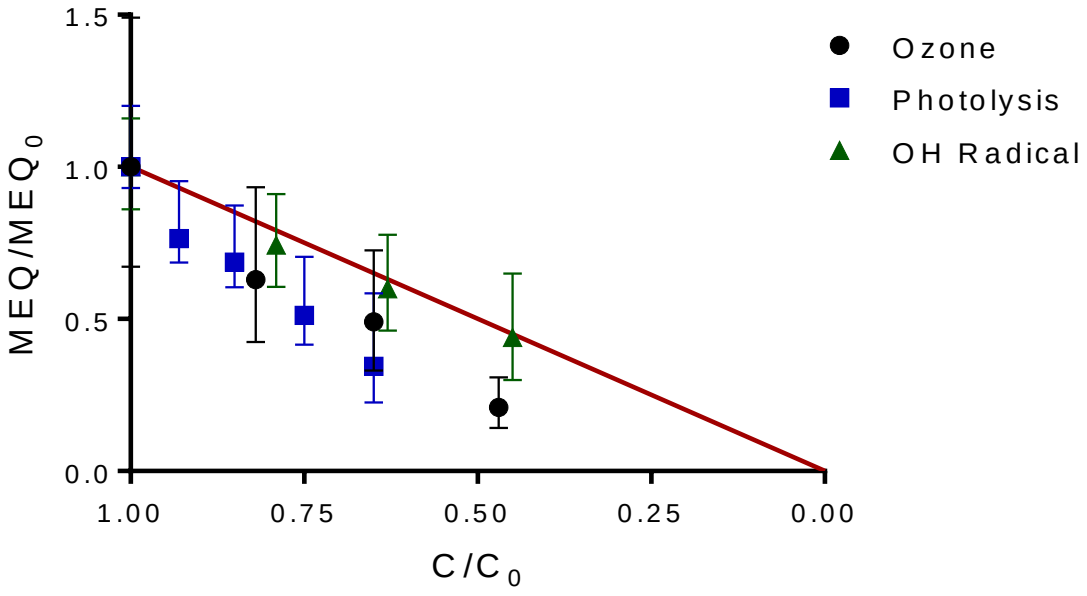


Figure 4.6 Mutagenic equivalents (MEQ) of cumene hydroperoxide in laboratory grade water treated with hydroxyl radical, direct photolysis, and ozone exposures.

The raw AMES toxicity data for 1,4-dioxane, oxiane-methyl, methyl-tert-butyl ether (MTBE), and cumene hydroperoxide can be found as Figure 4.7through Figure 4.12.

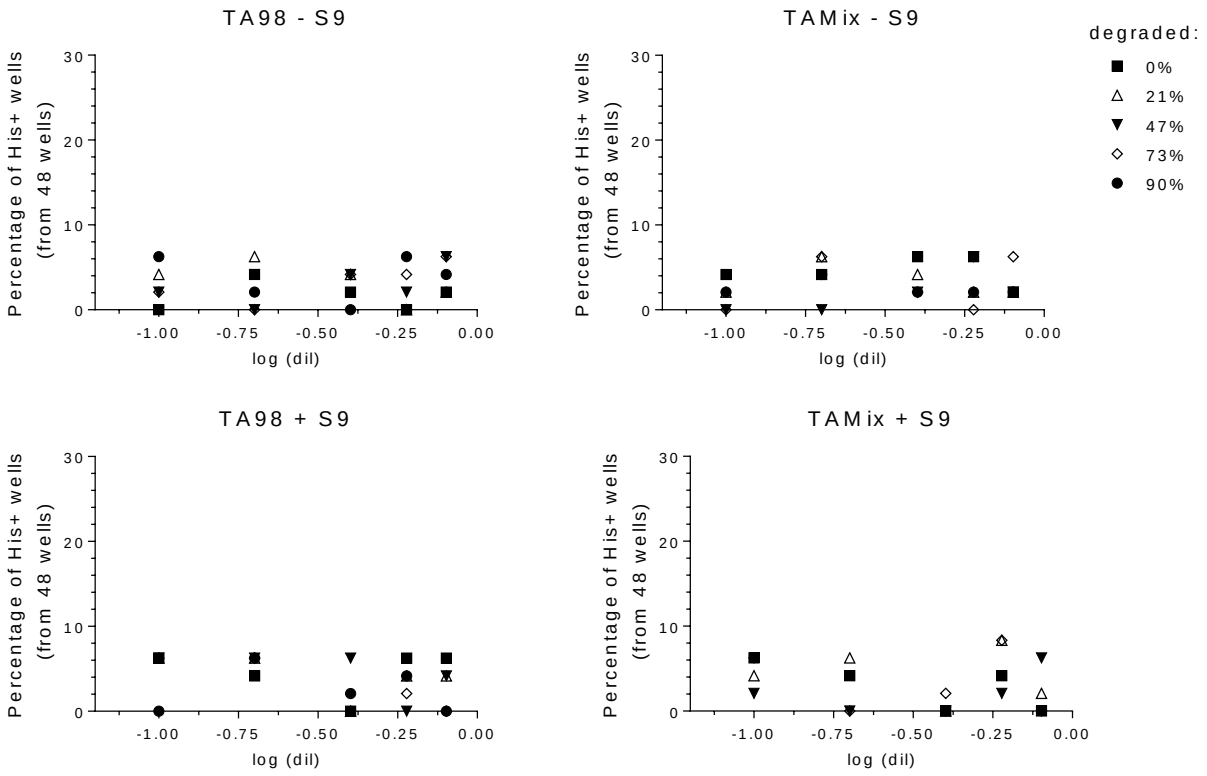


Figure 4.7 Mutagenicity in strains of Ames II during degradation of 1,4-dioxane by hydroxyl radical.

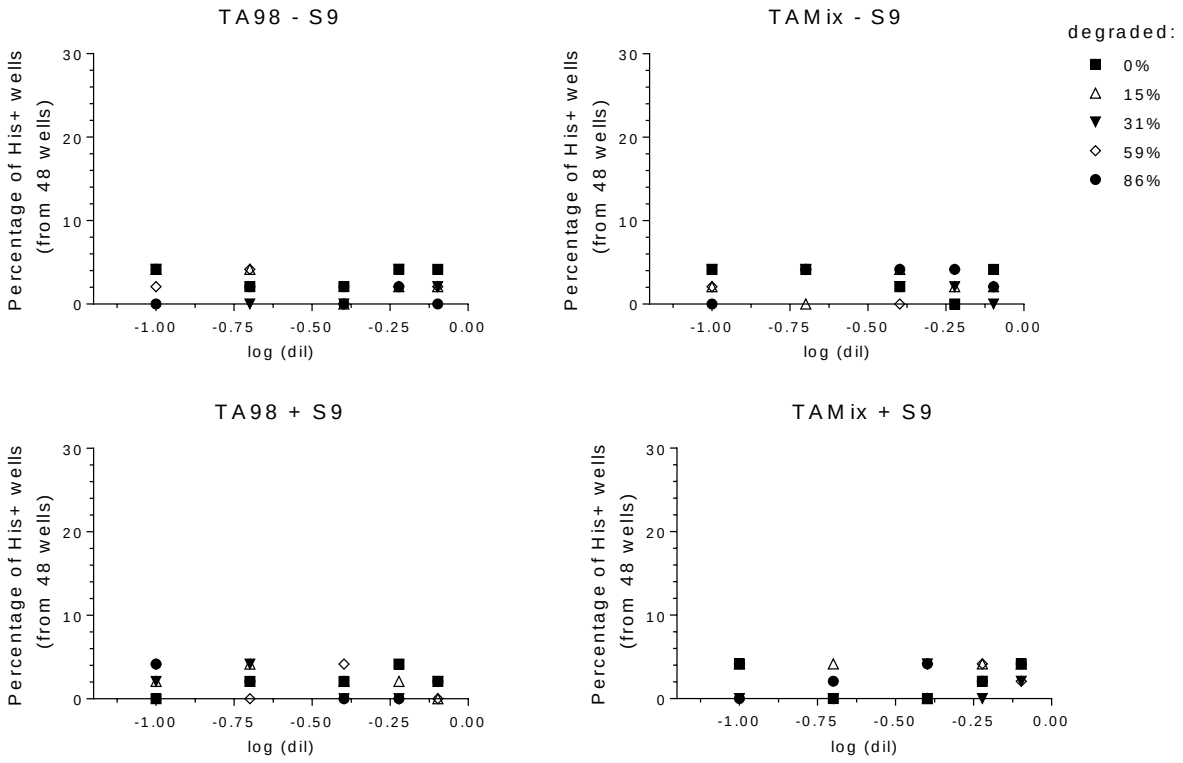


Figure 4.8 Mutagenicity in strains of Ames II during degradation of methyl-tert-butyl ether by hydroxyl radical.

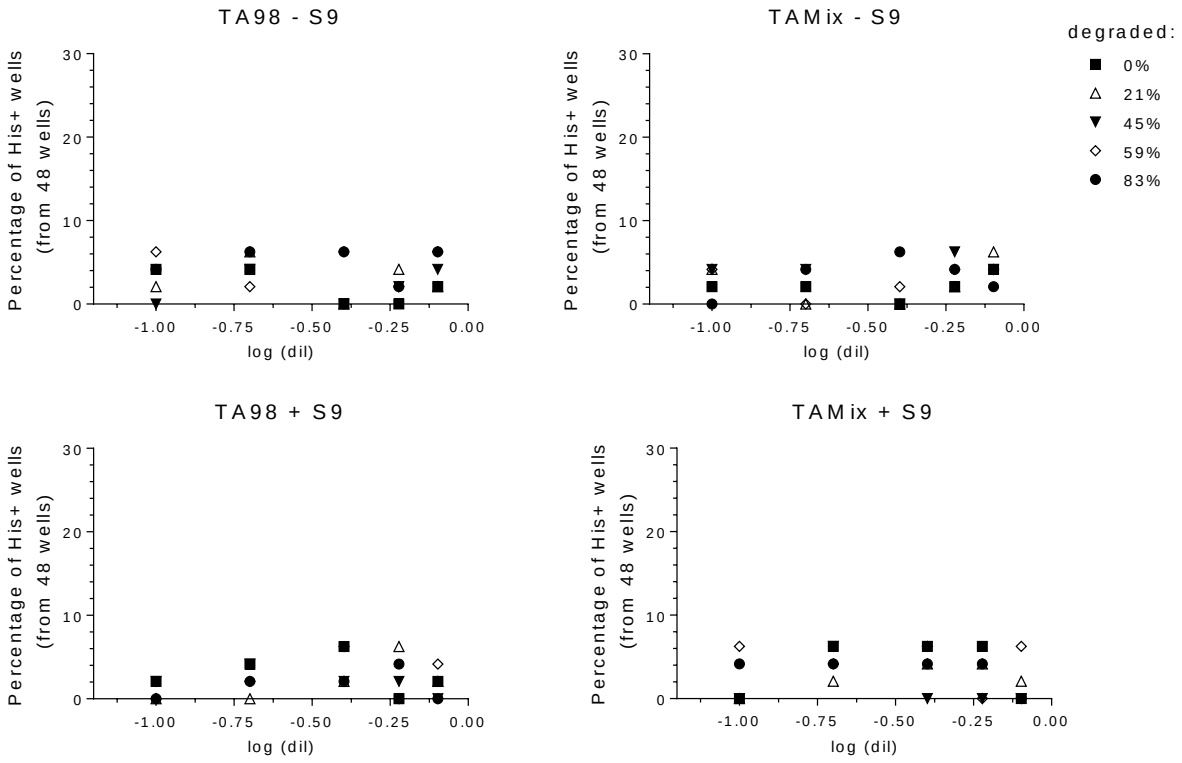


Figure 4.9 Mutagenicity in strains of Ames II during degradation of oxirane, methyl by hydroxyl radical.

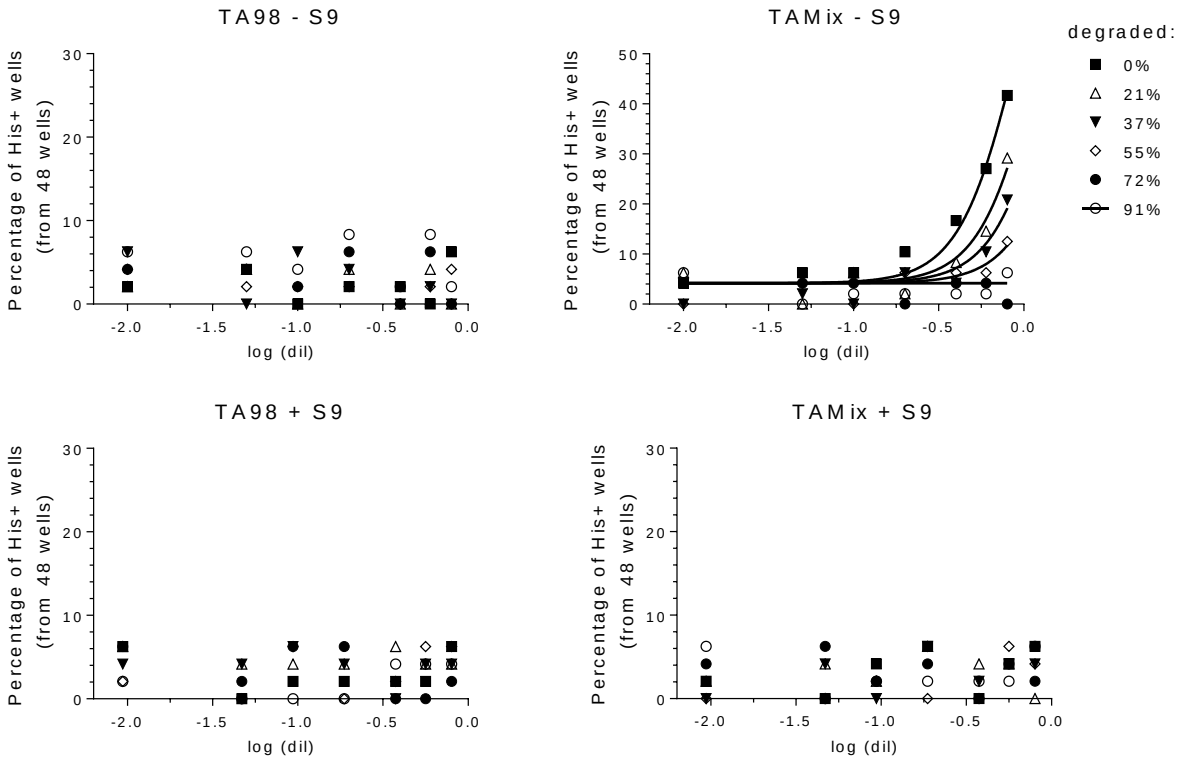


Figure 4.10 Mutagenicity in strains of Ames II during degradation of cumene hydroperoxide by hydroxyl radical.

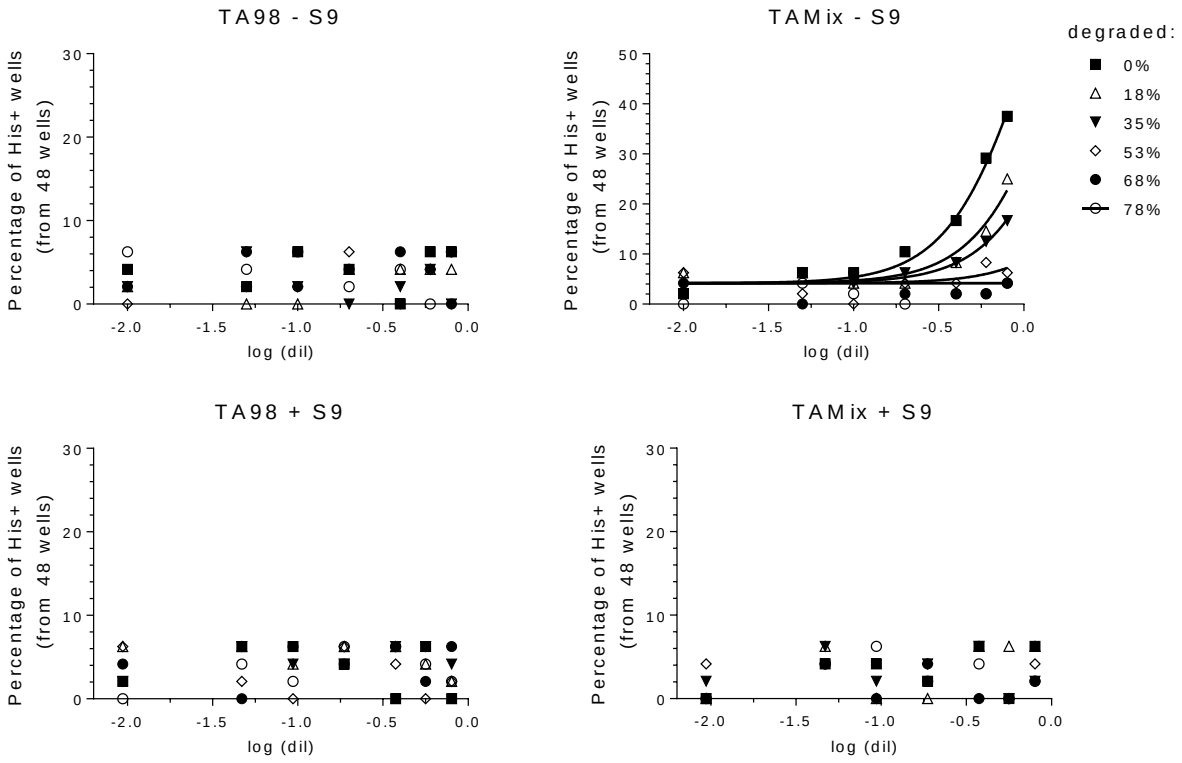


Figure 4.11 Mutagenicity in strains of Ames II during degradation of cumene hydroperoxide by ozone.

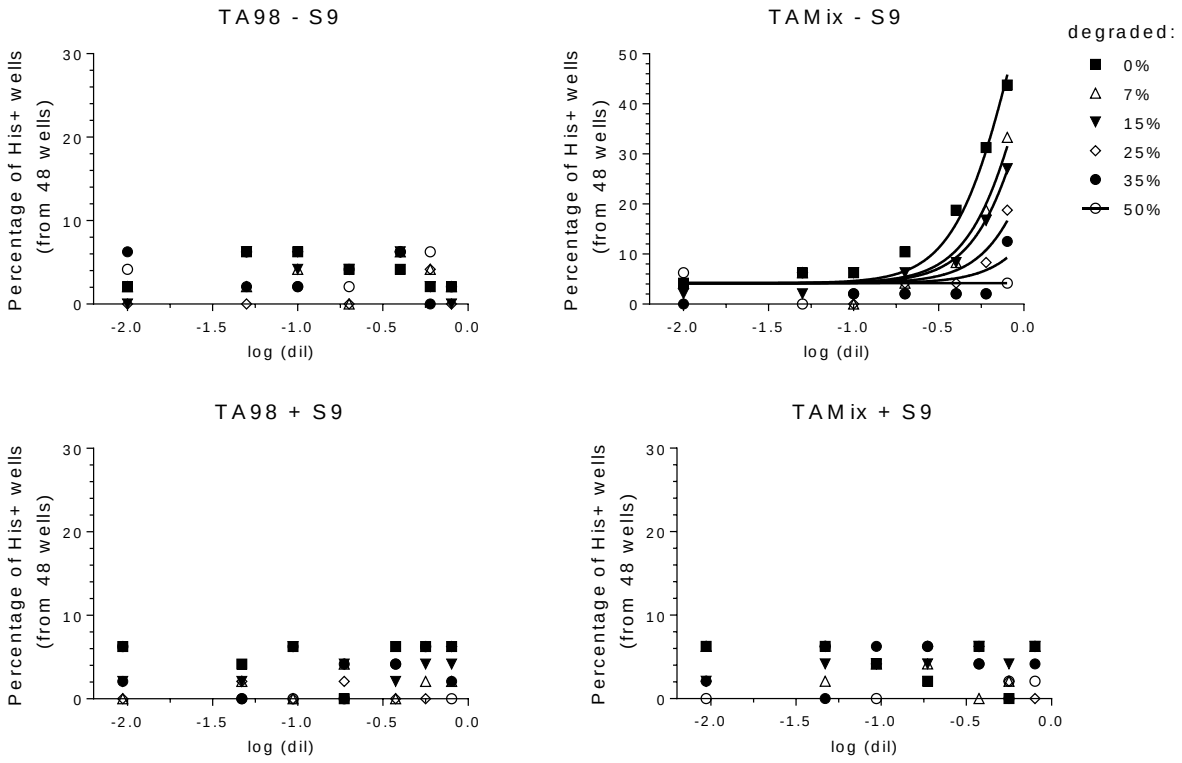


Figure 4.12 Mutagenicity in strains of Ames II during degradation of cumene hydroperoxide by direct photolysis.

4.4.7. **Nitrogen Containing Compounds**

During the direct photodegradation of aniline, degradation products, inducing mutagenicity, in the strain TA98 after metabolization by S9 are formed (Figure 4.13). Quinoline also showed an increase in mutagenic response after ozonation and hydroxyl radical based treatment.

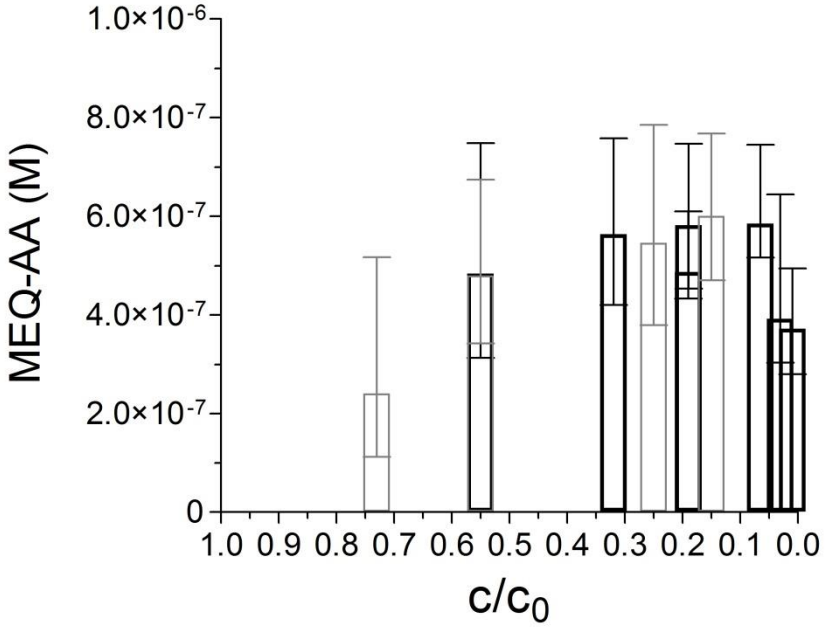


Figure 4.13 Mutagenic Equivalents of aniline during the degradation by hydroxyl radical with decreasing concentration of parent compound corresponding to increasing treatment (E).

The photolysis of aniline is leading to the formation of hydrazobenzene, 4-aminodiphenylamine and 2-aminidiphenylamine, benzidine and azobenzene (Zechner, Prangova et al. 1976). Benzidine has been recognized as a human carcinogen by US EPA and IARC and it was positively tested for mutagenicity in the strain TA98 in the presence of S9 (Sinsheimer, Hooberman et al. 1992; Chung, Chen et al. 2000). Azobenzene is also considered to be a human carcinogen (B2; probable human carcinogen) and tested positive in TA100 +S9 and

hydrazobenzene (Zeiger 1987). However, in this study it did not test positive when tested in the AMES II test with TAMix.

The main degradation products of aniline formed by oxidation with hydroxyl radical and ozone are quite different (benzoquinones, hydroquinone, nitrobenzene, nitrosobenzene, phenol, catechol (Brillas, Mur et al. 1998; Sauleda and Brillas 2001; Huang, Su et al. 2013)). The reaction with ozone is taking place mainly on the aromatic ring, so the yield of such a compound as azobenzene is negligible. The mutagenicity of benzoquinone in the traditional Ames strains is negative or provides an equivocal response (Hakura, Mochida et al. 1995). Nitrobenzene is negative in TA98 and TA100±S9 (Aßmann, Emmrich et al. 1997). Hydroquinone was negatively tested (Kamber, Fluckiger-Isler et al. 2009) and phenol weak or negative (Zeiger 1987; Kamber, Fluckiger-Isler et al. 2009) Catechol (IARC: possibly carcinogenic to humans) was reported non-mutagenic in Ames tests.

During oxidation of Q, mutagenic activity formation was detected in both strains (+/-S9) during oxidation by ozone, but only in the TA98 strain during oxidation by hydroxyl radical. Q itself exhibited a positive response for base-pair substitution in the Ames test in the TA100 strain when activated by S9 (Chen, Yang et al. 2005). However, the mutagenic potency of the parent compound was not detected in any strain used in this study. It seems that with the experimental conditions used in this study, the Ames test is not sensitive enough to detect the mutagenicity of the parent compound. Several possible oxidation products were shown to be mutagenic, *in vitro*. Mutagenic activity was detected in 5-OH-Q and 8-OH-Q, in the TAMix +S9 scenario, confirming results from plate incorporation tests where the mutagenicity of these two hydroxyquinolines were distinctly higher than others (Willems, Dubois et al. 1992). It was previously shown that 5-OH-Q and 8-OH-Q showed mutagenicity in TA98 and TA100 in the

presence of S9 (Willems, Dubois et al. 1992; Chen, Yang et al. 2005; Wang, Hu et al. 2008; Inami, Ishimura et al. 2010; Inami, Miura et al. 2012). The N-oxide of 8-OH-Q was between some others quinolone-N-oxide mutagenic in the TA100+S9 (Willems, Dubois et al. 1992) but no mutagenic activity was found in the previous study where mutagenicity of this compound was observed only in strain TA1538 (-/+S9). Therefore, it is concluded that the oxidation of mutagenic Q by ozone and hydroxyl radicals is leading to the formation of mutagenic degradation products.

4.4.8. Organophosphorous and Nitroso groups

Additional compound groups, namely the organophosphorous compounds and nitroso compounds are being prepared for publication as (Parker, Spangler et al. 2014) and (Mestankova, Bramaz et al. 2014).

4.4.9. Evolution of Estrogenic Activity

Table 4.4 shows the responsive results from the YES assay on the compounds tested. All additional compounds tested (without positive responses) can be found in supporting information as Table 4.9. Only compounds in the nitrogen containing class were responsive to YES bioassay estrogenicity measurements. Only compounds in the nitrogen containing class were responsive to YES bioassay estrogenic activity measurements. Aniline produced a negative response both before and after all treatments, 4,4’-methylenedianiline started with a positive parent compound response and decreased after treatment, and quinoline showed an increase in estrogenic response after being treated by AOP.

Table 4.4 Degradation results of YES with positive responses only in the nitrogen containing compounds group.

	oxidation by OH radical	reaction with ozone	direct photolysis
Nitrogen containing compounds: Anilines, amines, hydrazine, heteroaromatic compounds, isocynate			
Aniline	– → –	– → –	– → –
4,4'-methylenedianiline	+ ↘ -	+ ↘ -	–⊠→
Quinoline	- ↗ +	– → –	– → –

– → – Parent compound activity was negative, and after treatment sample was negative.

–⊠→ Insufficient degradation was achieved through treatment for testing.

+ ↘ - Parent compound activity was positive, and after treatment activity decreased

- ↗ + Parent compound activity was negative, and after treatment activity increased.

4.4.10. **4,4'-methylendianiline (MDA)**

Hamblen et al. (2003) previously found MDA to also have a weak positive response with EC50 1.9×10^{-4} M (using the recombinant yeast assay with 5 days of incubation) (Hamblen, Cronin et al. 2003). MDA, which is structurally related to bisphenol A, shows low estrogen binding affinity (Blair, Fang et al. 2000). The decrease of estrogenic activity of MDA was followed during its degradation with ozone and hydroxyl radicals. To evaluate the evolution of estrogenicity during degradation of compounds, the TEQ concept was applied and reported as estrogenic equivalents (EEQ). The ratio of the EEQ to its initial value (EEQ/EEQ_0) allows for quantitative evaluation during degradation of MDA, as shown in Figure 4.14.

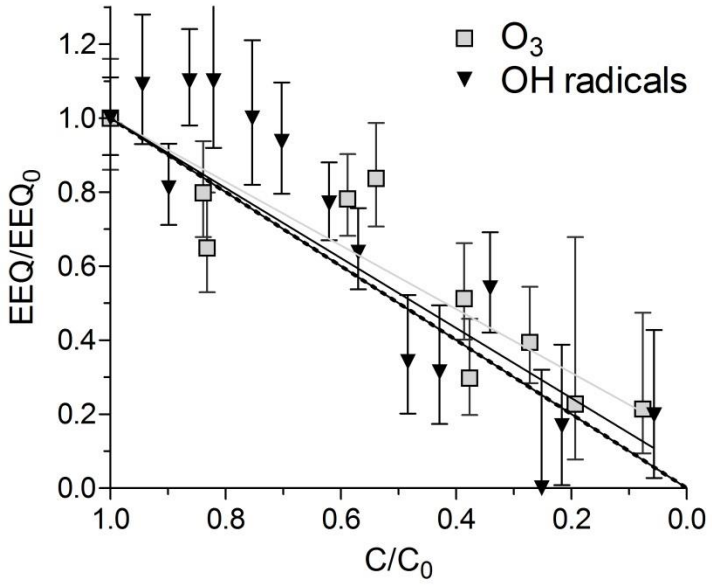


Figure 4.14 Degradation of 4,4'-methylendianiline (MDA) by ozone and hydroxyl radicals, followed in the YES bioassay, and results normalized to express the estrogenicity of the parent compound versus the treated byproducts.

Both processes (ozone and hydroxyl radical) lead to the decrease of the overall estrogenic activity, the EEQ equivalents decrease with decrease of the concentration of MDA.

$EEQ/EEQ_{bioassay,0}$ values lie close to a line with a slope of −1 and an intercept of 1 (note the inverse direction and origin of the x-axis). The slope equal to −1 is an ideal case; a decrease in concentration of MDA results in the corresponding stoichiometric loss of toxicity.

4.4.11. **Quinoline (Q)**

Quinoline (Q) is the only parent compound tested which gives a positive estrogenic response during its oxidation by hydroxyl radicals. The formation of estrogenic activity was confirmed with 2 different experimental set-ups: 1) a medium-pressure mercury lamp (including a band-pass filter in the wavelength range of 308-410 nm) in a solution containing 5 mM H_2O_2 and 2) with a low pressure lamp (emitting almost monochromatic light at 254 nm) with a lower concentration of H_2O_2, 1mM (Figure 4.15).

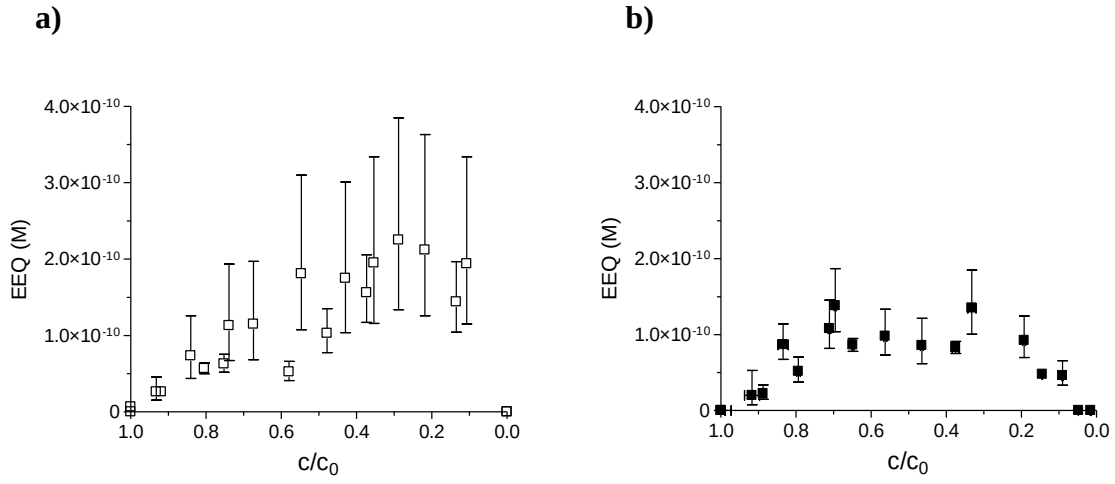


Figure 4.15 Estrogenic activity during degradation of Q by hydroxyl radicals: a) medium-pressure lamp in photoreactor with 5 mM H_2O_2; b) low-pressure lamp with 1 mM H_2O_2.

The formation of the estrogenic activity was also confirmed for a lower concentration of 1 µM, a concentration closer to the environmental conditions. The oxidation of 100 µM Q lead to a maximum estrogenic activity in the samples of approximately 2×10^{-10} M EEQ. Thus, of the concentrations of Q in water bodies are not likely to form a significant estrogenic activity during AOPs. During oxidation of Q by direct reaction with ozone, no estrogenic activity was observed. The direct photolysis of Q (slow process) did not lead to formation of significant estrogenic activity.

The degradation of Q by hydroxyl radical was studied in detail (Zhang, An et al. 2003; Nicolaescu, Wiest et al. 2005; Nicolaescu, Wiest et al. 2005; Lee, Yoon et al. 2007). Hydroxyl radical attack take occurs preferentially on the electron-rich benzene ring, leading to a formation of hydroxylated Q in position 5-7. Further oxidation of these products can lead to the formation of quinolone-5,8-dione. The pyridine ring is less attractive for the OH radical attack, but still occurs, as the products of its hydroxylation are also detected in systems where Q is degraded by OH radicals. The distribution of each degradation product depends on the system used for production of OH radicals. One of the main degradation products, 8-hydroxyquinoline, tested negative for estrogenic activity by the recombinant yeast assay (Miller, Wheals et al. 2001), but exhibited a positive response when tested in combination with other hydroxylated quinolines (6- and 7-OH-Q) for estrogenic activity by the YES 5-day assay (Schultz and Sinks 2002). Hydroxyquinolines with a hydroxyl group on the pyridine ring did not exhibit an estrogenic response. All hydroxyqinolines were tested by our procedure, and estrogenic activity was detected, as seen in

Table 4.5.

Table 4.5 EC50 values of estrogenic hydroxylated quinolines in yeast recombinant assay.

	Our study (M) (95% confidence interval)	5-d EC50 (M) (Schultz and Sinks 2002)
6-OH-Q	**$8.0\ 10^{-4}$** $6.5\text{-}9.7\ 10^{-4}$	$3.4\ 10^{-4}$
7-OH-Q	**$1.4\ 10^{-3}$** $1.1\text{-}1.6\ 10^{-3}$	$6.9\ 10^{-4}$
8-OH-Q	**$6.6\ 10^{-8}$** $3.1\ 10^{-8}\text{-}1.46\ 10^{-7}$	$3.4\ 10^{-7}$

The 8-OH-Q is the most potent from the estrogenic hydroxyquinolines, however, no *in vivo* data on its estrogenicity are available. The absence of the estrogenicity in samples taken during oxidation by ozone can be explained by the difference in the composition of the degradation products. Reaction of Q with ozone takes place on the benzene ring leading to the cleavage of 5,6 or 7,8 bonds (Mochizuki, Irving et al. 1980), and the formation of quinolinic acid, which can be further oxidized to give an N-oxide derivative. A minor ozone attack was observed on the pyridine ring, observed by the formation of N-oxide.

The structure of Q is present in a variety of natural and synthetic biologically active compounds, such as antimalarials and antiseptics (Eicher, Hauptmann et al. 2004). Q is naturally present in coal, petroleum and is or has been used as a solvent and chemical intermediate for the manufacturing of pharmaceuticals and dyes, which can result in relatively high concentrations being present in water bodies.

4.5. Overall Assessments and Conclusions

Advanced oxidation can be recommended for treatment of CCL3 compounds, as only 2 (1,2,3-trichloropropane and nitroglycerin) of the 25 compounds tested in this study contained hydroxyl radical degradation rate constants that were lower than the benchmark set at $5x10^8$ $M^{-1}s^{-1}$. From 45 treatment scenarios tested for mutagenic activity (hydroxyl radical, ozone, and direct photolysis), 9 cases led to the formation of mutagenic activity, meaning that 20% of treatment scenarios resulted in an increase in mutagenic activity.

From 23 compounds, 6 possessing a structural features leading to a formation of degradation products causing mutagenir or estrogenic effect detectable by *in vitro* assay. The most common mutations resulting from all treatment scenarios resulted in the TA98 strain (frameshift mutation). There was no correlation between metabolic activation with these increases in mutagenicity, with equal numbers of mutations happening with and without the addition of S9 enzyme. The most common result was no mutation detected with the parent compound and with the degradation products. Of 42 treatment scenarios tested using the YES bioassay, only 1 treatment resulted in an increase in estrogenic activity. Two scenarios resulted in a decrease in estrogenic activity, and only two parent compounds tested positive for estrogenic activity. It can therefore be concluded that mutagenic formation is more likely than an estrogenic formation (EEQ), regarding the CCL3 compounds tested in this study.

With the low level of toxic activity formation followed by promising treatment by advanced oxidation, it can be concluded that UV and ozone-based advanced oxidation technologies are a good technology for treating emerging contaminants found on the USEPA's CCL3 list.

4.6. Supporting Information

Table 4.6 Classes of compounds found on the CCL3 list and corresponding literature review or rationale regarding treatment by UV and ozone-based AOPs.

Class of compounds	Previous research of compound classes with AOP
Halogenated compounds	These compounds react slowly with ozone and •OH radicals. AOP is only feasible for compounds containing aromatic systems (von Gunten 2003). Perfluorinated compounds react much slower than chlorinated and brominated compounds(Vecitis, Park et al. 2009).
Olefines	Compounds are mostly fast reacting with ozone and •OH radicals. For this class of compounds, an ozone-based process would have a real advantage over other AOPs. The attack of the compound by ozone leads to a cleavage of the double bond and typically to smaller fragments, which are oxygen-rich and likely more biodegradable. The cyanotoxins have already been investigated in much detail in previous studies (Onstad 2005).
Nitro compounds	These compounds have reasonable rate constants for the reaction with •OH radicals, but only react slowly with ozone (see e.g. nitrobenzene). After reaction with •OH radicals, they will typically maintain some of the original structural properties, which give rise to their original toxicity. The hydroxylation of the aromatic ring yielding nitrophenolic compounds is the most important degradation pathway of this class of compounds. Selected compounds from this class include nitrobenzene and nitroglycerin.
Ethers, alcohols, phenols, aldehydes and other oxygen containing compounds	These compounds have moderate to fast rate constants with •OH radicals. With the exception of butylated hydroxyanisole (a phenol) they react typically slowly with ozone. Ethylene oxide reacts too slowly with •OH radicals to be treated with AOPs. Some of the smaller molecules are either directly biodegradable (methanol, formaldehyde) or their reaction products will be more biodegradable. MTBE has already been studied in much detail (Schmidt 2008). Among others 1,4-dioxane was selected from this class to demonstrate the effect of biological post-filtration on toxicology of degradation products. The degradation of this compound by hydroxyl radical leads to formation of primary products such as formaldehyde, methoxyacetic acid and a number of esters(Stefan and Bolton 1998).

Nitrogen containing compounds	All the compounds in this class have a high reactivity with •OH radicals; anilines and amines also react fast with ozone. In many cases, the attack occurs on the nitrogen, leading to nitrogen oxide, nitro- or nitroso-compounds.
Organophosphorous compounds	Only little kinetic information is available for this class of compounds. For a few compounds, rate constants for the reaction with •OH radicals are very high. Therefore, AOPs are suitable for the mitigation of this class of compounds. Because of the complicated structures of the molecules, it is likely that some of the fragments still have similar properties as the original compounds. The metabolites of fenamiphos, sulfoxide and sulfone were reported to be more potent inhibitors of nematode's AChE than fenamiphos itself; meaning that the toxicity after oxidation should be followed (Nordmeyer and Dickson 1990). Therefore, compounds from this class were selected for further testing.
Amides	With the exception of acetamide, all the compounds in this class react moderate to fast with •OH radicals. The reactivity with ozone is typically small. AOPs are feasible for the mitigation of these compounds. Acetanilide pesticides (alachlor, acetochlor and metalochlor) are often listed as probable or possible carcinogens to humans. Their metabolites, aniline compounds, also exhibit genotoxic activity (Osano, Admiraal et al. 2002). The estrogenic activity of this group is also of interest, as some of them possess estrogenic and/or anti-androgenic activity.
Nitroso compounds	The reactivity of these compounds towards •OH radicals increases with molecular weight. The smallest compound by molecular weight (NDMA) has low reactivity with •OH radicals, however, it can readily be photolyzed. Therefore, UV-based AOPs are more promising in this case. Nitroso compounds and especially nitrosoamines are of interest due to their probable carcinogenic activity. An attack of hydroxyl radicals can occur on the alkyl moieties and shorten the aliphatic chain. Since the carcinogenic activity of nitroso compounds increases with a decrease in aliphatic chain length, more toxic compounds can possibly be formed due to degradation by •OH radicals.
Aliphatic and aromatic compounds	The rate of the reaction of these compounds with •OH radicals is high. More oxygen rich compounds can be expected from the reaction with •OH radicals, which are generally more biodegradable and likely less toxic (von Gunten 2003; Hammes, Salhi et al. 2006; Ramseier and von Gunten 2009).
Metallorganic compounds	These compounds are expected to react readily with •OH radicals, but have high octanol-water partition coefficients.

Table 4.7 Analytical methods used for the identification of CCL3 compounds.

Compound	CAS #	Grade, Supplier	Column	Analytical Method Used
Group 1. Halogenated compounds				
1,2,3-trichloropropane	96-18-4	Analytical Standard, Sigma-Aldrich	Agilent DB-624	GC-MS: EPA Standard Method 504.1
Group 2. Olefines				
Dimethipin	55290-64-7	Analytical Standard, Sigma-Aldrich	Agilent DB-624	GC-MS: EPA Standard Method 525.3
Group 3. Nitro Compounds				
Nitrobenzene	98-95-3	PESTANAL® analytical standard, Sigma-Aldrich	Nucleodur C8 Gravity	HPLC: 35% acetonitrile 65% water, 265 nm
Group 4. Ethers, Alcohols, phenols, aldehydes and other oxygen containing compounds				
1,4-Dioxane	123-91-1	Analytical Standard, Sigma-Aldrich	Agilent DB-624	GC-MS: EPA Standard Method 522
Oxirane, methyl	75-56-9	Analytical Standard, Sigma-Aldrich	Agilent DB-624	GC-MS: CDC Standard method 1612
Methyl-tert-butyl ether	1634-04-4	Analytical Standard, Sigma-Aldrich	Agilent DB-624	GC-MS: EPA Standard Method 8020
Cumene hydroperoxide	80-15-9	Analytical Standard, Sigma-Aldrich	Agilent DB-624	GC-MS: EPA Standard Method 8260B
Group 5. Nitrogen containing compounds				
4,4' Methylenedianiline	101-77-9	Analytical Standard, Sigma-Aldrich	Lichrosphere 100Ò column RP-18 5	HPLC: 30% acetonitrile 70% phosphate buffer pH7 5 mM, 243 nm
Aniline	62-53-3	Analytical Standard, Sigma-Aldrich	Lichrosphere 100Ò column RP-18 5	HPLC: 15% acetonitrile 85% phosphate buffer pH 7 25 mM, 230 nm

Quinoline	91-22-5	Analytical Standard, Sigma-Aldrich	Lichrospher e 100Ò column RP-18 5	HPLC: 40% methanole 60% water, 225 nm
Group 6. Organophosphorous compounds				
Acephate	30560-19-1	Analytical Standard, Sigma-Aldrich	Agilent Eclipse XDB-C_8	HPLC: t=0 70% water + formic acid, 30% acetonitrile, t=5 30% water + formic acid, 70% acetonitrile, t=7 100% acetonitrile (EPA standard method 538)
Dicrotophos	141-66-2	Analytical Standard, Sigma-Aldrich	Agilent Eclipse XDB-C_8	HPLC: t=0 70% water + formic acid, 30% acetonitrile, t=5 30% water + formic acid, 70% acetonitrile, t=7 100% acetonitrile (EPA standard method 538)
Methamidophos	10265-92-6	Analytical Standard, Sigma-Aldrich	Agilent Eclipse XDB-C_8	HPLC: t=0 70% water + formic acid, 30% acetonitrile, t=5 30% water + formic acid, 70% acetonitrile, t=7 100% acetonitrile (EPA standard method 538)
Fenamiphos	22224-92-6	Analytical Standard, Sigma-Aldrich	Agilent Eclipse XDB-C_8	HPLC: t=0 70% water + formic acid, 30% acetonitrile, t=5 30% water + formic acid, 70% acetonitrile, t=7 100% acetonitrile (EPA standard method 538)
Group 7. Amides				
Metolachlor	51218-45-2	PESTANAL®, analytical standard, Sigma-Aldrich	Lichrospher e 100Ò column RP-18 5	HPLC: 50% acetonitrile 50% water, 215 nm
Metolachlor ethanesulfonic acid (ESA)	171118-09-5	PESTANAL®, analytical	Lichrospher e 100Ò	HPLC: t=0-4min 10% acetonitrile 90% phosphate buffer pH 7, t =

		standard, Sigma-Aldrich	column RP-18 5	11 min 40% acetonitrile, 215 nm
Metolachlor oxanilic acid (OA)	152019-73-3	PESTANAL®, analytical standard, Sigma-Aldrich	Lichrospher e 100Ò column RP-18 5	HPLC: t=0-4min 10% acetonitrile 90% phosphate buffer pH 7, t = 11 min 40% acetonitrile 60% phosphate buffer pH 7, 215 nm
Molinate	2212-67-1	PESTANAL®, analytical standard, Sigma-Aldrich	Lichrospher e 100Ò column RP-18 5	HPLC: 50% acetonitrile 50% water, 210 nm
N-Methyl-2-pyrrolidone	872-50-4	PESTANAL®, analytical standard, Sigma-Aldrich	Lichrospher e 100Ò column RP-18 5	HPLC: 15% methanol 85% water, 210 nm
Group 8. Nitroso compounds				
N–nitroso-diethylamine (NDEA)	55-18-5	Supelco analytical standard, Sigma-Aldrich	Nucleosil 100-5-C18 EC250/4.6	HPLC: 30% methanol: 70% 5mM phosphate buffer pH7, 230 nm
N- nitroso-dimethylamine (NDMA)	62-75-9	Supelco analytical standard, Sigma-Aldrich	Nucleosil 100-5-C18 EC250/4.6	HPLC: t= 0-4 min:10% methanole 90% phosphate buffer pH 7 t=6 min 90% methanol, 230 nm
N-nitroso-di-n-propylamine	621-64-7	Supelco analytical standard, Sigma-Aldrich	Nucleosil 100-5-C18 EC250/4.6	HPLC: t= 0-2 min:10% methanole 90% phosphate buffer pH 7 t=5-10 min 90% methanol, 230 nm
N- Nitroso-diphenylamine	86-30-6	Supelco analytical standard, Sigma-Aldrich	Lichrospher e 100Ò column RP-18 5	HPLC: 50% acetonitrile 50% water, 230 nm
N- nitroso-pyrrolidine (NPYR)	930-55-2	99%, Sigma-Aldrich	Nucleosil 100-5-C18 EC250/4.6	HPLC: t= 0-2 min:10% methanole 90% phosphate buffer pH 7 t=5-10 min 90% methanol, 230 nm

Table 4.8 Mutagenic and Estrogenic activity of parent compounds.

	Mutagenic Activity				Estrogenic Activity
strains:	TA98		TAMix		
metabolic activation:	-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: Anilines, amines, hydrazine, heteroaromatic compounds, isocynate					
Aniline	-	-	-	-	-
4,4'-methylenedianiline	-	-	-	-	+
Quinoline	-	-	-	-	-
6. Organo-phosphorous compounds					
Acephate	+?	-	-	-	-
Dicrotophos	-	-	+?	-	-
Methamidophos	-	+	-	-	-
Fenamiphos	-	-	+	-	+?
7. Amides					
Metolachlor	-	-	-	-	-
Metolachlor ethanesulfonic acid	-	-	-	-	-
Metolachlor oxanilic acid	-	-	-	-	-
2-methyl-2-pyrrolidone	-	-	-	-	-
Molinate	-	-	-	-	-
8. Nitroso compounds					
N-nitrosodimethylamine	-	-	-	-	NT
N-nitrosodiethylamine	-	-	-	-	NT
N-nitrosodi-n-propylamine	-	-	-	-	NT
N-nitrosopyrrolidine	-	-	-	-	-
N-nitrosodiphenylamine	-	-	-	-	-

NT Not Tested.
\+ Positive activity observed.
\- Negative or no activity observed.
+? Weak activity observed.

Table 4.9 Treatment of CCL3 compounds by OH radical, ozone, and direct photolysis and the corresponding estrogenicity results as measured in the YES assay.

	oxidation by OH radical	reaction with ozone	direct photolysis
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: Anilines, amines, hydrazine, heteroaromatic compounds, isocynate			
Aniline	– → –	– → –	– → –
4,4'-methylenedianiline	+ ↘ -	+ ↘ -	–⊠→
Quinoline	- ↗ +	– → –	– → –
6. Organo-phosphorous compounds			
Acephate	– → –	–⊠→	–⊠→
Dicrotophos	– → –	–⊠→	–⊠→
Methamidophos	– → –	–⊠→	–⊠→
Fenamiphos	– → –	– → –	– → –
7. Amides			
Metolachlor	– → –	–⊠→	– → –
Metolachlor ethanesulfonic acid	– → –	–⊠→	– → –
Metolachlor oxanilic acid	– → –	–⊠→	– → –
2-methyl-2-pyrrolidone	– → –	– → –	– → –
Molinate	– → –	– → –	– → –
8. Nitroso compounds			
N-nitrosopyrrolidine	– → –	–⊠→	– → –
N-nitrosodiphenylamine	– → –	– → –	– → –

– → – Parent compound activity was negative, and after treatment sample was negative.

–⊠→ Insufficient degradation was achieved through treatment for testing.

+ ↘ - Parent compound activity was positive, and after treatment activity decreased proportional to parent compound.

+↘ -? Parent compound displayed weak activity, and activity decreased with treatment, but was too weak to conclusively determine.

- ↗ + Parent compound activity was negative, and after treatment activity increased.

5. Exploring the Formation of AOC and the Use of Biofiltration Downstream of AOP

*All biofiltration laboratory experiments were completed by and partially published in Kate Dowdell's M.S. thesis (Dowdell 2013).

5.1. Introduction

Treatment of drinking water with ozone and AOPs results in the formation of lower molecular weight organic by-products from the partial degradation of natural organic matter (Andrews and Huck 1994; Hammes, Salhi et al. 2006). These compounds are easily utilizable by microorganisms and can result in the biological instability of the water. The biologically unstable compounds can be measured as a bulk parameter such as the assimilable organic carbon (AOC) or biodegradable organic carbon (BDOC). In order to cope with the AOC or BDOC, ozonation or AOPs are typically followed by a biological filtration step (Urfer, Huck et al. 1997; Hammes, Salhi et al. 2006; Kruithof, Kamp et al. 2007; Toor and Mohseni 2007; Sarathy and Mohseni 2009). Whereas in the AOP O_3/H_2O_2, hydrogen peroxide is used up in the course of the process, in the AOP UV/H_2O_2, a high residual hydrogen peroxide concentration remains, which can be removed by biological processes such as biologically activated carbon filtration (Urfer, Huck et al. 1997; Kruithof, Kamp et al. 2007).

5.1.1. Biological Filtration and Micropollutant Removal

Biological filtration processes are typically not optimized for micropollutant removal. It has been shown in several studies, that mainly natural compounds such as taste and odor compounds or cyanotoxins can be removed by a biological filtration processes (Elhadi, Huck et al. 2006; Ho, Hoefel et al. 2007). In the context of advanced oxidation followed by biofiltration, it is unclear whether degradation products of partially oxidized micropollutants are further degraded by

biological processes. It has been shown, that the presence of biodegradable organic matter such as AOC or BDOC can assist biodegradation of micropollutants in water treatment systems (Elhadi, Huck et al. 2006) and riverbank filtration (Grunheid, Amy et al. 2005) or rapid sand filtration after ozonation of secondary wastewater effluent. Based on the expected transformation products from ozonation and AOPs, namely, a higher oxygen content (carboxylic groups, aldehyde groups, keto groups, (Huber, Ternes et al. 2004; Ramseier and von Gunten 2009)) and/or smaller molecules (Sein, Zedda et al. 2008), it can be expected that these compounds are more biodegradable.

This theory was tested by utilizing methamidophos, which contained known mutagenic byproducts, from previous investigation (Chapter 3). The removal of toxicity following methamidophos byproducts was tested using the AMES assay, and biological sand and GAC filtration columns.

5.1.2. **Assimilable Organic Carbon Formation Following UV-AOP**

Formation of AOC is related to the nature and concentration of organics in the source water and can be effected by the water treatment processes employed. When treatment plant effluent AOC concentrations exceed 100 µg/L, in the presence of a secondary disinfectant, biological regrowth may occur in the distribution system (Volk and LeChevallier 2000). Carbon is believed to typically be the limiting nutrient for biological growth in distribution systems, especially with surface waters (Camper 2000). AOC is a known byproduct for ozone and ozone AOPs (Hammes, Salhi et al. 2006). However, less is known about AOC formation with UV AOPs. This research as well as other research has shown AOC formation following UV AOP can be minimal (Collins, Cotton et al. 2009). However, research has only been done on a limited basis and AOC formation with UV AOP may be possible depending on the organic matter composition of the

water matrix. When organic matter concentrations are low (e.g., groundwater), AOC formation has been shown to be limited to less than 100 μg/L with both UV and ozone AOPs (Collins, Cotton et al. 2011). Thus, the formation of AOC will depend on the treatment technology selected and the nature and concentration of the organic matter. AOC is not a regulated contaminant and the effect of increased AOC formation will be site specific and can depend on site specific characteristics such as background AOC levels, secondary disinfectant, and distribution system pipe materials.

The impact of AOC formation following UV AOP processes was investigated in this chapter. Both low and medium pressure bench scale UV experiments were performed along with low (LP) and medium pressure (MP) UV-H_2O_2 exposures. Natural water samples provided from a utility partner (utility A) currently operating a UV-H_2O_2 treatment process were used to measure the change in AOC concentration in a field operating system.

5.2. Methods

5.2.1. Biological Filtration Design

Biological sand media and influent water were shipped to the University of Colorado from project partner Greater Cincinnati Water Works in Cincinnati, OH to be used in biofiltration columns. Because it is desired that the peroxide residual following AOP is quenched before the biofiltration step, bovine catalase experiments, spiking catalase at 300 ug/L with peroxide at 4 mg/L were investigated as to the impact on the biological activity (phospholipid concentration) of the biological media.

Hydrogen peroxide quenching studies using a sacrificial granular activated carbon (GAC) pre-filter with a high flow rate were also studied, but showed results that were not promising in

both hydrogen peroxide quenching and excess contaminant removal. The studies in question, utilized bovine catalase which showed little effect on the biomass concentration to quench residual hydrogen peroxide prior to dosing with UV-AOP treated contaminant, as seen in Figure 5.1.

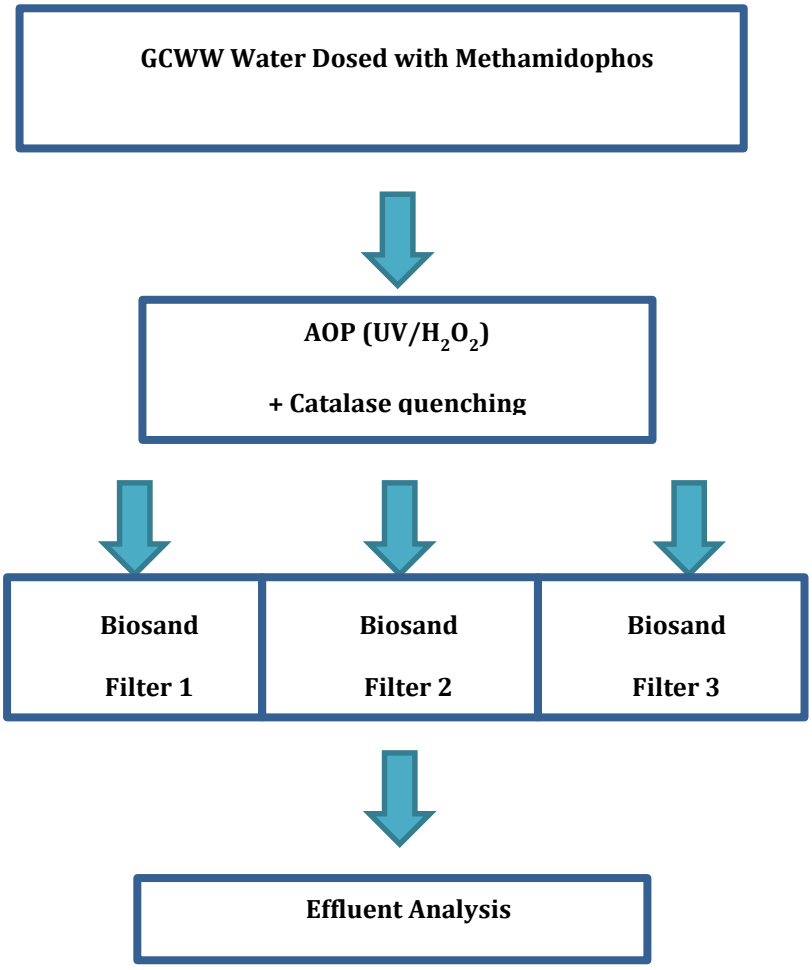


Figure 5.1 Experimental process diagram for biofiltration experiments from UV-AOP treatment through effluent analysis, including a sacrificial GAC column (Dowdell 2013)

The issue of biosand filter sensitivity to peroxide was addressed before this test began. Although data and literature are surfacing that show increased performance of biofilters in the

presence of residual peroxide (WaterRF 2013), our preliminary column testing revealed that sand biofilters with typical biomass concentrations (40 nmol PO_4/ dry gram media) experienced a 40 percent reduction in viable biomass when receiving peroxide influents as low as 1 mg/L with an empty bed contact time (EBCT) of 7.5 minutes. It was therefore necessary to develop a method of fully quenching the peroxide to prevent the biosand columns from being metabolically inhibited or suffering a decrease in microbial populations. Initially, attempts were made to use a GAC pre-filter to remove the peroxide. However, with adsorption losses of methamidophos parent compound as high as 20 percent, and peroxide quenching of only 20 to 30 percent, it was determined that the methamidophos losses outweighed the pre-filter efficacy of peroxide removal.

The best solution for quenching peroxide was found to be the addition of bovine catalase. There were concerns as to the impact of bovine catalase on the sand biofilters. Currently there is little data available on the impact of catalase on sand biofilters. At the same time, there were also concerns that the chosen influent concentration of methamidophos could negatively impact the microorganisms in the filters. A preliminary column test was designed to answer the following questions:

1. Does the use of bovine catalase to quench peroxide negatively impact biomass viability and metabolic processes?
2. Will biomass be negatively impacted by the influent of 100 ug/L methamidophos and daughter products resulting from UV/H_2O_2 processes?

5.2.2. **Biological Filtration Column Test**

A five column flow-through test was designed to examine the effects of bovine catalase, methamidophos, and methamidophos daughter products on the sand biofilter media. The test was divided into two, four hour segments. Part one of the test used water that was not treated with UV-AOP, and part two used UV-AOP treated water. The five columns used for this test were all fed an influent of dechlorinated tap water dosed with a TOC concentration of approximately 2 mg/L using an organic matter stock solution collected in Big Elk Meadows, Colorado and no UV-AOP treatment. Detailed influent water matrices compositions can be found in Table 5.1.

Table 5.1 Column test identification and influent composition of waters

Column	Influent Composition
1. Control Column	2 mg/L TOC
2. Catalase Control Column	2 mg/L TOC, 0.8 mg/L bovine catalase
3. Peroxide Column	2 mg/L TOC, 3.0 mg/L peroxide
4. Quenched Peroxide Column	2 mg/L TOC, 2.9 mg/L peroxide, 0.8 mg/L bovine catalase
5. Methamidophos Column	2 mg/L TOC, 3.0 mg/L peroxide, 0.8 mg/L bovine catalase, 0.1 mg/L methamidophos

A dose of 0.8 mg/L of bovine catalase was used because preliminary testing indicated that this was the approximate dose required for full quenching of peroxide at 3 mg/L. Testing of the influents for Columns 4 and 5 indicated that this dose fully quenched peroxide in the column influents. A peroxide dose of 3 mg/L was chosen because this was the expected peroxide concentration of column influent post-UV AOP treatment when the water is initially dosed with 4 mg/L before UV-AOP treatment. The methamidophos concentration of 0.1 mg/L was chosen because this will be the concentration of the parent compound and daughter products in the column influent after AOP treatment to 90 percent removal when initially dosed at 1 mg/L.

The columns were fed the influent water for 4 hours using a single-pass, pump-driven system. After four hours, the effluent was collected for TOC analysis and a small amount of sand was removed from the top of each column for biomass analysis. A sample of the media used to pack the columns was also analyzed for biomass for use as a baseline value.

5.2.3. Assimilable Organic Carbon Measurements

Laboratory grade water spiked with an acetate carbon solution, and previously characterized Suwanee River Natural Organic Matter (SRNOM) was used to simulate natural water for laboratory use. AOC was measured by utilizing the American Water method that involves the use of a luminescence plate reader and the bioluminescent bacterium NOX and P-17 (Weinrich, Giraldo et al. 2009). The NOX and P-17 bacterium chosen in the development of this assay are representative of the heterotrophic bacterial population that can utilize AOC to meet their nutritional requirements (Kooij 1992). This assay provides an indication of heterotrophic bacterial regrowth potential in the distribution systems. The experiments in this chapter were designed to test the heterotrophic regrowth potential following direct UV and UV-AOP systems with varying doses of both UV light and hydroxyl radicals (i.e. hydrogen peroxide concentration). Experimental dosing parameters can be seen presented as Table 5.2.

Table 5.2 Experimental matrix for monitoring low and medium pressure UV and UV-AOP experiments

Sample	LP UV Doses (mJ/cm^2)	MP UV Doses (mJ/cm^2)	H_2O_2 concentration (mg/L)
Natural Water	0, 100, 250, 500, 750, 1000	0, 100, 250, 500, 750, 1000	0, 5, 10, 15
DI + SRNOM	0, 100, 250, 500, 750, 1000	0, 100, 250, 500, 750, 1000	0, 5, 10, 15

Both LP (low pressure - monochromatic light) and MP (medium pressure –polychromatic light) exposures were conducted according to Bolton and Linden 2003 (Bolton and Linden

2003), with a collimated beam bench scale setup. The matrices were sampled and plated in duplicate and measured for luminescence for 1 to 3 hours in order to calculate the change in AOC concentration.

5.3. Results and Discussion

5.3.1. TOC and Biomass Analysis

The purpose of measuring TOC was to ensure that the metabolic processes in the columns were not limited or increased by the catalase, methamidophos, or daughter products. The purpose of biomass analysis was to ensure that biomass viability was not affected as a result of the influent composition. Results showed that there was little variation between the TOC removals in the control columns and those dosed with methamidophos and catalase, and variability fell within the error of the instrument. These results indicate that there were no short-term decreases in metabolic activity in the columns due to the influents.

The biomass results also indicated that compounds in the influent did not decrease viable cell populations. Table 5.3 contains the results of biomass analysis. The chemical extraction method used to measure biomass was developed by Wang et. al. (1995) (Wang, Miltner et al. 1995).

Table 5.3 Biomass analysis results from column test showing biomass concentration as a result of catalase, peroxide, and methamidophos influents

Column	Biomass Concentration (nmol PO_4/g dry media)	Percent Change in Biomass v. Control	95 Percent Confidence Interval (nmol PO_4/g dry media)
Part One			
Pre-Test Media	65	----	±6
Column 1: Control	84	----	±11
Column 2: Catalase Control	78	-7%	±5
Column 3: Peroxide	68	-19%	±4
Column 4: Quenched Peroxide	69	-17%	±18
Column 5: Methamidophos	81	-3%	±24
Part Two			
Column 1: UV Control	58	----	±8
Column 5: UV Methamidophos	76	+31%	±30

Results presented in Table 5.3 show that the media was not significantly harmed by the influent compounds in question. The columns of greatest interest, Columns 2 and 5, were not impacted in a statistically significant way by the catalase or pesticide compounds. Therefore, it was concluded that the three week, four column methamidophos test could proceed as designed.

5.3.2. AMES Bioassay Analysis: biofiltration following UV-AOP treated methamidophos

The toxicity of 90% treated UV-AOP methamidophos was followed through the biofiltration columns (both sand and GAC), and showed an increase in toxicity (base pair mutation in the presence of S9 enzyme) following biological sand filtration (Figure 5.2). The non-acclimated sand columns showed complete removal of toxicity from the influent degraded methamidophos.

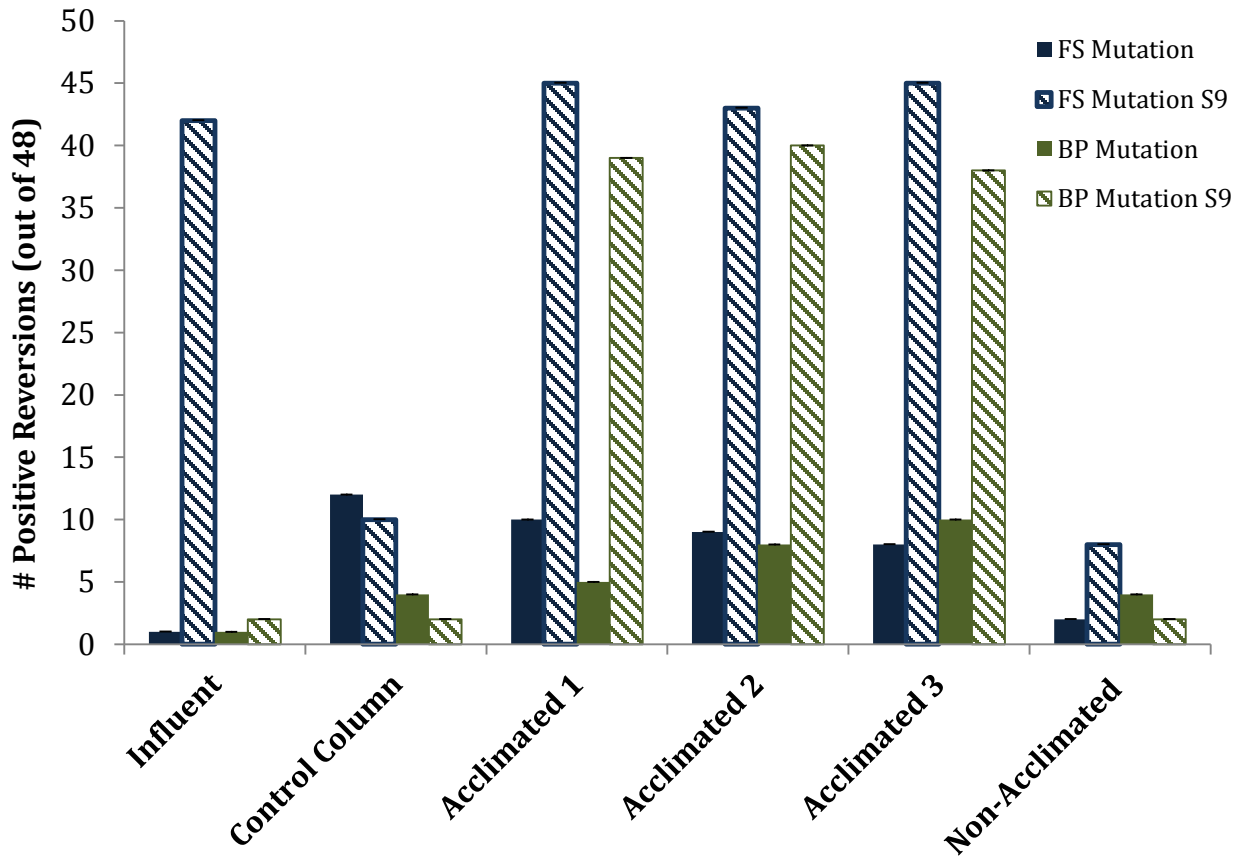


Figure 5.2 AMES results of 90% UV-AOP treated methamidophos using biological sand filtration

Experiments were repeated using biological granular activated carbon (GAC) with the same experimental design as the biological sand experiment. Biological GAC removed all of the measurable toxicity of methamidophos for all 4 types of mutations measured (Figure 5.3).

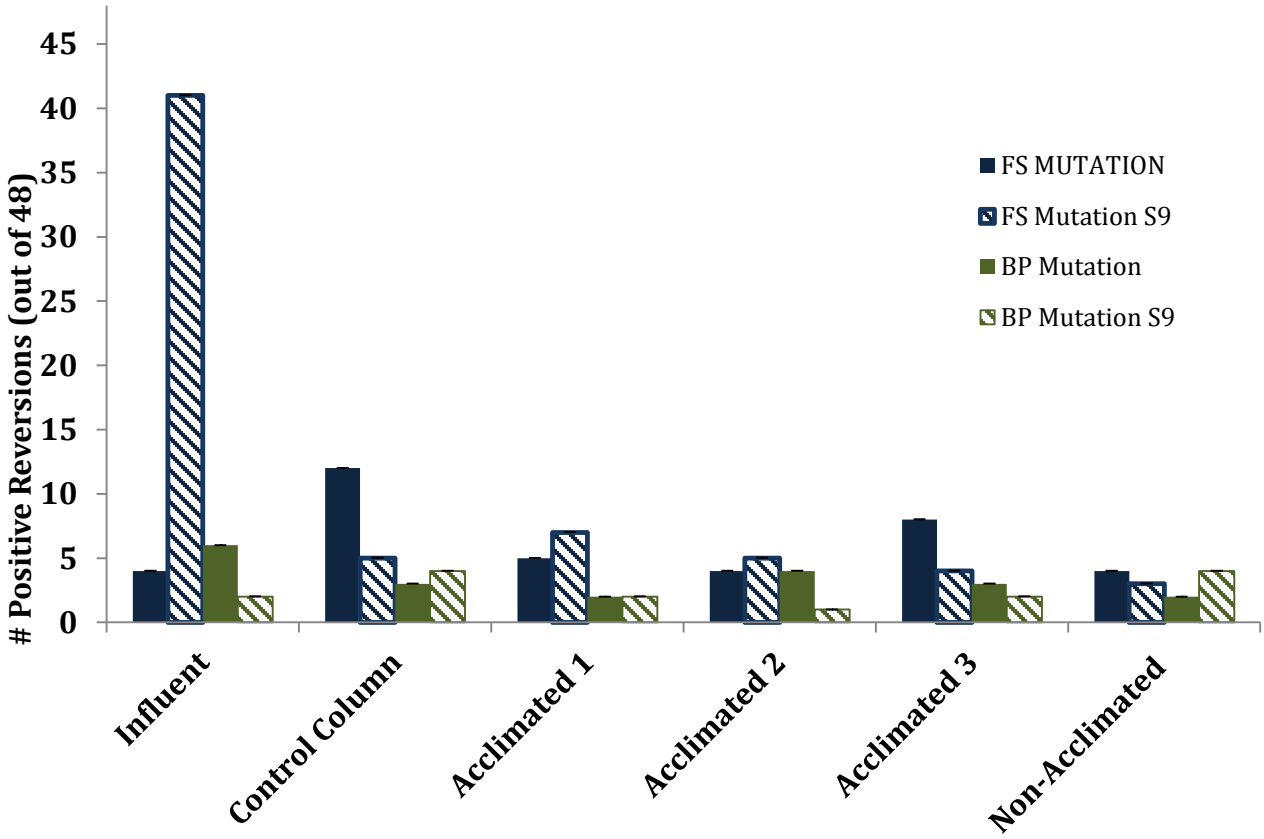


Figure 5.3 AMES results of 90% UV-AOP treated methamidophos using biological GAC filtration

5.3.3. Assimilable Organic Carbon Formation Results

As a first step in the bioluminescence assay validation, a correlation between the luminescence reading in relative light units (RLU) and the concentration of acetate carbon stock was made (Figure 5.4). This correlation provided a calibration curve that was used to interpret luminescence data from natural water samples.

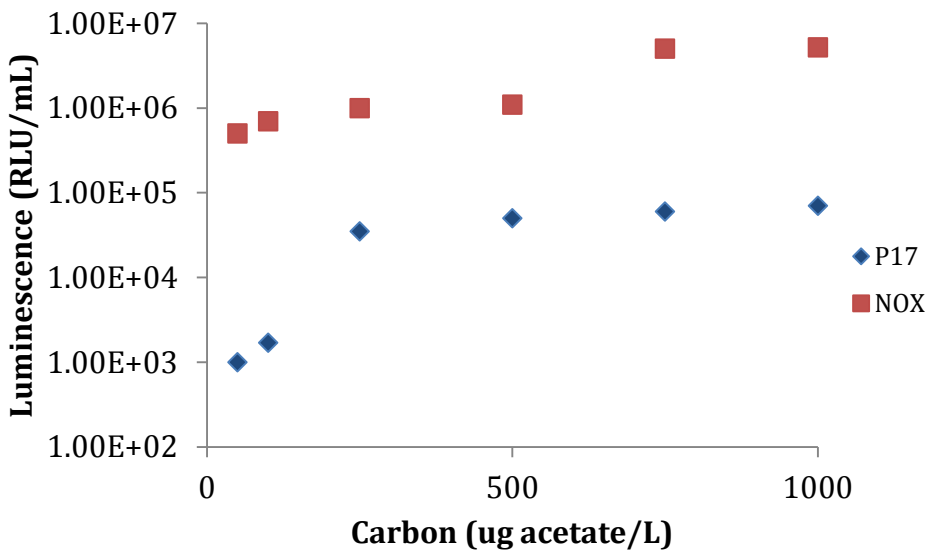


Figure 5.4 Assimilable Organic Carbon (AOC) validation of P17 and NOX bacterial response to acetate carbon concentrations, measured by luminescent response

No significant increase in AOC formation was seen following the degradation of the natural water tested with UV photolysis at 254 nm (low pressure) and low pressure AOP at varying doses of hydrogen peroxide, Figure 5.5. Polychromatic photolysis (medium pressure) showed a small correlation between high doses of UV and a slightly higher AOC formation rate with hydroxyl radical exposure; however, this did not correlate to the amount of hydroxyl radicals present, as shown in Figure 5.6. At higher doses of hydrogen peroxide, no significant AOC formation was observed compared to the lower dose of 5 mg/L. It appears that hydroxyl radical exposure alone in combination with polychromatic UV light shows a correlation towards increased AOC formation.

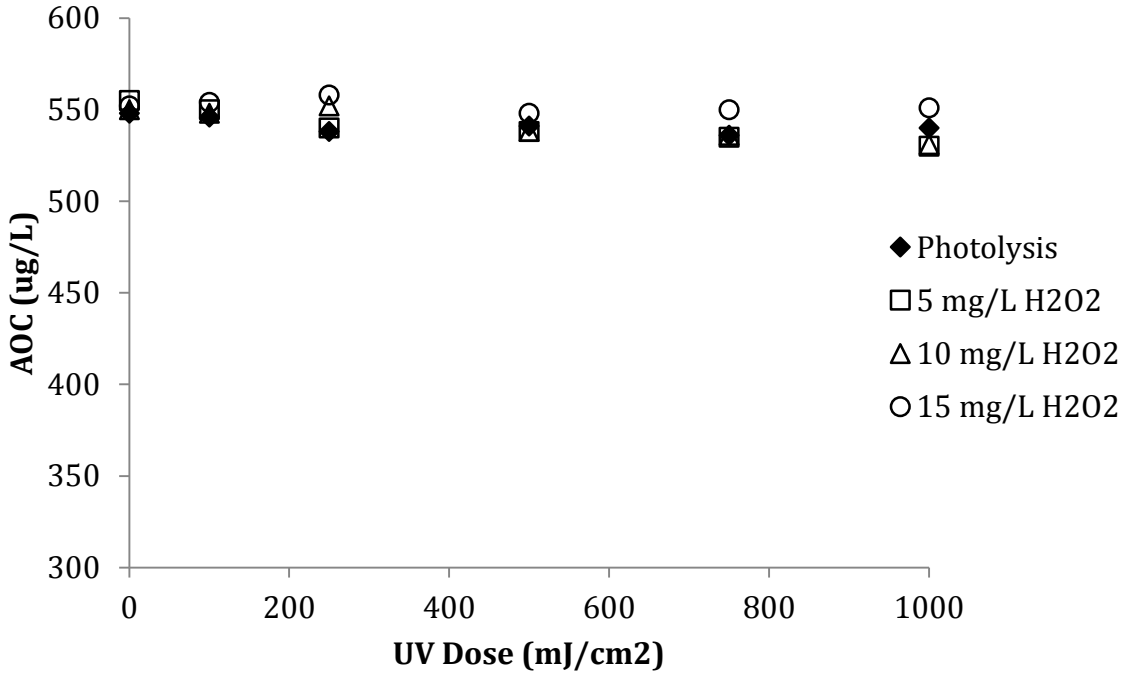


Figure 5.5 Assimilable Organic Carbon (AOC) concentration (ug/L) versus the dose of low pressure UV light (mJ/cm^2) in post-riverbank filtration influent water from utility partner A

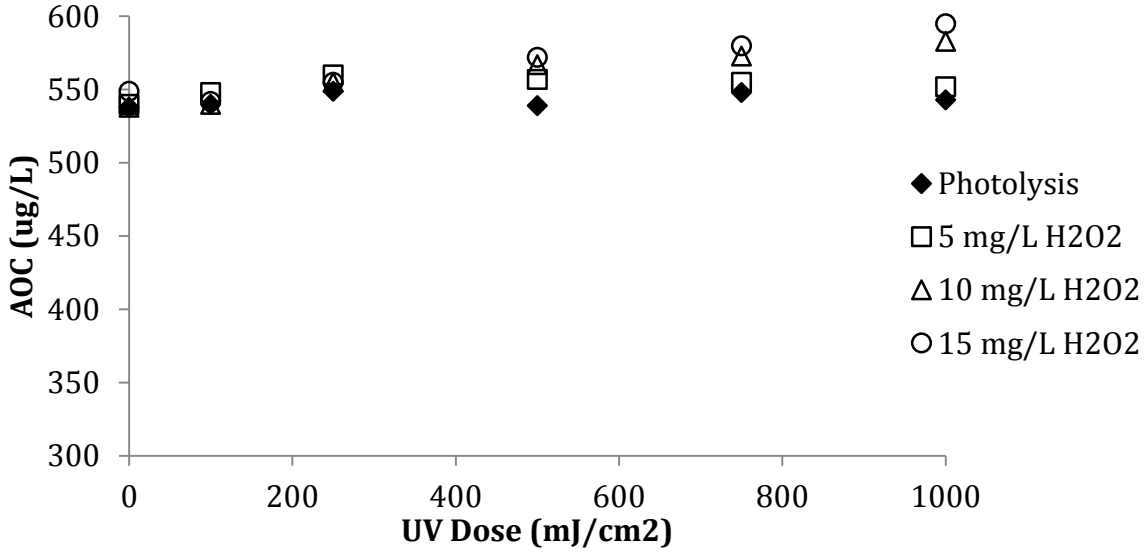


Figure 5.6 Assimilable Organic Carbon (AOC) concentration (ug/L) versus the dose of medium pressure UV light (mJ/cm^2) in post-riverbank filtration influent water from utility partner A

Figure 5.7 and Figure 5.8 show the effect of low and medium pressure photolysis and combined UV-H_2O_2 processes on AOC formation in a laboratory water matrix. The laboratory water matrix consisted of ultrapure milli-q water, 500 ug/L acetate carbon stock solution, and 2 mg/L of Suwanee River natural organic matter (SRNOM). No significant increase in AOC was observed following the low pressure process at any hydrogen peroxide dose. In the case of the medium pressure experiments, a similar result was found as to that of low pressure. No significant increase in AOC formation was observed. It does appear with the medium pressure experiments that the hydroxyl radical exposure might play a role in the small amount of AOC formation present in the laboratory grade water.

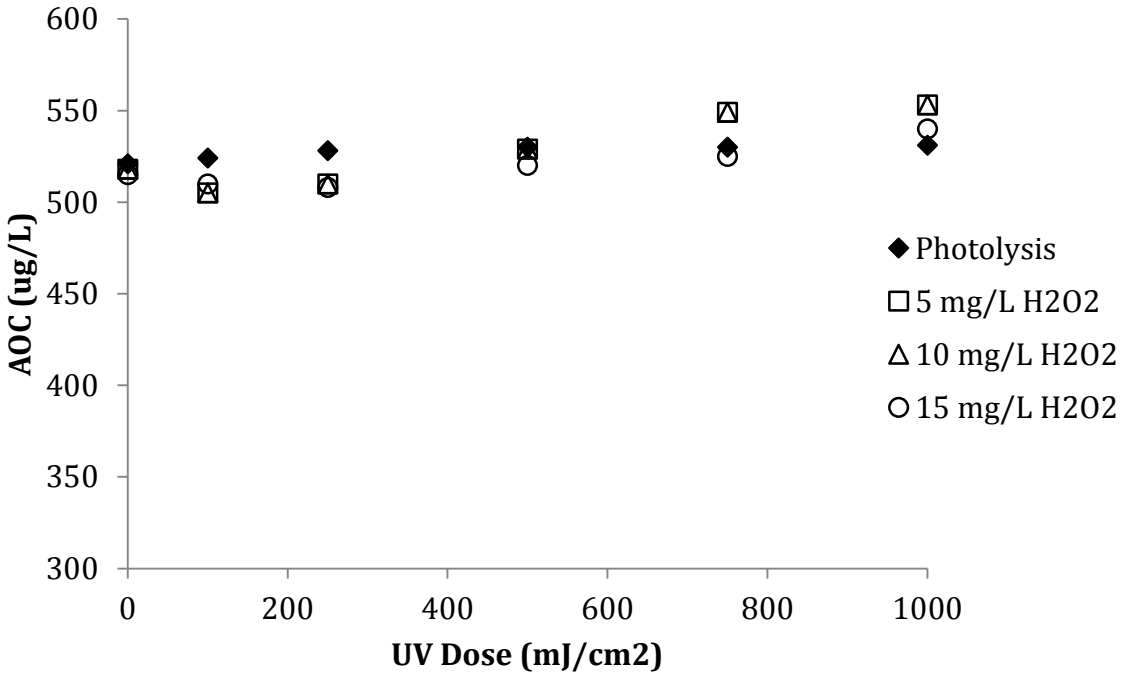


Figure 5.7 Assimilable Organic Carbon (AOC) concentration (ug/L) versus the dose of low pressure UV light (mJ/cm^2) in milli-q water dosed with acetate carbon at 500 ug/L and Suwanee River NOM

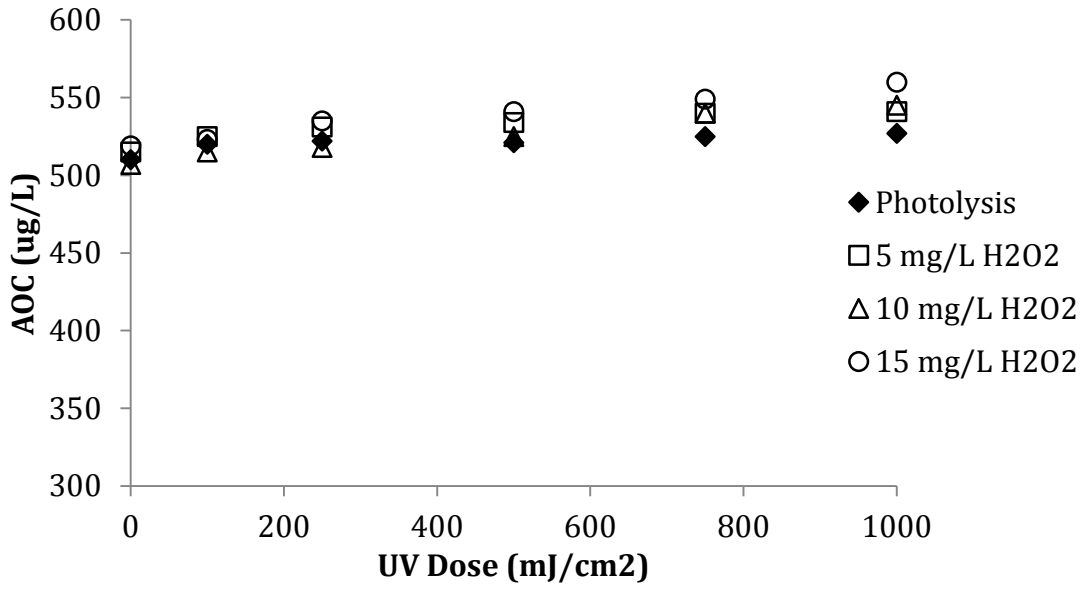


Figure 5.8 Assimilable Organic Carbon (AOC) concentration (ug/L) versus the dose of medium pressure UV light (mJ/cm^2) in milli-q water dosed with acetate carbon at 500 ug/L and Suwanee River NOM

5.4. Conclusions

5.4.1. Biological Filtration

The reason behind the increase in toxicity following biological filtration (sand only) could potentially be a result of hydrolysis of methamidophos by-products during the 3 week acclimation period being more toxic than that of the parent compound or UV-AOP byproducts that are formed. This could also be a result of biological transformations in the acclimated columns resulting from a resistance built up by bacteria during the three weeks of acclimation. In line with previously reported literature, the biological GAC resulted in complete removal of all mutagenicity tested.

5.4.2. **AOC Formation**

No correlation was found between levels of AOP treatment and increases in AOC formation. Post-AOP treatment may be required if AOC or toxicity increases are observed. AOC formation can be reduced but not eliminated through upstream NOM removal. However, toxicity increases can result from the incomplete oxidation of the parent compound and cannot be reduced through upstream treatment. Biological filtration was shown to be an effective post-AOP treatment process for reducing AOC and toxicity. Biological filtration can provide multiple benefits including byproduct control, hydrogen peroxide quenching, and filtration.

6. The Third Contaminant Candidate List (CCL3): Exploring New Frontiers in Determining Contaminants for Potential Regulation

6.1. Abstract

The 1996 Safe Drinking Water Act (SDWA) amendments established a process to identify and evaluate new contaminants for potential regulation, starting with a list known as the Contaminant Candidate List (CCL). The CCL is used as the starting point for regulatory decisions for emerging contaminants in drinking water. Each contaminant on the Third CCL (CCL3) was assigned attribute scores by the US Environmental Protection Agency to quantify it's health and occurrence data relative to other contaminants. Regulating by groups was explored in this study by considering the structure and biological mode of action of contaminant groups as well as the contaminants use or purpose. This included weighing the contaminants attribute scores within each group, and looking at the spread in data to show the variation that would be used to inform regulatory decisions by grouping. An alternative approach to consideration of emerging contaminants is proposed, using effect-directed analysis (EDA) and the use of biological monitoring tools. An EDA approach would require input and investment from three key stakeholders; the USEPA, the drinking water utilities, and the public or drinking water consumers.

6.2. Regulatory Background

The 1996 Safe Drinking Water Act (SDWA) amendments established a three-step regulatory development process for identifying and evaluating new contaminants for potential regulation:

1. The Contaminant Candidate List (CCL); the starting point of the regulatory development process that includes identifying contaminants of potential concern from a regulatory perspective;
2. Regulatory Determinations; i.e., the decision to regulate (or not), or issue guidance or determine if more research is needed;
3. The setting of the Maximum Contaminant Level Goal (MCLG), the health-based goal, and the Maximum Contaminant Level (MCL), the enforceable standard. In cases where the contaminant cannot be appropriately measured, a Treatment Technique (TT) can be set in lieu of the MCLG/MCL.

CCLs and Regulatory Determinations are supposed to be on-five-year cycles, as mandated in the SDWA amendments. However, some of these actions are now stretching beyond the five-year cycles. The U.S. Environmental Protection Agency (USEPA) published the First Contaminant Candidate List (CCL1) and the Second Contaminant Candidate List (CCL2) in 1998 and 2005, respectively (USEPA 1998; USEPA 2005). USEPA published the current Third Contaminant Candidate List (CCL3) in 2009 (USEPA 2009). The CCL3 consisted of 116 (104 chemical and 12 microbial) contaminants.

After a chemical or microbial contaminant is listed on the CCL, it may or may not be subsequently regulated through decisions known as Regulatory Determinations. USEPA published the First and Second Regulatory Determinations in 2003 and 2008, respectively (USEPA 2003; USEPA 2008). With these determinations, USEPA decided to not regulate 20 of the contaminants, as a national regulation would not provide a "meaningful opportunity for health risk reduction" as required by the SDWA. In 2011, USEPA decided to regulate

perchlorate in an "off-cycle" (separate from the traditional five-year cycles) Regulatory Determination (USEPA 2011). The preliminary Third Regulatory Determination is scheduled to be published sometime in 2014.

The first three CCLs and two Regulatory Determinations illustrate some of the challenges that USEPA faces in identifying new contaminants for potential regulation. Section 1412(b)(1)(A) of the SDWA specifies the three criteria that must be met for USEPA to make a determination to develop a national regulation for a contaminant as:

1. The contaminant may have an adverse effect on the health of persons;
2. The contaminant is known to occur or there is a substantial likelihood the contaminant will occur in public water systems with a frequency and at levels of public health concern; and
3. In the sole judgment of the USEPA Administrator, regulation of the contaminant presents a meaningful opportunity for health risk reductions for persons served by public water systems.

The gaps in health effects, occurrence, and analytical methods data for the CCL3 contaminants illustrate the challenges that USEPA faces in satisfying the above three criteria. In the CCL3 *Federal Register* notice, USEPA identified the Regulatory Determination data/information needs for the CCL3 chemicals. At that time, in October 2009, only 16 of the 104 (15%) CCL3 chemicals had sufficient data/information to continue moving these chemical forward in the regulatory development process. At a June 2011 stakeholder meeting, USEPA

presented information on 32 (31%) chemicals for which they had enough information such that they were continuing in the Regulatory Determination process.

The majority of CCL3 contaminants have data/information gaps that preclude them from advancing further into the Regulatory Determination process. Combined, these needs represent a research investment that is not feasible with current allocations of federal resources. Therefore, some innovative, less time intensive, and less costly alternative approaches are needed to identify and evaluate contaminants for potential regulation within the framework of Section 1412(b)(1)(A) of the SDWA.

6.1. Contaminant Attribute Scoring

USEPA assigned scores for the 104 chemical contaminants on the CCL3 in four attribute categories: Potency (P), Severity (S), Prevalence (Pr), and Magnitude (M) as part of its process to develop CCL3. Scores were assigned from 1 to 10 with a lower number corresponding to a lower response in each attribute column, and the analysis reported herein used these scores as the foundation for potential grouping methods. All 104 chemical contaminants, and their corresponding attribute scores are reported in Table 6.1.

Potency (P) and severity (S) correspond to the potential of contaminants to cause adverse health effects, and prevalence (Pr) and magnitude (M) correspond to how likely the contaminant is to be found in drinking water [7]. By taking these scores (assigned by the USEPA) and separating out the occurrence and health effects data, regulating by groups such as by use or treatment technology, can be explored using a normalized system of attribute scoring across all contaminants.

A variety of grouping methods could be used in a regulatory context, including: the chemical structure and biological mode of action of the contaminant, the contaminants use (and/or) purpose as defined by the toxic release inventory (TRI), analytical methods, and possible treatment technologies.

Table 6.1 104 CCL3 chemical contaminants with corresponding attribute scores assigned by the USEPA (USEPA 2009).

CCL3 #	Chemical Name	Potency (P)	Severity (S)	Prevalence (Pr)	Magnitude (M)
1	1,1,1,2-Tetrachloroethane	5	8	3	6
2	1,1-Dichloroethane	4	8	7	7
3	1,2,3-Trichloropropane	7	8	3	6
4	1,3-Butadiene	7	8	10	9
5	1,3-Dinitrobenzene	7	3	1	8
6	1,4-Dioxane	5	8	9	8
7	17alpha-estradiol	7	6	9	3
8	17α-ethynylestradiol	7	6	9	3
9	17β-estradiol	8	8	10	5
10	1-Butanol	4	5	10	10
11	2-Methoxyethanol	6	7	9	7
12	2-Propen-1-ol	5	6	8	8
13	3-Hydroxycarbofuran	7	7	2	7
14	4,4'-Methylenedianiline	7	8	7	7
15	Acephate	6	5	10	7
16	Acetaldehyde	5	3	10	8
17	Acetamide	5	8	7	9
18	Acetochlor	5	7	1	1
19	Acetochlor ethanesulfonic acid (ESA)	5	3	1	1
20	Acetochlor oxanilic acid (OA)	5	3	1	1
21	Acrolein	6	9	3	7
22	Alachlor ethanesulfonic acid (ESA)	4	3	9	3
23	Alachlor oxanilic acid (OA)	5	8	9	3
24	alpha-Hexachlorocyclohexane	7	8	4	3
25	Aniline	5	6	9	8
26	Bensulide	5	5	10	6
27	Benzyl chloride	6	8	7	5
28	Bromochloromethane (Halon 1011)	5	3	5	6
29	Bromomethane (Methyl bromide)	6	6	6	7

30	Butylated hydroxyanisole	7	3	8	4
31	Captan	4	8	10	8
32	Chlorate	5	6	10	10
33	Chlorodifluoromethane (HCFC-22)	5	5	10	10
34	Chloromethane (Methyl chloride)	5	8	8	7
35	Clethodim	5	4	10	6
36	Cobalt	5	4	4	8
37	Cumene hydroperoxide	4	9	8	8
38	Cyanotoxins (3)* - Microcystin-LR	9	3	10	4
38	Cyanotoxins (3)* - Cylindrospermopsin	8	3	5	10
38	Cyanotoxins (3)* - Anatoxin-a,	6	9	9	8
39	Dicrotophos	7	5	8	6
40	Dimethipin	5	6	8	5
41	Dimethoate	6	5	10	7
42	Disulfoton	7	5	1	1
43	Diuron	6	4	4	7
44	equilenin	7	6	9	5
45	equilin	7	6	8	5
46	Erythromycin	6	3	10	4
47	estriol	7	6	10	3
48	estrone	7	6	9	3
49	Ethoprop	7	3	7	3
50	Ethylene glycol	3	9	10	10
51	Ethylene oxide	6	8	10	8
52	Ethylene thiourea	7	6	4	1
53	Fenamiphos	7	3	8	6
54	Formaldehyde	4	6	10	8
55	Germanium	6	6	4	10
56	Hexane	4	3	10	10
57	Hydrazine	7	8	9	7
58	Mestranol	8	3	9	4
59	Methamidophos	7	5	10	6
60	Methanol	3	6	10	10
61	Methyl tert-butyl ether	4	8	5	8
62	Metolachlor	4	3	6	6
63	Metolachlor ethanesulfonic acid (ESA)	2	1	6	6
64	Metolachlor oxanilic acid (OA)	2	1	6	6
65	Molinate	6	7	1	8
66	Molybdenum	5	5	9	8
67	Nitrobenzene	6	3	1	10
68	Nitroglycerin	7	6	7	6
69	N-Methyl-2-pyrrolidone	3	5	10	10
70	N-nitrosodiethylamine (NDEA)	9	8	1	2

71	N-nitrosodimethylamine (NDMA)	8	8	10	2
72	N-nitroso-di-n-propylamine (NDPA)	7	8	2	2
73	N-Nitrosodiphenylamine	5	6	2	1
74	N-nitrosopyrrolidine (NPYR)	7	8		
75	Norethindrone (19-Norethisterone)	8	7	10	4
76	n-Propylbenzene	6	3	4	6
77	o-Toluidine	6	8	7	5
78	Oxirane, methyl-	6	8	10	8
79	Oxydemeton-methyl	7	5	9	5
80	Oxyfluorfen	5	8	10	6
81	Perchlorate	6	1	9	8
82	Perfluorooctane sulfonic acid (PFOS)	8	3	10	7
83	Perfluorooctanoic acid (PFOA)	6	3	10	6
84	Permethrin	4	8	10	7
85	Profenofos	7	3	8	6
86	Quinoline	7	8	7	5
87	RDX (Hexahydro-1,3,5-trinitro-1,3,5-triazine)	6	8	5	5
88	sec-Butylbenzene	5	5	3	6
89	Strontium	3	5	10	10
90	Tebuconazole	5	7	9	6
91	Tebufenozide	5	6	9	5
92	Tellurium	5	7	4	9
93	Terbufos	7	3	1	1
94	Terbufos sulfone	7	3	1	1
95	Thiodicarb	5	8	10	6
96	Thiophanate-methyl	5	8	10	6
97	Toluene diisocyanate	5	8	10	7
98	Tribufos	6	3	9	8
99	Triethylamine	6	5	10	9
100	Triphenyltin hydroxide (TPTH)	8	8	10	6
101	Urethane	6	9	7	6
102	Vanadium	6	5	10	8
103	Vinclozolin	5	8	10	5
104	Ziram	5	8	10	7

6.2. Regulating by Groups

6.2.1. Background

In March 2010, then USEPA Administrator Lisa Jackson announced a new drinking water strategy with four components, one of which was regulating contaminants by groups as opposed to regulating them individually. USEPA identified several potential classification frameworks for regulating by groups:

- Similar health effects;
- Co-occurrence;
- Common analytical method(s);
- Common treatment or control processes.

While regulating by groups initially sounded like a framework to make regulations more efficient, it may be more complicated than originally thought. In February 2011, USEPA Administrator Jackson announced that carcinogenic Volatile Organic Compounds (cVOCs) would be the first group to be regulated. In the February 2011 announcement, USEPA identified eight currently regulated cVOCs as well as eight unregulated cVOCs from the Third Contaminant Candidate List (CCL3) that were being considered for inclusion in a group cVOC Rule.

Clearly, as a group, cVOCs would have the same health effect (cancer) and should have some additional commonalities with analytical methods and treatment technologies. However, the potential commonalities for the cVOCs are less than one might have initially thought [8]. In order to determine the applicability of group contaminant regulation of cVOCs, this study applied USEPA assigned attribute scores to various groups and evaluated several groups using quantifiable scoring methods.

6.2.2. **Grouping and Scoring**

The 104 chemical contaminants on the CCL3 were divided into groups considering their structure and biological mode of action, and their contaminant use (and/or) purpose. The contaminants were then evaluated by using the attribute scoring metrics, assigned by the USEPA, shown individually in Table 6.11. Each group of contaminants were then quantified following the health effects only (PxS), occurrence only (PrxM), and combined health effects and occurrence (PxS + PrxM). These scores were normalized within each scoring method, by dividing the total score by the maximum possible score in each method. Grouping by contaminant structure (corresponding to biological mode of action) and contaminant use (and/or) purpose are shown below as

Figure 6.1 and Figure 6.2, respectively. Ideally, if a particular grouping method was valid across all contaminants in that group, a very small spread in data would be seen. This small variation in data in each group would indicate a measure of confidence in regulating by groups in the same categories.

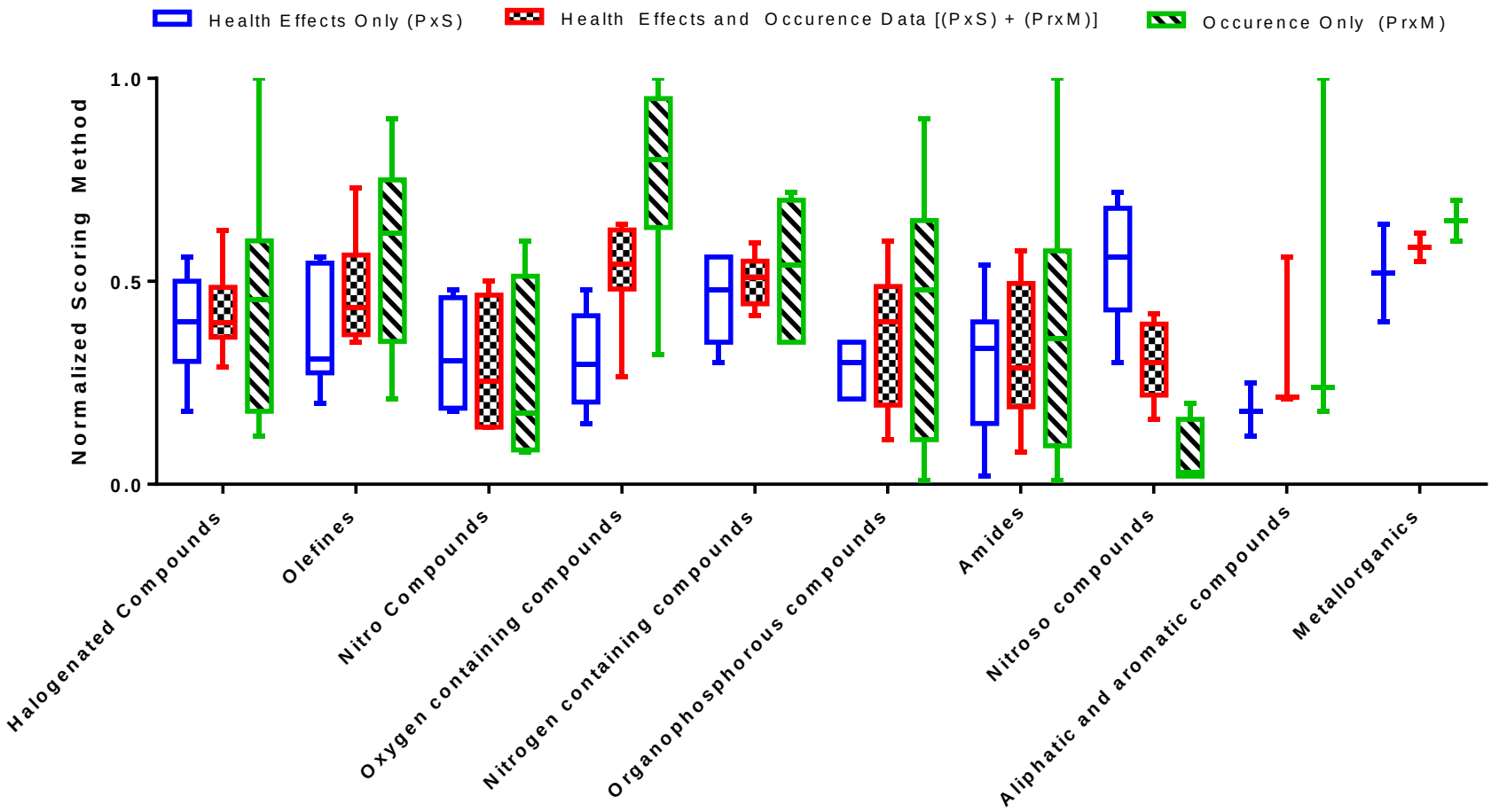


Figure 6.1 Normalized scoring data for grouping by structure and biological mode of action; PxS = health effects only data (blue), PrxM = occurrence only data (green), and (PxS) + (PrxM) = health effects and occurrence equal weighting data (red).

124

Contaminants were grouped by their structure (corresponding to their major biological mode of action), with each contaminant falling into only one category. These categories were then scored according to the attribute scoring methods discussed, and their average and data spread are shown in Figure 6.1. An example of this method would be to take the compound 1,2,3-tricholoropropane (CCL #3) with attribute scores of P = 7, S = 8, Pr = 3, and M = 6. First, this compound was placed into a group of contaminants that contained similar structures, the 'halogenated compounds' group. Next, the health effects only category was calculated for this compound; multiplying potency and severity (PxS = 56). Then, the occurrence only data was scored; multiplying prevalence and magnitude (PrxM = 18). The total attribute score for health and occurrence data was then calculated as PxSxPrxM = 1008. These scores for 1,2,3-trichloroprpane were then averaged with the other attribute scores in the halogenated compounds group, and then all individual compound scores were normalized out of the total amount possible for each category (PxS = 100, PrxM = 100, and PxSxPrxM = 10,000).

In each figure, the middle line in the box of each data group represents the average attribute score, and the whiskers represent the spread (lowest and highest) of the additional attribute scores of the group. The health effects only data, which includes only potency and severity, varied with each group and category; the aliphatic and aromatic compounds averaging the lowest, and the nitroso compounds having the highest average. When the occurrence data is included, the aliphatic and aromatic compounds remain the lowest, but with a very large span in data due to the large range in occurrence data. The highest structural category when all attributes are taken into account is the oxygen containing compounds. The span of data surrounding the categories is quite large, so taking only the average into account could lead to some potential gaps in the regulation of particular contaminants.

125

The range in data based on contaminant groups was similar when the contaminants were categorized by their use and/or purpose (Figure 6.2) when compared to structure and biological mode of action. Averages alone give potential insight into regulation of categories of groups by comparing groups amongst themselves in each method. For example, if several grouping methods were used and similar compounds were consistently causing high scores, this could allow regulators to determine which compounds are the major offenders relative to others. Comparative insight could be beneficial; however, the range in data is still quite large. The nitrosamines class had the highest average with respect to health effects, and the disinfection byproducts provided the highest average for occurrence data. The solvents category showed the largest variation in data in the occurrence category, and manufacturing compounds showed the most variation when health and occurrence data were combined.

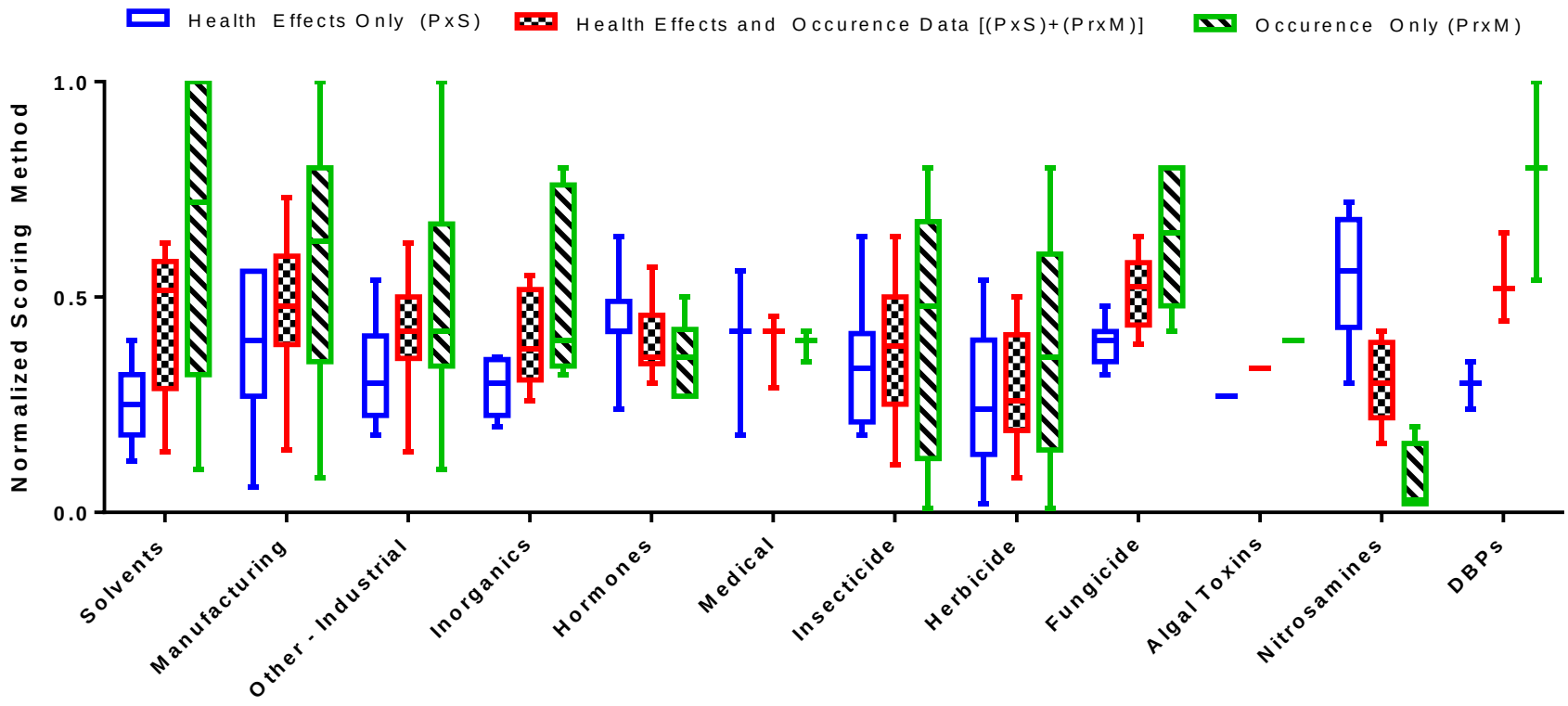


Figure 6.2 Normalized scoring data for grouping by contaminant use and purpose; PxS = health effects only data, PrxM = occurrence only data, and (PxS) + (PrxM) = health effects and occurrence equal weighting data.

Lack of continuity in the data supports a different approach be taken to regulating emerging contaminants other than grouping. Alternate approaches would need to be able to capture a wide array of contaminants with different health-effects endpoints, and would ideally not depend on occurrence only as a factor. Regulating by health effects endpoints, or bioassay analysis was then explored as this approach would possibly be able to capture a wide array of contaminants, without the need to quantify or group them as individuals.

6.3. Regulating by Health-Effect Endpoint

New contaminants not listed on the CCL are being continually manufactured and released. Additionally, the transformation of known emerging contaminants, listed on the CCL or the Toxic Release Inventory (TRI), can happen naturally in the environment prior to the influent of a drinking water facility or can happen in an engineered process. Transformation processes, such as biological degradation or advanced oxidation (AOPs), work by transforming a contaminant from its parent compound into smaller, potentially less biologically active, byproducts. These transformation products are not currently regulated, and are also mostly uncharacterized. There is a need to better understand and quantify the level of biological activity from transformation products and emerging contaminants alike, which could potentially be done using bulk in-vitro bioassay analysis utilizing the concept of effect-direct analysis.

6.3.1. Effect-Directed Analysis

Effect-directed analysis (EDA) is a method used to quantify the toxic potential of unknown byproducts in matrices, such as water matrices with many unknown byproducts. EDA is an environmental toxicology tool that has been explored and applied in a research setting for some time [9-11]. The basis of EDA is a combination of fractionation procedures, biological testing,

and chemical analysis. EDA is most commonly used to detect toxic compounds in unknown mixtures, thus can be directly applied to water matrices where there are many unknowns, including a variety of regulated and unregulated emerging contaminants.

Biological testing usually consists of a combination of biological assays (human cell-line based and bacteria based) with varying toxicological endpoints. With regard to drinking water treatment applications, the toxicological endpoints provide indications of human-health effects. The USEPA uses biological screening assays to help determine the Health Effects attribute scoring discussed in the grouping section. ToxCast™ is the program through which the USEPA uses all of the screening assays [12]. This biological screening data helps the USEPA to narrow the Preliminary CCL (PCCL) down to the final CCL. More data obtained through biological screening assays and ToxCast™ on a variety of chemicals could aid in moving towards a monitoring and modeling approach to emerging contaminant regulation, rather than regulating on a contaminant-by-contaminant basis.

The use of biological screening assays at a utility level is not an entirely new concept. Bioassays have been implemented in the field of ecological toxicology for several years in order to monitor wastewater release post-treatment [13, 14]. Several of the concepts gained from this wastewater release work could be applied to drinking water treatment standards and human-health indicators. With wastewater reuse becoming important in water stressed areas, and increasing concern over drinking water influent water quality, the idea of biological-endpoint-based regulation being used in the drinking water field might be a logical progression.

6.3.2. **Stakeholder Analysis**

Three main stakeholders should be engaged prior to the decision to implement bioassay endpoints for regulatory consideration: regulators (USEPA: US and states), drinking water utilities, and the public drinking water consumers. The USEPA would be in charge of the scientific development and validation of bioassays that can be implemented into research and water quality laboratories, as to not burden utilities with significant further equipment costs. The USEPA would also be setting "minimum acceptable level of toxicity" standards. It is possible that several assays will need to be paired together, in order to capture several biological endpoints, and the question of whether or not to regulate each type of toxicity individually, or combine them could be a similar analysis to that of regulating contaminants by grouping. Ensuring accurate monitoring, reporting, and general compliance monitoring of bioassay standards will be an additional challenge to overcome.

Multiple benefits to USEPA are possible:

- An easier way to regulate a variety of emerging contaminants,
- A large amount of monitoring data of toxicological endpoints that could potentially help with compliance of the TRI monitoring system, and
- Increased confidence of environmental and human-health protection.

From USEPA's perspective, a key decision point would be whether to use EDA in its research program to identify new contaminants for potential regulation, or whether to push EDA "into the field" for compliance monitoring for a regulation in the future. These two paths are quite different simply from the number of facilities that would be impacted. The number of research laboratories (both USEPA's and contract laboratories) needed for the regulatory

development process would be much smaller than the several hundred commercial and water utility laboratories that would be needed for compliance monitoring.

From a utility perspective, the additional internal cost that would come with bioassay monitoring includes materials, employee-training, and frequency. The ability to meet this type of compliance standard might be difficult for some utilities if they cannot afford the cost of biological monitoring; however, this cost might be offset by the need for less analytical monitoring if commercially available products are affordable. Depending on the resources involved, utilities would have to make the decision on whether to run these tests "in-house" or ship samples to commercial laboratories for EDA testing.

Public interpretation of biological data will also be of concern to utilities. The customers of most utilities have become acclimated to the idea of chemical concentrations in drinking water. The numerical standards provide a relatively simple (notwithstanding the underlying complicated risk assessment and risk management processes) "bright line" for customers to understand that the water is safe to drink. Convincing their customers that there can be for example, a "mutagenic signal" in their drinking water, that it is not harmful, is a public outreach challenge that will need to be overcome. The American Water Works Association (AWWA) could help to develop communication guidelines for to aid utilities in this type of outreach.

Potential benefits to the utility include:

- An increase in consumer confidence that accompanies additional "advanced" monitoring;
- An additional barrier for water quality confidence regarding emerging contaminants; and
- Increased confidence in advanced treatment technologies.

EDA could also be potentially integrated, along with enhanced customer complaint systems and other technologies, into the concept of 'early warning system' for drinking water treatment.

It is likely that the financial burden of this type of regulation would be applied to the public in the form of an increase in water rates. However, the consumer could benefit from this type of regulatory approach. Additional monitoring data would provide consumers with increased confidence in their drinking water. Biological screening data also has the potential to capture unknown byproducts in drinking water that are not regulated, and also are not currently known. The monitoring data would only serve to help the protection of human-health, which is the ultimate goal in drinking water distribution with regard to water quality and contaminant regulation.

6.4. Conclusions

The "regulatory roadmap" for identifying and evaluating new contaminants for potential regulation that is mandated by the SDWA is not a simple one for USEPA to navigate. The CCL and Regulatory Determinations processes are sound and logical, but the resources needed for health effects, occurrence, and treatment research are enormous and pose challenges under the ongoing federal fiscal environment. Outside of perchlorate, no new contaminants have been regulated through the CCLs and Regulatory Determinations, and new approaches need to be considered to optimize the regulatory development process.

Regulating emerging contaminants by groups does not account for the wide array of data concerning their potential health effects. Group contaminant regulation also does not capture any toxicologically relevant unknown compounds or transformation products at the influent and effluent of the drinking water facility. EDA is a new approach that should be explored for contaminant regulation. This approach would capture these unknowns in the water, and could directly apply the information to potential chronic human-health indicators, like mutagenicity or

other forms of toxicity. In order to regulate contaminants based on effect-directed analysis, the USEPA would need to do further research and provide guidance on standard methods and monitoring protocols. Utilities will be required to comply, taking time and training, but the public and drinking water consumers would benefit from this extra monitoring, which would increase consumer confidence in drinking water in the United States.

7. Thesis Conclusions, Applications and Future work

7.1. Conclusions

The research aims/objectives of this thesis were outlined in Chapter 1, and supporting evidence was reported as Chapters 2 – 6. Conclusions from each objective or set of objectives can be found below:

7.1.1. Objectives 1 and 2

1. Assess the **treatability of CCL3 contaminants** selected by AOP. (Chapters 3 and 4)
2. Assess the **toxicity** (by defined endpoints) **of CCL3 contaminants** before and after degradation by AOP. (Chapters 3 and 4)

The important information gathered in this section included the knowledge gaps present within the CCL3 contaminant literature regarding AOP treatment. The knowledge gaps were highlighted, and conclusions were drawn pertaining to the structure of the contaminants in question. The log Kow presented with all CCL3 contaminants was discussed as a surrogate parameter to indicate whether or not the contaminant would make it to the end of the treatment plant where AOPs are likely found. The grouping of contaminants by structure, and their resulting partition coefficients gives insight into where the treatment needs are, pertaining to AOPs and the CCL3 list.

The use of bioassay screening as a way to track contaminant treatability using AOPs was explored. A thorough discussion of bioassay applications, toxicity endpoints, and the relationship of microbial assay indicators to human health indicators was presented. This information gathered and presented with regards to bioassay application and the CCL3 list provides a framework for effect-directed analysis being used in water treatment plants.

Hydroxyl radical and ozone degradation rate constants were experimentally determined where the data gap or knowledge was missing in the literature. Most organic contaminants react with hydroxyl radical on the order of magnitude of 10^9 $M^{-1}s^{-1}$ making AOP a viable treatment technology for organic contaminant degradation. Of the 25 compounds chosen for this study, 23 were found to be amenable for AOP treatment, and readily degraded by hydroxyl radicals.

Two distinct toxicity assays, the Ames II and YES bioassay were selected to screen the compounds tested and provide information on toxicity pre and post AOP treatment. In addition, the acetylcholinesterase inhibition assay (AChE) was used as a specific toxicity assay for testing of organophosphorous compounds, which are known neurotoxins. The mutagenic activity from the AMES test over 45 treatment scenarios, resulted in 9 cases, or 20%, of increased mutagenicity. The most common mutations, 6 of the 9, were the result of a frameshift mutation. The results from both the estrogenic and neurotoxic assays illustrated almost no increase in toxicity with only 1 of 42 treatment scenarios resulting in an increase in estrogenic activity, and no increased neurotoxicity identified following AOP treatment.

7.1.2. **Objectives 3 and 4**

3. Investigate the use of **biological filtration** for the removal of toxic/unknown byproducts following AOP. (Chapter 5)
4. Investigate the **formation and mitigation of AOC** following AOP treatment. (Chapter 5)

Granular activated carbon biological filtration (GBAC) was shown to be an effective post-AOP treatment process for reducing AOC and toxicity. Biological filtration proved to provide multiple benefits including byproduct control, hydrogen peroxide quenching, and filtration. Sand-only biofiltration lead to an increase in toxic activity following the further degradation of

methamidophos metabolites in the biofiltration columns. This toxic activity was not seen when the columns were not acclimated, or when BGAC versus sand alone was used. The formation of AOC following UV-AOP conditions was investigated, and no significant formation of AOC following UV-AOP conditions occured following exposure either from low or medium pressure UV lamps.

7.1.3. Objective 5

5. Explore the **policy implications** of using transformation processes (i.e. advanced oxidation) for the treatment of CCL3 contaminants. (Chapter 6)

Emerging contaminant regulations could potentially begin utilizing bioassays as indicators of potential human-health hazards, or as treatment breakdown indicators in basic water quality laboratories in water treatment plants for contaminant monitoring. A data analysis study utilizing attribute scores provided by the US EPA showed that grouping of contaminants was not an ideal way to regulate if the goal was to capture and represent health and occurrence data of each contaminant. A stakeholder analysis was provided that delved into the issues surrounding using biological tools, such as bioassays, as an "early warning system" for water treatment plants with regard to chemical contaminant regulation.

7.2. Applications

The results obtained throughout this study have led to some recommendations that can provide water utilities, engineers, and others in the water profession with guidance regarding use of AOP treatment and oxidative transformation product mitigation. These recommendations include:

1. Advanced oxidation processes can confidently be recommended for the treatment of CCL3 emerging contaminants. Contaminants are treatable by hydroxyl radical, and lead to minimal harmful byproduct formation.
2. The formation of AOC following UV-AOP treatment should be tested on a pilot or bench scale for each site in order to appropriately select downstream technologies.
3. When evaluating AOPs and other technologies for the treatment of CCL3 or other contaminants, both cost and non-cost factors should be evaluated.
4. The toxicity testing approach utilized in this research should be considered by utilities who have or are considering implementing a transformation based treatment process in order to assess finished water quality that includes unknown transformation products.
5. If AOC or AOP byproducts are formed in a site-specific location, an additional treatment step (e.g. BAC) should be considered to meet regulatory or utility goals.
6. Grouping CCL contaminants structurally or by their use or purpose may not be the best method for contaminant regulation.
7. Additional cost-effective regulations for emerging contaminants should be explored that include the ability to monitor for transformation products and unknown contaminants in the drinking water supply.

7.3. Future Work

As a continuation of the thesis work presented here, several projects could be explored in the future including testing more compounds through the test battery recommended in this study and comparing the results. Adding to the body of knowledge of unknown rate constants with advanced oxidation processes, including hydroxyl radical rate constants for various

contaminants, would be beneficial in guiding treatment design decisions. Delving further into the formation and mitigation of AOC following UV based AOPs in a wider range of water matrices would be helpful not only in the US, but also in other countries where there is often no disinfection residual. Developing a framework for policy decisions using health-effect endpoints is also a necessary follow-up to this research if this type of policy decision is to be implemented. Engineering considerations relating to AOP implementation for emerging contaminant mitigation should be explored pertaining to the target contaminants, water quality criteria, hydrogen peroxide dosing and quenching, byproduct formation, and regulatory complexity.

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