IN VITRO TOXICOLOGY OF COMPLEX MIXTURES FROM DRINKING WATER DISINFECTION AND AMINE-BASED CARBON CAPTURE SYSTEMS

BY

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THESIS

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Abstract

In nature, exposure to chemical insults and toxic agents never occurs in isolation. Any number of concurrent exposures can result from environmental factors. The complex network of integrated organ systems and metabolic pathways are altered by the presence of different agents, so it is possible that, in combination, the biological response to a series of agents might not be simply expressed as the sum of individual exposures. A single-compound paradigm, as has been the historical norm in both toxicological research and regulatory policy, might be inadequate to effectively assess the environmental and health hazards associated with chemical, industrial, or engineering processes. A focus on a select few priority compounds may hide the underlying toxicities of a given set of samples if we are unable to effectively identify or measure the important constituents. Toxicological research into complex mixtures may help to elucidate information on the unknowns in a given system and better identify hazards that might not be obvious by a narrower, single-compound focus. In this thesis, the toxicology of two complex mixture sources was investigated. The first are complex mixtures of drinking waters contaminated with a common pharmaceutical, resulting in the generation of iodinated disinfection by-products (DBPs), a set of DBPs that are currently unregulated but have been found to be more toxic than their analogs with other halogens. The second investigates a series of priority nitrogenous materials derived from a carbon capture process and the complex mixtures of by-products. In this comparison, the bulk of toxic effect could not be explained by simply the compliment of priority contaminants investigated, but rather the complex mixtures provided unexpected insight into more environmentally benign alternatives that would not have been realized had the short list of single compounds been the only focus of the research. The research demonstrates that, to effectively assess the risks presented by by-products of any of a number of processes, complex mixture analysis is essential.
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CHAPTER 1

Introduction to Complex Mixtures Toxicology

1.1 Introduction

Through the past several decades, toxicologists have recognized that most toxicology research struggles with a very profound challenge – exposure is not an isolated event. Chemical insults can be acute or chronic in nature and, outside a laboratory setting, never occur as single exposures, yet around 95% of toxicological research has focused on single chemical exposures [1]. Other stressors present have the potential to alter the potency or effects of individual agents. Environmental and organismal factors play important roles in the potential damage of a toxicant. Most tangibly, however, exposure to a toxicant might potentially be altered by the presence of other toxicants [2]. Interactions can be additive, synergistic, or antagonistic [1]. Humans are constantly bombarded with an array of synthetic and natural chemicals – complex mixtures of exposure in our daily lives [3]. Toxicology has traditionally focused on the effects of single chemical constituents because testing procedures and regulatory oversight are generally focused on a handful of important or easy to characterize constituents. This single-compound approach can potentially miss yet-to-be characterized components and important biological endpoints resulting from chemical interactions [2]. Ultimately, testing of complex mixtures could potentially provide more accurate descriptions of our daily experiences with the chemicals around us.
1.2 Individual Contaminants to Complex Mixture Analysis

1.2.1 Early Regulations

In the United States, Congress has given regulatory authority to a number of executive agencies, charging them to establish and enforce standards for contaminants in food, water, and occupational exposures. For example, the Safe Drinking Water Act, enacted in 1974, requires the United States Environmental Protection Agency (U.S. EPA) to compile a list of priority contaminants in drinking water, referred to as the Contaminant Candidate List [4]. This list is used to help establish regulatory and research priorities to ensure a safe drinking water supply.

Contaminants can be either microbial or chemical and include industrial chemicals, pesticides, disinfection by-products, and water-borne pathogens. After investigation, the U.S. EPA can establish maximum permissible levels of contaminants, referred to as maximum contaminant levels (MCL) that, above these concentrations, action can be taken against a violation.

Identification of priority contaminants begins the regulatory process and is followed by regulations on maximum concentrations of individual (or classes of, as is the case for trihalomethanes and haloacetic acids) compounds. This regulatory feature fits the historical paradigm of focusing on a single agent or class of agents as a proxy for the net toxicological impact of a given drinking water.

This “bottom-up” approach, where individual compounds are expected to provide sufficient information to characterize the complete mixture, may prove to be inadequate [1]. Drinking water disinfection chemically alters constituents of the drinking water to new, unexpected chemicals [5]. Half of all halogenated materials in chlorine-treated drinking water remain unidentified [6], preventing a single-compound research methodology from even beginning to assess the toxicology of potentially important chemical actors [7]. These new
chemical species are by-products of the disinfection process – drinking water disinfection by-products or DBPs – and have their own implications for long-term public health [8]. These effects, however, have not received sufficient attention to thoroughly understand their significance. Epidemiological studies have correlated certain cancers and adverse reproductive outcomes to DBP exposures. Individual DBP agents have been determined to be genotoxins, mutagens, teratogens, and carcinogens [8-11].

DBPs are the result of the chemical reactions of naturally-occurring organic matter, present in all source waters, and the strong oxidants such as chlorine, chloramine, hydrogen peroxide, or ozone. Many DBPs are halogenated. The first major drinking water disinfection by-products were determined in the 1970s and included the simple halogenated methyl groups. These trihalomethanes (THMs) were the first DBPs that were regulated under the Stage 2 rules promulgated by the U.S. EPA, under the authority of the Safe Drinking Water Act, and much of the existing literature uses this class to estimate the total toxicity and DBP levels of waters [12].

1.2.2 Unintended Consequences

In addressing these early DBPs, the Stage 2 Rules had their own unintended consequence. To meet the new compliance standards for total concentrations of DBPs, certain utilities have begun to move away from the traditional free chlorine systems of disinfection for alternative oxidants such as chloramine. Though the net THM load and the total amount of halogenated materials in treated waters is reduced with this alternative oxidant, new classes of compounds are more represented and a large percentage of by-products have not been characterized at all [13, 14]. The ultimate result of this transition is unknown, but a toolbox of toxicological assays can help to understand the implications of an increased burden of other
classes of compounds such as the nitrogenous DBPs and of complex mixtures of the treated waters as a whole, more accurately mimicking human exposure to these agents [15].

Drinking water disinfection is changing as new challenges are discovered. Disinfection and its byproducts are influenced by the materials present in the source of the water, whether natural or anthropogenic. Humic acids, a variety of biological molecules, salts, and metals are all present at different levels in even the most pristine of source waters. Especially in surface waters, variation in ecosystem activity cause levels of these materials to vary seasonally. New pressures on water supplies have resulted in a variety of additional materials to these mixtures, including increasing levels of nitrogenous materials from wastewater effluents that are recycled through treatment to be used again, rising levels of pharmaceuticals and personal care products, and a slew of industrial and agricultural contaminants. These new base materials alter the type and levels of DBPs in impacted waters [1, 16, 17]. With additional attention to the regulated DBP levels causing utilities to seek out novel technologies to come into compliance with the law, new alternatives to chlorine disinfection are producing new reaction conditions that alter the DBPs that are generated. Addressing the health effects of emerging DBPs is becoming ever more dynamic and complex.

1.2.3 Emerging Regulatory Authority on Complex Mixtures

Continuing this story of contaminants, the regulatory authority governing monitoring and control of drinking water has evolved over time through a series of amendments allowing for additional protection of source waters, practices to reduce certain contaminants from treatment to tap, and additional enforcement powers granted to the U.S. EPA. In 1996, amendments to the Safe Drinking Water Act required the Agency to:
develop new approaches to the study of complex mixtures, such as mixtures found in drinking water, especially to determine the prospects for synergistic or antagonistic interactions that may affect the shape of the dose-response relationship of the individual chemicals and microbes, and to examine noncancer endpoints and infectious diseases, and susceptible individuals and subpopulations.

Essentially, U.S. EPA must develop methods of analysis to address the potential hazards of complex mixtures in a way that departs from the historical single-compound approach to risk assessment and public health goals [18]. Research into “top-down” toxicology – that is, an assessment of the whole mixture sample, in opposition to the “bottom-up” approach of picking constituents and making inference of the total, or top-level toxicology – may help to determine other indicators of risk and respond more efficiently to emerging challenges [1, 17, 19].

This initiative has not been limited to drinking water. In the same year, Congress passed the Food Quality Protection Act of 1996. This act required investigation of complex mixtures of residues on foods that are believed to have similar mechanisms of action and therefore possibly resulting in enhanced toxicity when agents are combined [20]. The act focuses on pesticide residues in foods, but the shift in toxicological and risk assessment moves in the same direction as with the amendments to the Safe Drinking Water Act – a recognition that single-chemical analysis might be insufficient to protect human health and that analysis of complex mixtures might potentially provide more relevant insights to protect human health and the environment.

Initiatives like these have helped to establish research agendas related to complex mixtures. This thesis continues that priority. By selecting important mixtures – the organic fraction of disinfected drinking water containing a common contaminant or a large volume industrial waste stream – toxicologists can more effectively assess the environmental hazards presented by emerging technologies or possible hazards in a comparative manner. This research allows for interdisciplinary work with fellow scientists and engineers to determine the important factors at play for higher or lower toxicological impacts and allow for better development of less
toxic alternatives and ultimately inform best practices and truly guide protections for public and environmental health. The cases presented here assess generalizable biological endpoints in a series of different complex mixtures and some individual chemical agents to identify important sources of toxicological impact and allow for a better understanding of the public and environmental health risks presented by important water contaminants.

1.3 Complex Mixtures in Toxicology

1.3.1 Advantages of Analyzing Mixtures

Regulatory attention has turned towards investigating the potential health hazards of complex mixture exposures. There are a number of benefits associated with assessing complex mixtures of environmental and industrial origin.

- Complex mixtures can account for detrimental biological endpoints resulting from materials present in environmental samples that are unable to be characterized through conventional identification assays due to low concentration levels or high molecular weights.

- Mixtures allow for the assessment of multiple chemical insults at a time, providing a complete response to the mixture rather than extrapolating from a few select, though not necessarily representative, chemical constituents.

- Mixtures present a more relevant picture of exposure than individual compound assays. Ultimately, regulations are intended to protect biological systems and organisms; therefore, testing more relevant exposures like complex mixtures provide more relevant insight to biological endpoints of concern.
These are the driving reasons behind complex mixture assessment as an additional research focus to single-compound toxicological assays [1, 19, 21, 22].

Any combination of chemical agents, known or unknown, qualitatively or quantitatively, can be a mixture. Early toxicological work on mixtures involved two or more agents in defined concentrations. These well-defined mixtures with a limited number of species are considered to be “simple” mixtures. This type of testing can be useful if a specific pathway or mechanism is expected to be at work for a given biological endpoint. The better characterized a mixture is, the more easily its action can be teased out of experimental data. These experiments are also typically not limited by sample size, as a new stock can be made by mixing chemical standards. Because simple mixtures are more easily controlled, they are generally easier to work with [1].

Mixtures with ten or more chemical constituents are generally considered “complex.” Frequently, concentrations of individual chemical species in complex mixtures are unknown. If the mixture contains undefined constituents, it is also considered to be a complex mixture. Environmental samples that contain too many different constituents to characterize can be studied as complex mixtures and can provide important insight into the “real world” effects of contamination. Complex mixtures can more accurately represent the multitude of exposures organisms face at any time [1].

1.3.2 Difficulties of Testing Complex Mixtures

Unfortunately, there are some difficulties associated with analyzing complex mixtures.

- Complex mixtures tend to be less stable than well-defined, simple ones.
- Extraction efficiency can present problems when environmental samples are being prepared for analysis.
• Environmental samples are snapshots of dynamic systems and processes. Chemical constituents are ever-changing in a dynamic environmental system. Seasonal changes, meteorological patterns, and unknown factors can all influence the uncharacterized portion of an environmental sample used for testing. A representative sample from one time point may change dramatically between samplings. Samples taken from even the most controlled of industrial settings may have variables that are impossible to control for, generating greater variability than single-compound or simple mixture samples [23].

• Sample size can present a challenge. Environmental or industrial samples must be taken all at one time, which can be limited by the size of a reactor or the vessel for sampling. For testing and repeats, this must be a substantial volume. Ideally, 10-20 L of water is necessary for a single mixture to complete a battery of assays in efficient in vitro systems. If volumes of this scale are unavailable, the statistical power of the work can be limited. Whole animal studies of complex mixtures require incredible volumes of material that hinder their capacity to achieve statistical significance (for an important design, see [16]).

• Toxicological assays are conducted to determine generalizable conclusions, yet the more complex the mixture, the more specific the results become. Determining “how similar is similar” is an additional challenge to complex mixtures that is less of an issue in simple mixture or single compound analyses.

Despite these research challenges, the benefits of complex mixtures’ insight into public health risks and outcomes outweigh the drawbacks and limitations of this type of research [22].
1.4 Research Projects

This thesis focuses on two projects of interest. The first addresses the issue of emerging contaminants, either recently detected or the result of new chemical contaminants or processes, in drinking water supplies and the unexpected outcomes of drinking water disinfection. Iopamidol, a widely-used X-ray contrast medium and common pharmaceutical contaminant of surface and ground waters, is investigated as a potential source of highly toxic iodinated disinfection by-products. The specific chemistry of iopamidol’s degradation is still unknown. The biological feedback of toxicological assays of these complex mixtures allows for profiles of toxicity to be compared even without the identities of all of the individual degradation products. Different methods of disinfection are explored to more broadly determine the patterns of by-product generation. Here, differences in complex mixtures allow us to glean important chemical characteristics for further exploration in a way that would be impossible in a traditional single-chemical assay.

In the second project, the industrial waste generated by an amine-based carbon capture project is examined for its cytotoxicity and genotoxicity from both the complex mixture and single agent methods. Effluent from the combustion reaction is treated with amine mixtures at variable conditions to optimize for carbon capture efficiency, resulting in different end mixtures of chemicals. Special attention was given to high volume nitrogenous materials that were expected to be major contributors to the effluents’ overall cytotoxicity and genotoxicity. The results of the single-compound nitrosamines and nitramines provide references applicable to other mixtures containing nitrogenous materials, while the results of the complex mixture assessments help to determine whether or not these priority contaminants are actually driving the toxicological responses seen in the total effluent. The complex mixtures also provided a means
of comparison between different solvents and reactor conditions, allowing for the company to
determine which of the processes and proprietary solvents would be the least toxic of its options.

1.5 Research Objectives

- Assess, in a comparative manner, the organic fractions from these sample complex
  mixtures
- Compare generalizable biological endpoints (cytotoxicity, genotoxicity, mutagenicity) across chemical treatment groups to identify key components that
  might serve as measurable indicators of enhanced toxicity
- For the nitrogenous materials in the second project, assess the toxicity of important
  individual constituents to determine whether or not these chemical agents are the
  primary contributors to the overall toxic response
- Assess chemical conditions of samples (choice of disinfectant, presence or absence of
  important contaminants, reaction conditions) to identify process-based toxicological
  outcomes
- Provide clear, meaningful feedback to utilities or industry so as to inform engineering
  processes with biological information, relying on measures that have been used
  previously such as “toxic equivalence factors” [24]
Literature Cited

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CHAPTER 2
Formation of Toxic and Iodinated Disinfection By-Products from Compounds used in Medical Imaging\textsuperscript{1,2}

2.1 Abstract

Iodinated X-ray contrast media (ICM) were investigated as a source of iodine in the formation of iodo-trihalomethane (iodo-THM) and iodo-acid disinfection by-products (DBPs), both of which are highly genotoxic and/or cytotoxic in mammalian cells. ICM are widely used at medical centers to enable imaging of soft tissues (e.g., organs, veins, blood vessels) and are designed to be inert substances, with 95\% is eliminated in urine and feces unmetabolized within 24 h. ICM are not well removed in wastewater treatment plants, such that they have been found at elevated concentrations in rivers and streams (up to 100 µg/L). Naturally occurring iodide in source waters is believed to be a primary source of iodine in the formation of iodo-DBPs, but a previous 23-city iodo-DBP occurrence study also revealed appreciable levels of iodo-DBPs in some drinking waters that had very low or no detectable iodide in their source waters. When 10 of the original 23 cities’ source waters were re-sampled, four ICM were found—iopamidol, iopromide, iohexol, and diatrizoate—with iopamidol most frequently detected, in 6 of the 10 plants sampled, with concentrations up to 2700 ng/L. Subsequent controlled laboratory reactions of iopamidol with aqueous chlorine and monochloramine in the absence of natural organic matter (NOM) produced only trace levels of iodo-DBPs; however, when reacted in real source


\textsuperscript{2} For this paper, I was responsible for the cytotoxicity and genotoxicity assays conducted on sample sets with chloramination as the method of disinfection.
waters (containing NOM), chlorine and monochloramine produced significant levels of iodo-THMs and iodo-acids, up to 212 nM for dichloroiodomethane and 3.0 nM for iodoacetic acid, respectively, for chlorination. The pH behavior was different for chlorine and monochloramine, such that iodo-DBP concentrations maximized at higher pH (8.5) for chlorine, but at lower pH (6.5) for monochloramine. Extracts from chloraminated source waters with and without iopamidol, as well as from chlorinated source waters with iopamidol, were the most cytotoxic samples in mammalian cells. Source waters with iopamidol but no disinfectant added were the least cytotoxic. While extracts from chlorinated and chloraminated source waters were genotoxic, the addition of iopamidol enhanced their genotoxicity. Therefore, while ICM are not toxic in themselves, their presence in source waters may be a source of concern because of the formation of highly toxic iodo-DBPs in chlorinated and chloraminated drinking water.

2.2 Introduction

In a previous 23-city occurrence study, we measured the widespread presence of iodinated disinfection by-products (iodo-DBPs)—iodo-acids and iodo-trihalomethanes (iodo-THMs)—in chloraminated and chlorinated drinking water in the United States and Canada, up to 10.2 µg/L and 1.7 µg/L for individual iodo-THMs and iodo-acids, respectively [1]. Iodo-DBPs are highly genotoxic and cytotoxic, with iodoacetic acid being the most genotoxic DBP identified to-date [1, 2]. The primary source of iodine in iodo-DBPs is believed to be from natural iodide in source waters, and most plants in this previous study showed increasing levels of iodo-DBPs with increasing levels of natural iodide, which is to be expected, based on known iodide/chloramination chemistry [3, 4]. However, natural iodide levels were very low or not detected in some cases (e.g., Plants 2, 4, 11, and 15), such that iodo-DBP formation could not be
completely accounted for by natural iodide concentrations in the source waters (Table A.1, Supporting Information [SI]). Therefore, we investigated other potential sources of iodine that could contribute to iodo-DBP formation.

Iodinated X-ray contrast media (ICM) are widely used to enable medical imaging of soft tissues (e.g., organs, veins, blood vessels). ICM are large molecules (~600-700 Da) with triiodobenzoic acid analogues in their basic structures (Figure 2.1). Global consumption of ICM is approximately $3.5 \times 10^6$ kg/year; a single application can be up to 200 g. ICM are designed to be inert, with 95% unmetabolized and eliminated in urine and feces within 24 h [5]. Individual ICM differ mainly in their side chains, which contain hydroxyl, carboxyl, and amide moieties to impart elevated polarity and aqueous solubility [6].

Due to their incomplete removal in wastewater treatment plants, ICM have been found at elevated concentrations in rivers and streams [7-11]. Concentrations as high as 100 µg/L have been detected in a creek containing more than 50% wastewater [12]. ICM have also been found in groundwater and drinking water because they are somewhat recalcitrant during soil-aquifer passage and are not completely removed by activated carbon filtration or ozonation [10, 13-18]. ICM are primary contributors to the total organic halogen burden in clinical wastewater [19]. More than 90 % of the adsorbable organic iodine in wastewater and surface water can be attributed to ICM [9, 11, 14, 19-21]. Recently, Schulz et al. [22] and Kormos et al. [23, 24] identified 46 biotransformation products (TPs) of the nonionic ICM, iopromide, iohexol, iomeprol, and iopamidol from aerobic soil–water and sediment–water systems. These TPs, which still contain iodine in their structures, were subsequently found in municipal wastewater effluents [22-25].

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DBPs are generally formed by the reaction of disinfectants with natural organic matter (NOM), but anthropogenic contaminants can also react with disinfectants to form DBPs. Contaminant DBPs have been reported for pharmaceuticals, personal care products, estrogens, pesticides, textile dyes, alkylphenol surfactants, UV filters, and diesel fuel [26]. Contaminants with activated benzene rings or other functional groups that can react with chlorine and other oxidants are potential DBP precursors. Because of the widespread presence of ICM and the relatively high levels observed in surface waters, we investigated them as a potential source of the iodine in iodo-THM and iodo-acid DBPs in chlorinated and chloraminated drinking water.

2.3 Materials and Methods

2.3.1 Chemicals and Reagents

Iopamidol, iomeprol, iopromide, iohexol, and sodium diatrizoate were purchased from U.S. Pharmacopeia (Rockville, MD), Polysciences (Warrington, PA), Schering (Berlin, Germany), or Byk Gulden (Konstanz, Germany) at purities of 100, 97, 97.9, 100, and 100%, respectively. Iodo-THMs (dichloroiodomethane, bromochloroiodomethane, dibromoiodomethane, chlorodiiodomethane, bromodiiodomethane, and iodoform) and iodoacids (iodoacetic acid, bromoiodoacetic acid, (Z)-3-bromo-3-iodo-propenoic acid, (E)-3-bromo-3-iodo-propenoic acid, and (E)-2-iodo-3-methylbutenedioic acid) were purchased at the highest level of purity from Orchid Cellmark (New Westminster, BC, Canada), CanSyn Chem. Corp. (Toronto, ON, Canada), and Sigma-Aldrich. Commercial 10-13% sodium hypochlorite (NaOCl), purchased from Aldrich (Milwaukee, WI), contained equal-molar amounts of OCl⁻ and Cl⁻. All other organic and inorganic chemicals were certified ACS reagent grade and used without further purification.
2.3.2 Controlled Laboratory Reactions

Aqueous stock solutions and experiments utilized purified water (18 MΩ cm\(^{-1}\)) from a Barnstead ROPure Infinity™/NANOPure™ system (Barnstead-Thermolyne Corp., Dubuque, IA). The pH was monitored with an Orion 940 pH meter equipped with a Ross combination electrode (Thermo Scientific, Waltham, MA). For pH adjustment, either 1 N H\(_2\)SO\(_4\) or NaOH was used prior to oxidant addition. Glassware and polytetrafluoroethylene (PTFE) septa were soaked in a concentrated free chlorine solution (6%) for 24 h, rinsed with deionized water, and dried prior to use. Source waters (Oconee River) were obtained from the Athens-Clarke County (ACC) Drinking Water Treatment Plant in Athens, GA, and filtered (using 5.0 and 0.45 µm filters, Millipore Corp., Billerica, MA) prior to use. Source water characteristics are shown in Table A.2. Chlorination kinetic experiments were conducted under pseudo first-order conditions with total chlorine, [Cl\(_2\)]\(_T\), to iopamidol molar ratios of 5:1, 6.7:1, 10:1, 20:1, and 40:1. Chlorine was added to solutions under rapid-mix conditions using a magnetic stir plate and a PTFE-coated stir bar. Reactions were conducted over 72 h and in duplicate unless otherwise stated. The pH was maintained using 10 mM of phosphate (pH 6.5 and 7.5), borate (pH 8.5), or carbonate (pH 9.0) salts. Aqueous chlorine solutions were diluted to 250 mM and added to the aqueous solution containing iopamidol and buffer in a 500 mL Erlenmeyer flask. Four aliquots were then placed into 120 mL amber reaction vessels with PTFE septa and stored in the dark at 25 °C. Monochloramine experiments followed the same experimental protocol, and utilized additions of preformed monochloramine to avoid potential artifacts caused by reactions of excess free chlorine that may briefly exist if monochloramine was formed \textit{in-situ} (27). Monochloramine solutions were prepared by mixing 5.64 mM ammonium chloride with 3.7 mM hypochlorous
acid to achieve the desired 0.7 Cl/N molar ratio. The solution was allowed to react and equilibrate for 30 min in 10 mM bicarbonate buffer, pH 8.5, prior to use.

Oxidant residuals were quenched with 120 µM sodium sulfite (20% excess of initial chlorinated oxidant concentration; prepared fresh in deoxygenated water). The stability of iodo-DBPs in the presence of sodium sulfite was examined and is discussed in Supporting Information. Samples were extracted immediately after quenching to eliminate the chance for potential degradation of iodo-DBPs. At the end of each experiment, residual aqueous oxidant concentrations were determined using the N, N-diethyl-p-phenylenediamine titrimetric method [28].

2.3.3 ICM Measurements

Source waters were collected from drinking water treatment plants from 10 of the 23 cities in the 23-city iodo-DBP occurrence study [1]. Samples were collected in 2-L Teflon bottles (headspace free) and shipped on ice packs (2-day delivery) to the Federal Institute of Hydrology (Koblenz, Germany). Water samples were extracted using solid-phase extraction and analyzed using liquid chromatography (LC)/electrospray ionization (ESI)-mass spectrometry (MS)/MS, according to a previously published method [12]. Further details are also available in SI.

2.3.4 Iodo-THM, Iodo-acid, Iodate, and Iodide Measurements

Iodo-THM measurements were carried out using liquid-liquid extraction and gas chromatography (GC) with electron ionization-MS; iodo-acid measurements were carried out using liquid-liquid extraction, diazomethane derivatization, and detection by GC/negative
chemical ionization (NCI)-MS, according to a previously published method with minor modification [1]. Iodide and iodate were measured using ion chromatography (Dionex LC30 chromatography oven, ASRS ion suppressor, AD25 absorbance detector). Further details are available in SI.

2.3.5 Total Organic Carbon and SUVA Measurements

Total organic carbon (TOC) was measured using a Shimadzu TOC 5000 (Shimadzu Scientific, Columbia, MD). UV absorbance was measured using a Shimadzu UV1601 spectrophotometer.

2.3.6 Mammalian Cell Cytotoxicity and Genotoxicity

Mammalian cell cytotoxicity and genotoxicity measurements were conducted on organic extracts of source waters (20 L each) spiked with iopamidol (5 µM) and treated with chlorine or monochloramine. Controls included raw source waters spiked with iopamidol (no oxidant) and raw source waters treated with chlorine or monochloramine (no iopamidol). Treated waters were concentrated using XAD resins (40 mL XAD-8 over 40 mL XAD-2), as described in a previously published procedure [29]. Chinese hamster ovary (CHO) cells, line AS52, clone 11-4-8 were used for the cytotoxicity and genotoxicity analyses of the water concentrates [30]. These assays have been described in the literature [31, 32]; detailed descriptions of each assay are presented in SI. For chronic cytotoxicity (72-h exposure), a series of concentrations were analyzed with 4-8 replicates per concentration. A concentration-response curve was generated and regression analysis used to calculate the %C½ value. This value is analogous to LC₅₀ and is the concentration that induced a cell density at 50% of the negative control. A one-way analysis
of variance (ANOVA) test was conducted to determine whether the water concentrate induced a significant level of cell killing. If a significant $F$ value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted. The power of the test statistic was maintained as 0.8 at $\alpha = 0.05$. To determine the acute genotoxicity of the water concentrates, single-cell gel electrophoresis (SCGE) was employed; it quantitatively measures genomic DNA damage induced in individual nuclei of treated cells [32]. CHO cells were exposed for 4 h at 37°C, 5% CO$_2$. Each experiment included a negative control, a positive control (3.8 mM ethylmethanesulfonate), and 9 water extract concentrations. The concentration range was determined by measuring acute cytotoxicity with a vital dye. After treatment, cells were harvested, embedded in an agarose microgel, and lysed; the DNA was denatured and electrophoresed under alkaline conditions. Using Komet 3.1 software, the primary measure of DNA damage was the % tail DNA which is the amount of DNA that migrated from the nucleus into the agarose gel. Within the concentration range that allowed for 70% or greater viable cells, a concentration-response curve was generated, and a regression analysis was used to fit the curve. The SCGE genotoxic potency value was determined as the midpoint of this curve. The % tail DNA value for each microgel was determined, and the data were averaged among all microgels for each water extract concentration. The % tail DNA values were analyzed with an ANOVA test. If a significant $F$ value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted. The power of the test statistic was maintained as 0.8 at $\alpha = 0.05$. 
2.4 Results and Discussion

2.4.1 Discovery of ICM in Source Waters

When source waters for 10 of the original 23 cities in the iodo-DBP occurrence study were reexamined for the presence of five commonly used ICM, four of them were detected: iopamidol, iopromide, iohexol, and diatrizoate (Table 2.1). Iopamidol was the ICM most frequently detected, in 6 of the 10 plants sampled, up to 2700 ng/L.

2.4.2 Controlled Laboratory Reactions

As a result, controlled laboratory reactions were initiated to determine whether iopamidol could be a source of iodine in the formation of iodo-THM and iodo-acid DBPs in drinking water. When iopamidol was reacted with free chlorine in purified water (in the absence of NOM), only trace levels of iodo-DBPs were observed (e.g., dichloriodomethane ranged from 2.2 to 10.7 nM and iodo-acids were not detected; Table A.3). However, when iopamidol was dissolved in actual source waters (river water), chlorination produced significant levels of iodo-DBPs, up to 212 nM for dichloriodomethane and 3.0 nM iodoacetic acid (Figure 2.2; Table A.3). Dichloriodomethane and chlorodiiodomethane were the predominant iodo-THMs formed, and iodoacetic acid was the predominant iodo-acid formed. Other iodo-acids were occasionally detected, but were always below the minimum quantification limit (0.5 nM). Iodate was also formed with maximum formation at pH 7.5 and 8.0 (Figure A.1). In contrast, no iodo-DBPs were detected in a control of the same raw source water reacted with chlorine in the absence of iopamidol. It is evident that both NOM and iopamidol are important precursors in the formation of iodo-DBPs and that iopamidol was likely the source of iodine in the structures of these iodo-DBPs. In general, iodo-THMs were increased in formation with chlorine at higher pH (pH 7.5
and 8.5, relative to pH 6.5), with up to 212 nM of dichloroiodomethane at pH 8.5 vs. 43.7 nM at pH 6.5.

Monochloramine also produced iodo-DBPs from iopamidol in NOM-containing source waters, but the pH trend was the opposite of chlorine, such that increased iodo-DBPs were found at lower pH (6.5) (Figure 2.2, Table A.3). Overall, iodo-THM concentrations were much lower with monochloramine as compared to chlorine.

Because this is the first report of DBPs from an ICM, we investigated the potential for iodide or other impurities in the iopamidol that could be responsible for iodo-DBP formation. While the iopamidol was reported by the manufacturer to be 100% pure, a 2 g/L solution was prepared in purified water and analyzed for the presence of iodide impurities with ion chromatography. With detection limits for iodide (and iodate) of 1.0 µM, no inorganic iodide compounds were observed. In addition, we dissolved iopamidol in methanol and analyzed by full-scan GC/MS for potential impurities, as well as extracted iopamidol in purified water with ethyl acetate and analyzed this extract by full-scan GC/MS. Half of this extract was also derivatized with diazomethane to investigate for carboxylic acid impurities. When these samples were compared to their corresponding solvent/derivatization blanks, no impurities were detected. A similar check by LC/MS/MS revealed no signs of impurities in the iopamidol. In addition, the formation of iodo-DBPs and/or disappearance of iopamidol were consistently observed with different lots of iopamidol and with two different source waters (Oconee River, Athens, GA, and Rhine River, Koblenz, Germany) collected over separate times of the year. In separate experiments conducted with Suwannee River fulvic and humic acid as the NOM source in purified water (SI), iodo-THMs and iodo-acids were formed at increasing levels with increasing fulvic/humic acid doses and behavior was comparable to that observed with real source waters.
(Figure A.2). Therefore, all data pointed to the iodo-DBPs being formed by the iopamidol itself (along with NOM) and did not appear to be an artifact from potential impurities.

Iodo-DBP formation was also investigated as a function of increasing iopamidol dose and time. Figure 2.3 shows the formation of dichloroiodomethane as a function of increasing iopamidol dose and time at pH 8.5. From these data, it is evident that iodo-DBPs increased in formation with increasing levels of iopamidol. To understand the reaction of iopamidol with aqueous chlorine, iopamidol degradation experiments were conducted in the presence of excess aqueous chlorine over the pH range of 6.5-9 without NOM present. A plot of the pseudo first-order rate constant vs. pH shows an increase in reaction rate of chlorine with iopamidol from pH 6.5 to 9.0, with the rate leveling off at pH 8.0-9.0 (Figure A.3). The pH dependence suggests that OCl\(^-\) may be the primary reactive species with iopamidol (pKa of 7.5 for dissociation of HOCl to OCl\(^-\)) (Figure 2.4). In reactions involving monochloramine, we also propose that OCl\(^-\) may be the primary reactive species. Below pH 8.5, where increased iodo-DBP levels were observed, monochloramine is less stable and hydrolyzes to form HOCl and NH\(_3\). When excess concentrations of ammonia were applied (chlorine:ammonia molar ratios of 0.7, 0.5, 0.1, 0.05, and 0.025 mol/mol) at pH 8.5, concentrations of iodo-DBPs and iodate were monitored as a function of time. Iodate was not formed, and the concentrations of iodo-DBPs were reduced by 60\% or greater as function of increasing residual ammonia concentrations. Excess ammonia favors the formation of NH\(_2\)Cl and inhibits the hydrolysis back-reaction to form HOCl (and OCl\(^-\)) (Figure 2.4). Hypochlorite ion may be the reacting species, but not by the conventional known iodide oxidation pathway [3, 4]. OCl\(^-\) is known to act as a nucleophile towards tetrahedral phosphorus and carbonyl functional groups [33, 34]. From preliminary investigations of high molecular weight reaction species, it appears that OCl\(^-\) may be initially attacking one of
the amide side chains; however, the detailed mechanism is not known at this time. Complete product studies of iopamidol in the presence of aqueous chlorine are currently in progress.

2.4.3 Comparison Reactions with Iodide

In separate experiments, raw source waters were spiked with 5 µM iodide and reacted with chlorine or monochloramine to compare with the iopamidol reactions. As expected from the iodo-DBP occurrence study [1] and from a previous controlled laboratory study that measured iodo-THMs [3], chloramine plus iodide formed much higher levels of iodo-DBPs than chlorine plus iodide (Figure 2.5). Dichloroiodomethane and iodoacetic acid were formed at a maximum of 579 and 39 nM, respectively, in the chloraminated water, and up to 114 and 3.7 nM, respectively, in the chlorinated water. The behavior was quite different from reactions with iopamidol, where chlorine forms higher levels than monochloramine. It should be noted that in these experiments with iodide, chlorine was completely consumed at 24 h, while monochloramine had measurable residuals at 72 h. This is different from reactions with iopamidol, where chlorine residuals were still detectable at 72 h. The rapid reaction of chlorine with iodide is consistent with the rapid sequential oxidation of iodide to iodate (Figure A.4), which happens with chlorine, but not with monochloramine, due to much slower rates of oxidation of hypoiodous acid (HOI) to iodate [33, 34]. Iodo-THM and iodo-acid levels measured in the chlorine reactions at 24, 48, and 72 h were essentially the same, such that there was no degradation (e.g., hydrolysis) over 72 h at pH 6.5, 7.5, and 8.5.
2.4.4 Mammalian Cell Cytotoxicity and Genotoxicity

Because iodo-THMs and iodo-acids are known to be cytotoxic and/or genotoxic, we investigated the mammalian cell cytotoxicity and genotoxicity of the reaction mixtures of chlorine or monochloramine with iopamidol and NOM. Mammalian cell cytotoxicity and genotoxicity results supported the formation of toxic iodo-DBPs from iopamidol. Concentration-response curves for experiments that measured chronic CHO cell cytotoxicity with chlorine or chloramine as the disinfectant are presented in Figure 2.6A and 2.6B, respectively. Comparative chronic CHO cell cytotoxicity demonstrated that extracted ACC water was one of the least toxic samples ($%C_{50} = 158.1\times$, Table 2.2), as well as an extraction of a pure water blank (data not shown). ACC water with iopamidol from two different sampling experiments expressed an average $%C_{50}$ value of 127.4$\times$, which suggests that these organic extracts are slightly more cytotoxic than the ACC source water extracts (Table 2.2). Disinfection with chlorine or chloramine increased the cytotoxicity of the extracts by 4.5-fold or 7.1-fold, respectively, based on their $%C_{50}$ values (Figure 2.6A, 2.6B, Table 2.2). Finally, extracts from reaction mixtures containing iopamidol and chlorine in ACC water was slightly more cytotoxic than the corresponding source waters treated with chlorine (Figure 2.6A). For chloramine disinfection, there appears to be little effect on chronic cytotoxicity with or without iopamidol (Figure 2.6B, Table 2.2).

Experiments that measured genomic DNA damage with chlorine or chloramine as the disinfectant are presented in Figures 2.6C and 2.6D, respectively. Organic extracts from ACC water alone or ACC water with iopamidol were negative or very weakly genotoxic. After disinfection by chlorine or chloramine, the ACC source water extracts expressed significant genotoxicity because of DBP formation. Notably, the addition of iopamidol in ACC water
disinfected with chlorine or chloramine resulted in a 1.7-fold or 1.3-fold increase in genotoxicity, respectively (Figure 2.6C, 2.6D, Table 2.2).

In Table A.4 (SI), we calculated the weighted impact by the analyzed iodo-DBPs that resulted in the toxicity observed in Figure 2.6. These data indicate that the descending rank order of the iodo-DBPs that contributed to the greatest cytotoxicity response for the chlorinated water sample (with iodopamidol) was iodoacetic acid (IAA) > ClI₂ > Cl₂I > I₃ > ClBrI. The descending rank order for the chloraminated water sample (with iopamidol) was IAA > ClI₂ > Cl₂I. Clearly, the major driver of chronic cytotoxicity was the presence of IAA. IAA was also implicated as the iodo-DBP agent inducting the largest level of genotoxic response in these water samples.

During the past decade, we demonstrated that iodinated DBPs are generally more cytotoxic and genotoxic than their brominated or chlorinated analogs. This trend holds true for DBP classes including the THMs [1, 31], haloacids [1, 2, 35], haloacetonitriles [36], and haloacetamides [37]. Recent comparative human cell toxicogenomic analyses of the monohaloacetic acids demonstrated that iodoacetic acid modified the expression of more human genes associated with adverse health outcomes than bromo- or chloroacetic acid [38]. The fact that iopamidol can generate iodo-DBPs after disinfection and that iodo-DBPs demonstrate higher levels of toxicity support concerns that ICM may have adverse impacts upon public health and the environment when they are released into wastewaters.

2.5 Future Research and Implications

As indicated in the previous 23-city iodo-DBP occurrence study, natural iodide is probably still the most important source of iodine in the formation of iodo-DBPs, especially for
chloraminated drinking waters [1]. However, it is evident from the current study that the ICM, iopamidol, can also be a source of iodine in these DBPs for both chlorinated and chloraminated drinking water.

Of the ICM investigated to-date, iopamidol appears to be the most important contributor to iodo-DBP formation. Preliminary experiments were conducted using two other ICM (iohexol and iopromide) that were found in three of the 10 source waters re-sampled from the 23-city iodo-DBP occurrence study. Similar to iopamidol, iohexol and iopromide formed only trace levels of iodo-DBPs when chlorination and chloramination reactions were carried out in purified water (in the absence of NOM). But, unlike iopamidol, which produced significant levels of iodo-DBPs in real source waters (containing NOM) treated with chlorine and monochloramine, iohexol and iopromide did not form appreciable levels (Tables A.5 and A.6, SI). Further research is underway to understand this behavior and also to establish a detailed mechanism of formation of iodo-DBPs from iopamidol, including the site of initial reaction with chlorine/monochloramine, high molecular weight iopamidol intermediates, and toxicity resulting from these reactions.

2.6 Acknowledgements

We would like to thank Michael Bartlett of the University of Georgia for the generous use of his mass spectrometer for early experiments, as well as the drinking water treatment plants for providing us source waters. The toxicology was supported by the Center of Advanced Materials for the Purification of Water with Systems, a National Science Foundation Science and Technology Center, under Award CTS-0120978 and Illinois/Indiana Sea Grant R/WF-09-06. Although this work was reviewed by EPA and approved for publication, it may not necessarily
reflect official Agency policy. Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the U.S. EPA.

2.7 Supporting Information

Additional information on experimental methods, including additional figures and tables follows in Appendix A.
Table 2.1 ICM in U.S. Drinking Water Sources (ng/L)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Iopamidol</th>
<th>Iomeprol</th>
<th>Iopromide</th>
<th>Iohexol</th>
<th>Diatrizoate</th>
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### Table 2.2 CHO Cell Chronic Cytotoxicity and Acute Genotoxicity of Water Concentrates

#### CHO Cell Chronic Cytotoxicity Results

<table>
<thead>
<tr>
<th>Water Samples</th>
<th>Conc. Factor Range (x-Fold)</th>
<th>%C½ Value (LC₅₀)</th>
<th>R²</th>
<th>Lowest Toxic Conc. Factor</th>
<th>ANOVA Statistic</th>
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</thead>
<tbody>
<tr>
<td>ACC Water + Iopamidol</td>
<td>10 - 200</td>
<td>85.9x</td>
<td>0.98</td>
<td>50.0x</td>
<td>F₁₀,₁₁₇ = 58.1; P ≤ 0.001</td>
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<tr>
<td>ACC Water + HOCl</td>
<td>5 - 50</td>
<td>35.0x</td>
<td>0.96</td>
<td>30.0x</td>
<td>F₁₂,₁₆₈ = 34.3; P ≤ 0.001</td>
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<td>23.5x</td>
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<td>10.0x</td>
<td>F₁₀,₁₀₅ = 159; P ≤ 0.001</td>
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<td>ACC Water + Iopamidol</td>
<td>10 - 350</td>
<td>168.9x</td>
<td>0.99</td>
<td>100x</td>
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<td>ACC Water + NH₂Cl</td>
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<td>22.4x</td>
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<td>20x</td>
<td>F₁₀,₇₇ = 378; P ≤ 0.001</td>
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<td>ACC Water + Iopamidol + NH₂Cl</td>
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<td>23.5x</td>
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<td>75x</td>
<td>F₁₀,₇₇ = 185; P ≤ 0.001</td>
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#### CHO Cell Acute Genomic DNA Damage (SCGE) Results

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<th>Water Samples</th>
<th>Conc. Factor Range (x-Fold)</th>
<th>SCGE Genotox Potency Value</th>
<th>R²</th>
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<th>ANOVA Statistic</th>
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<tr>
<td>ACC Water + Iopamidol</td>
<td>40-1000</td>
<td>NG</td>
<td>NA</td>
<td>NS</td>
<td>F₁₃,₂₀ = 1.39; P = 0.25</td>
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<td>ACC Water + HOCl</td>
<td>40 - 480</td>
<td>285x</td>
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<td>ACC Water + Iopamidol + HOCl</td>
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<td>166x</td>
<td>0.95</td>
<td>160x</td>
<td>F₁₀,₃₉ = 21.2; P ≤ 0.001</td>
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<td>ACC Water + Iopamidol + NH₂Cl</td>
<td>120-1000</td>
<td>588x</td>
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<td>ACC Water</td>
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<td>NA</td>
<td>NS</td>
<td>F₀,₅₀ = 1.69; P = 0.12</td>
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</tbody>
</table>

---

a Athens-Clarke County (ACC) source water (river water) with and without disinfection and with and without iopamidol (IPM). b The %C½ value is the concentration factor of the extract determined from a regression analysis of the data that induced a cell density of 50% as compared to the concurrent negative control. c The coefficient of determination for the regression analysis upon which the %C½ value was calculated. d Lowest toxic concentration factor of the water concentrate in the concentration-response curve that induced a significant reduction in cell density as compared to the negative control. e The degrees of freedom for the between groups and residual associated with the calculated F-test result and the resulting probability value. f Source waters were collected at different times of the year for these two sets of experiments involving either chlorination or chloramination. g The SCGE genotoxic potency value is the concentration factor that was calculated, using regression analysis, at the midpoint of the curve within the concentration range that expressed above 70% cell viability. h The coefficient of determination for the regression analysis upon which the genotoxic potency value was calculated. i Lowest genotoxic concentration factor of the water concentrate in the concentration-response curve that induced significant genomic DNA damage as compared to the negative control. j Non-genotoxic. k Not significant.
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<td>Diatrizoate</td>
<td>737-31-5</td>
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**Figure 2.1** Chemical structures of ICM commonly used for medical imaging.
Figure 2.2  Iodo-DBP formation at 72 h of reaction mixtures containing iopamidol, aqueous chlorine or chloramine, and natural source waters.  [Cl₂] or [NH₂Cl] = 100 µM, [iopamidol] = 5 µM, [Buffer] = 10 mM, temperature = 25 °C, and [TOC] = 2.1 mg/L.  Cl₂I = dichloroiodomethane, ClBrI = bromochloroiodomethane, Br₂I = dibromoiodomethane, ClI₂ = chlorodiiodomethane, BrI₂ = bromodiiodomethane, I₃ = iodoform, IAA = iodoacetic acid, BrIAA = bromoiodoacetic acid, Z = (Z)-3-bromo-3-iodopropenoic acid, E = (E)-3-bromo-3-iodopropenoic acid, diacid = (E)-2-iodo-3-methylbutenedioic acid.
Figure 2.3 Dichloroiodomethane formation as a function of time at pH 8.5 in reaction mixtures containing iopamidol, natural source waters, and (A) aqueous chlorine, or (B) monochloramine. [Cl₂]ₜ = 100 µM, [Buffer] = 10 mM, temperature = 25 °C, and total organic carbon [TOC] = 2.1 mg/L.
Figure 2.4 Proposed pathway for formation of iodo-DBPs from iopamidol.
Figure 2.5  Iodo-THM formation at 24 h of reaction mixtures containing iodide, NOM, and aqueous chlorine (A) or monochloramine (B). \([\text{Cl}_2]_T = 100 \mu\text{M}, [\Gamma] = 5 \mu\text{M}, \text{[Buffer]} = 10 \text{mM}, \text{temperature} = 25 ^\circ\text{C}, \text{and total organic carbon [TOC]} = 2.1 \text{mg/L.} \) \text{Cl}_2\text{I} = \text{dichloroiodomethane, } \text{ClBrI} = \text{bromochloroiodomethane, } \text{Br}_2\text{I} = \text{dibromoiodomethane, } \text{ClI}_2 = \text{chlorodiiodomethane, } \text{BrI}_2 = \text{bromodiiodomethane, } \text{I}_3 = \text{iodiform, } \text{IAA} = \text{iodoacetic acid, } \text{BrIAA} = \text{bromoiodoacetic acid, } \text{I}_2\text{AA} = \text{diiodoacetic acid, } Z = (Z)-3\text{-bromo-3-iodopropenoic acid, } E = (E)-3\text{-bromo-3-iodopropenoic acid, } \text{diacid} = (E)-2\text{-iodo-3-methylbutenedioic acid.}
Figure 2.6  (A) Concentration-response curves of CHO cell chronic cytotoxicity of organic extracts of Athens-Clarke County (ACC) source water with iopamidol (IPM), ACC water after chlorination, and ACC water plus iopamidol plus chlorination (for reactions carried out at pH 7.5).  (B) Concentration-response curves of CHO cell chronic cytotoxicity of organic extracts of ACC source water with IPM, ACC water after chloramination, and ACC water plus IPM plus chloramination.  (C) Concentration-response curves of CHO cell acute genotoxicity of organic extracts of ACC water with IPM, ACC water after chlorination, and ACC water plus IPM plus chlorination.  (D) Concentration-response curves of CHO cell acute genotoxicity of organic extracts of ACC water with IPM, ACC water after chloramination, and ACC water plus IPM plus chloramination.  Note:  ACC source waters for the chlorination and chloramination experiments, respectively, were collected at two different times in the year.
Literature Cited


3.1 Introduction

Toxicology has applications to nearly any biological or engineering question. Exposure to physical and chemical agents in the environment can damage the biological integrity of tissues or organisms, causing adverse consequences for biological systems and organisms. A growing concern about global climate change and greenhouse gas emissions has encouraged engineers and energy utilities to investigate possible measures to limit greenhouse gas emissions to the atmosphere. While the enthusiasm for this innovation is needed, a successful response to the challenge of climate change must not ultimately cause sufficient harm to the environment so as to simply exchange one problem for another. By integrating biological toxicology into engineering decisions surrounding the development of amine-based carbon capture technologies, scientists and engineers can work together to develop and improve best practices associated with carbon capture systems. Currently, Statoil ASA in Norway is developing the largest commercial-scale carbon sequestration facility attached to a fossil fuel power plant at Mongstad. With supporting analytical biological work, the toxicology data can help to direct this facility and subsequent carbon capture plants towards the safest optimal procedures.

3.2 Carbon Capture Process and Chemistry

The amine-based carbon capture systems being used in pilot facilities today and proposed for the Statoil facility in Mongstad are not a new idea. Amine solvents have been used for decades to remove acid gasses, including CO$_2$ and H$_2$S, from natural gas. Efforts to use amines
specifically for the capture of carbon were piloted during the late 1970s and early 1980s in an attempt to provide an economical source for carbon dioxide to be used for enhanced oil recovery, but the process did not prove to be profitable and the technology was not introduced to commercial-scale power plants [1]. Additional applications include using amines to capture dilute carbon dioxide from air supplies on submarines [2]. With the advent of carbon taxes and an increased attention to global climate change, interest in carbon capture has been renewed for economic and environmental reasons. This type of emissions control is especially appealing because the amine-capture technology has already been proven effective at capturing 70-90% of CO$_2$ and can be adopted by existing coal-fired power plants by retrofitting with absorber and regenerator columns in a manner similar to the addition of sulfur controls [1].

The CO$_2$ is removed from flue gas by the addition of a number of steps following the combustion controls already in place. The emissions from the combustion (energy generating) stage are scrubbed for removal of acidic gases that are already subject to regulations. Rather than being released, the gas is modified, to alter the temperature or other gaseous constituents, for example, in order to enhance the efficiency of capture. The modified stream is sent through a secondary scrubber to strip CO$_2$ from the effluent. The aqueous amine solution is sprayed over the gas and collects at the base of the column. This material is then recovered and undergoes physical alterations to release the CO$_2$ for collection and regenerate the solvent for reuse (Figure 3.1). Side reactions during both CO$_2$ collection and release can result in unexpected by-products and loss of solvent. Understanding these processes are essential for informed assessment of both the efficiency of the process as well as any potential hazards it presents to human health and the environment.
While a variety of amines can be used in the carbon capture process, the fundamental chemistry remains unchanged. When the amine solution is introduced to the flue gas stream in the absorber column (Figure 3.2), the acidic carbon dioxide gas reacts with the basic amine solution and forms a water-soluble product. When heated in the regenerator column, the reaction reverses, releasing the carbon dioxide and regenerating the amine to be recycled through the absorber column again [3, 4]. A number of different but related mechanisms have been proposed but can be simplistically summarized in Figure 3.3. After the carbon dioxide is captured, changes in heat and pressure liberate the CO$_2$ and give off a stream of water and carbon dioxide. The gas must first be dried before the CO$_2$ can be condensed and stored.

While much of the solvent is regenerated, there is some loss every cycle. The amines used are very sensitive to other materials typically found in flue gas from coal-fired power plants. SO$_X$ is a main concern, as SO$_2$ levels of less than 10 ppm are advisable but typical concentrations for flue gases from coal-fired power plants range from 700-2500 ppm [1]. NO$_X$ are also constituents of concern, with NO$_2$ being the most important species for side reactions. Oxygen, fly ash, and carbon monoxide are also believed to be responsible for fouling of the solvent and producing heat-stable amine salts that can promote further corrosion, but these reactions have not been thoroughly investigated [1]. Generalizations about the performance of amine carbon capture systems on coal-fired power plants is further complicated by the variation in type and quality of coal used by a given facility, resulting in varying compositions of flue gas from source to source [5, 6].

The reactive amines can also cause corrosion problems, placing an upper limit on the total concentration of the amine in solution of 20-30% amine by weight. The process’s efficiency is also strained because flue gasses from many power plants are relatively lean in carbon dioxide,
comprising less than 15% of the stream and leaving an abundance of materials that can potentially react in undesirable ways with the amine. The low concentrations require high rates of solvent cycling. Coal flue gas is typically released at ambient pressure and relatively high temperatures, encouraging degradation of the amine.

Solvent degradation is more complex and less well understood than the reactions that are believed to govern the capture chemistry. A number of pathways have been put forward, but a common feature is the production of ammonia and vinyl or an organic acid that serve as important intermediates to the mixture of organics recovered in wash water [Figure 3.4] [4]. Other mechanisms emphasize a series of radical reactions catalyzed by iron (III) [1, 7]. Harsher conditions for absorption and recovery have typically resulted in greater volumes and higher toxicity of waste. It is believed that the ammonia generated results from the absorption of O₂ present in the flue gas. An alternative scheme focuses on oxygen [Figure 3.5] [8].

Only two studies attempted to identify actual materials present in the waste water following solvent regeneration. A number of organics were identified in the water at the bottom of the reclamer at the IMC Chemicals Facility in Trona, CA [4]. While this work provides a basic guideline for chemical constituents in a carbon capture system, cursory investigations for the Mongstad facility indicate important differences, such as the detection of nitrosamines. A summary table of the more prominent constituents found by Strazisar and colleagues is presented in Table 3.1 [4].

This diverse array of products certainly qualifies as a complex mixture. Because of the variety of materials produced in side reactions with the solvents involved in the carbon sequestration systems, toxicology testing must be done on complex mixtures. In assessing these complex mixtures, constituents expected to be major contributors to the overall toxicity of the
samples are being investigated as well. Assays can be developed to capture enough information about a given mixture to compare its relative toxicity and potential to harm the environment to other alternatives. In this way, the analytical biology can direct a technology towards a more benign process.

### 3.3 Priority Individual Agents

Nitrosamines have been identified as a primary concern as byproducts of the carbon sequestration process. As a chemical class, nitrosamines have been shown to be highly carcinogenic. Of the over 300 nitrosamines tested for carcinogenicity, approximately 90% were positive [9]. Tests of individual nitrosamines have shown a number of interesting features, including an observable carcinogenic effect on all species tested and a high degree of organ specificity in carcinogenic activity. The nitrosamines are promutagens and require cytochrome P450-mediated metabolic activation to induce DNA damage. Most respond to a rat liver microsomal fraction such as S9.

Nitramines are expected to be important by-products of carbon capture systems. Structurally, the nitramines are very similar to the nitrosamines. The nitramines have an additional oxygen atom bonded to the secondary nitrogen instead of the nitrosamine’s single oxygen, providing resonance stabilization that helps to explain the lower reactivity of the chemical class. Nitramine stability has historically meant less attention in the literature, as the nitrosamines more frequently deliver positive toxicity results. While less reactive, their stability gives the nitramines a longer persistence in the environment. Without a thorough investigation of their toxicity, the assumptions on relative safety of nitramine constituents in the effluent have not
been confirmed. This study represents the first systematic, analytical, comparative toxicity studies of the two chemical classes together.

Beyond the scope of the immediate project, nitrosamines and nitramines are independently important environmental contaminants. Both are nearly ubiquitous and have many routes of exposure to humans. These nitrogenous materials are found in as diverse media as cooked or processed foods, personal care products, tobacco smoke, and drinking water. Detection in drinking waters is on the rise as more utilities convert from chlorine disinfectant to chloramine to come into compliance with the Stage 2 rules. Further impacted source waters for drinking water utilities, caused by increased development and rising populations, also provide additional available nitrogen that can be converted into these contaminants [10]. Nitrosamines can even be generated endogenously when nitrites encounter the acidic environment in the stomach [11]. The research described in this thesis has broad relevance both within and beyond the project at hand. Individual agents identified for analysis and their structures are presented in Table 3.2 and Figure 3.6, respectively.

3.4 Materials and Methods

3.4.1 Reagents

General reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St. Louis, MO). Media and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Logan, UT) or from Fisher Scientific Co. (Itasca, IL).
3.4.2 Safety

All experiments were conducted in certified stage 2 biological safety cabinets. Waste was stored of in designated “Toxic Waste” bins and disposed of in accordance with the University of Illinois’s safety policies.

3.4.3 Mammalian Cell Culture

Chinese hamster ovary (CHO) cells have been widely employed in *in vitro* toxicology studies. An image of CHO cells is presented in Figure 3.7. As a mammalian cell line, they more closely parallel human and animal tissues than the bacterial models such as *E. coli* and *S. typhimurium*. CHO are also very stable models – they express normal morphology and cell contact inhibition, contain a stable chromosome complement, and have a consistent cell doubling time. These features are often not true of transformed or tumor cell lines, making data from the neoplastic cell lines less consistent than with CHO studies. Transgenic CHO cell line AS52 was derived from the parental CHO K1-BH4 line [12]. Isolated clone 11-4-8 is used here [13, 14].

Cells were maintained by growing on glass culture plates with Hams F12 medium and 5% fetal bovine serum (FBS) and incubated at 37°C in a humidified atmosphere of 5% CO₂. When plates became confluent, cells were trypsinized and transferred to a new plate with fresh medium to continue the line.

3.4.4 CHO Chronic Cytotoxicity

The CHO chronic cytotoxicity assay measures cell density as a factor of the concentration of a test agent after a 72 h (three cell cycles) exposure. The assay was conducted in a 96-well, flat-bottomed microplate. Each of the columns represented a different treatment
Column 1 was loaded with the negative control, $3 \times 10^3$ CHO cells in 200 µL F12+FBS without any test agent. Column 2 was used for a blank with no cells but 200 µL of F12+FBS and used to measure the intrinsic absorbency of the plate. Ten concentrations of agent and $3 \times 10^3$ cells were loaded in a volume of 200 µL of F12+FBS in columns 3-12. 8 rows (A-H) provided technical repeats and helped to establish statistical power. An illustration of the plate set-up is included as Figure 3.8. After all treatments were added to the plate, wells were covered with sterile AlumaSeal™ and incubated for 72 h at 37ºC.

After the 72 h exposure period, the cells progressed through three cell cycles and, if no toxicity was observed, reached a density of approximately $2.4 \times 10^4$. The supernatant was aspirated off the wells and transferred to a designated waste container for disposal. The cells were fixed to the plate with the addition of 50 µL of methanol per well. After 10 min, the methanol was shaken out to dryness and 50 µL of a 1% crystal violet (1:1 methanol:water v/v) was gently added to each well. Wells were stained for 10 min, washed with tap water, and tapped dry, leaving the stained cells adhering to the plate. Into each well, 50 µL of a 75% DMSO 25% methanol solution was added and the plate was incubated at room temperature for a final 10 min. The plate was analyzed for light absorbance at 595 nm on a BioRad microplate reader. Absorbance data for each well was recorded and transferred to an Excel spreadsheet. The data for each well were corrected by subtracting off the average “blank” well absorbance value, and normalized as a percentage of the negative control.

A one-way analysis of variance (ANOVA) test was used to determine if the agent or complex mixture induced a statistically significant level of killing. If a significant $F$ value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted. The power of the test statistic was maintained as 0.8 at $\alpha = 0.05$. A concentration-
response curve was generated and regression analysis was used to calculate a %C½ (LC₅₀) value or the concentration where the cell density is equal to 50% of the negative control. The LC₅₀ was used to compare the rank order of toxicity between different compounds or mixtures.

### 3.4.5 CHO Genotoxicity

Acute genotoxicity was measured with single-cell gel electrophoresis (SCGE). SCGE quantitatively measures genomic DNA damage in cell nuclei [15]. The day before an experiment, 4×10⁴ cells and 200 µL F12+FBS were added to 11 wells of a clean, UV-sterilized 96-well microplate and incubated overnight at 37°C in a humidified, 5% CO₂ incubator. On the day of treatment, the medium was aspirated from the wells and the cells were washed twice with Hank’s Balanced Salt Solution (HBSS). Dilutions of chemical agent were made from stock solutions in DMSO (stored at -20°C). For experiments requiring metabolic activation, an S9b150 mix was prepared [16]. Experiments consisted of 11 treatment groups, including a negative control, nine concentrations of test agent or mixture, and a positive control. For non-S9 experiments, the positive control was 3.8 mM of ethylmethanesulfonate and all treatments were conducted in a final volume of 25 µL F12. With S9, 750 mM NDMA was used as a positive control and the final volume of treatment groups was 100 µL, 20 µL of which was the S9 mixture. After the addition of the treatments, the wells were covered with sterile AlumaSeal™ and incubated for 4 h at 37°C.

Following the incubation, the AlumaSeal™ was removed and the medium aspirated from the wells. Cells were again washed twice with HBSS and then harvested using trypsin. To check for acute cytotoxicity, a 10 µL sample of the cells was collected and stained with Trypan Blue vital dye and cells were manually counted to determine a percent acute toxicity. For analysis, cell
viability was kept at 70% or greater. The remaining mixture was embedded in an agarose microgel and the cell membranes were lysed. After lysing, the microgels were incubated at 4°C in alkaline electrophoresis buffer for 20 min, allowing the DNA to denature. The microgels were electrophoresed for 40 min. The microgels were neutralized with neutralization buffer and either dried in methanol for future analysis or immediately stained with ethidium bromide and analyzed using Comet IV software. Genomic DNA damage was measured as % tail DNA, or the amount of DNA that has migrated from the nucleus into the agarose during electrophoresis [15]. A summary of this procedure is presented in Figure 3.9. The average % tail for each microgel was used as the unit of measure. Data were analyzed in SigmaPlot, a concentration-response curve was generated and a regression analysis was used to fit a curve to the data. Genotoxic potency was measured as the midpoint of the % tail DNA curve. An ANOVA test was used to determine statistical significance of treatment groups. If a significant $F$ value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted. The power of the test statistic was maintained as 0.8 at $\alpha = 0.05$.

3.4.6 CHO Cytotoxicity

The general measure of cytotoxicity was determined with a chronic assay. CHO cells were uniformly loaded in a 96-well microplate with a concurrent negative control and blank, and treatment wells were exposed to a range of concentrations of a test agent for a 72 h incubation period. After incubation, the supernatant was evacuated, the remaining cells were fixed to the wells and dyed with crystal violet stain and the absorbance of each well is measured. This assay has proven to be a consistent, accurate, and rapid way of assessing the relative toxicity of direct-acting agents of interest [15].
The nitrosamines and nitramines, however, require metabolic activation to be metabolized into toxic agents. This consideration is important, as exposure to a potential toxicant will result in processing by the metabolic systems in the liver and potentially leading to the formation of problematic secondary products. The 72 h chronic cytotoxicity assay, however, is unable to accurately measure the toxicity of agents that require metabolic activation for a variety of reasons. For in vitro assays, an additional S9 mixture, a system of salts, cofactors, and microsomes isolated from rats, must be added to the supernatant of cells and test agent to mimic the metabolic activities of an in vivo exposure. Due to the high salt content of the activation system, exposure to the S9 resulted in enhanced toxicity to cells if extended past a few hours, eliminating the possibility of a chronic test.

The materials in the S9 mix also precipitated out of the supernatant over a short time period, coating the cells at the bottom of the wells. This extra layer of material caused an increase in the absorbance of individual wells in a manner not related to the relative concentration of the dye being measured. The cells themselves are significantly less likely to detach in the presence of S9 and the S9 probably was not efficiently washed from wells. Dead cells remaining in the wells after washing will absorb crystal violet dye into their membranes and will be measured as if they were live cells, confounding the results.

Instead of using crystal violet, efforts were made to modify an XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt) to measure cytotoxicity. This approach was applied to a short-term exposure to a test agent for a time period used in the SCGE measures of genotoxicity employed in the rest of this study. XTT is a dye that, when reduced in the mitochondria, undergoes a color change from yellow to orange. The absorbance of this material provides a measure of mitochondrial activity and gives an indication of the survival
of the exposed cells. Cells exposed to the toxicant will show less viability at concentrations that cause killing, ending their ability to convert the XTT dyes to the orange color and providing a signal in the supernatant of mitochondrial activity. The supernatant can then be moved to fresh wells, unimpeded by the precipitates from the S9, and absorbance can be measured.

As a proof of concept, a variable number of cells were loaded onto a 96-well microplate and incubated overnight, allowing them to attach to the wells. The following day, the cells were washed twice with HBSS and exposed to F12 and S9 and allowed to incubate for 4 h. After treatment, the cells were washed twice more and XTT/menadione mixture in filter-sterilized HBSS was added. The cells were incubated for an additional 3 hours before absorbance was measured on a spectrophotometer at 450 nm. The total volume of supernatant from each well was then transferred to a clean well and the absorbance was measured again. From these data, a blank absorbancy associated with the XTT/menadione-only wells was subtracted from the total absorbance of wells of that volume. The data for these volumes was then transformed to a measure of the percentage of the highest (confluent) loading of cells. Curves for each concentration selected indicated that the XTT assay can still be utilized in the presence of S9 and provides relatively consistent data at all but the highest volumes of XTT. The experiment was repeated, with the additional step of taking the XTT materials aspirated from the treatment wells and moving them into centrifuge tubes, where they were then spun down for 3 minutes at 3000 rpm before being carefully aspirated again into clean wells. This additional step to remove any carry-over precipitates enhanced accuracy of measurement, albeit slightly.

While the modified XTT assay could provide accurate and repeatable data when known cell counts were added to wells, it ultimately provided little additional information for this project. After using the maximum possible concentrations of test agent as limited by the DMSO
percentage in the final well volume, no cytotoxicity was observed. It was inferred that the nitrosamines and nitramines, while active mutagens and genotoxins, were not sufficiently acutely toxic to show a depressed survival in these cells over the 4 h treatment times at these concentrations. The XTT toxicity assay modifications in this research project provided a promising approach to other scenarios in assays that also have measurement interference with absorption.

3.4.7 *Salmonella typhimurium* Mutagenicity Assay

*S. typhimurium* tester strain YG7108 (*hisG*46, *rfa*; △(*chl, uvrB, bio*) △*ada*ST; *ogt*ST; *Cm*′; *Amp*′) was used in this assay due to its sensitivity to nitrosamines and other alkylating agents. The strain was provided by Dr. T. Nohmi, National Institute of Health Sciences, Tokyo, Japan [17]. Test agents or mixtures that induce base pair substitution mutations will cause a mutation at *hisG*46 (Figure 3.10) to return the bacteria to the wild type phenotype, allowing it to grow on a selective VB medium (Figure 3.11).

A master plate was used to maintain the bacterial culture. The bacteria were streaked across an LB plate and grown overnight in a humidified 37°C incubator, then sealed with parafilm and stored at 4°C until needed. The day before treatment, a single-colony isolate was grown in a 100 mL LB medium with 25 μg/mL kanamycin and 10 μg/mL chloramphenicol at 37°C and shaken at 200 rpm [17]. On the day of treatment, the culture was centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the bacterial pellet was gently suspended in 50 mL 100 mM potassium-phosphate buffer (PPB). The material was centrifuged again, the supernatant discarded, and the pellet suspended in 2.5-3 mL of PPB. A 50 μL aliquot of the bacteria suspension was added to 4.95 mL PPB and vortexed. The solution’s optical density was
measured at 660 nm. The optical density corresponds to the concentration of bacteria in the tube. The bacteria from the suspension were used to make a titer of $6 \times 10^9$ bacteria/mL in a volume determined by the size of the experiment. The titered bacteria were stored on ice until the time of treatment.

Dilutions of the complex mixtures or individual agents were prepared in PPB. For assays utilizing mammalian metabolic activation systems, an S9 complex was mixed and included 50 mM PPB pH 7.4, 10 mM MgCl2, 5 mM glucose-6-phosphate, 30 mM KCl, and 4 mM NADP plus 200 µL/mL of Aroclor 1254-induced rat hepatic microsomal suspension (S9), with the final S9 concentration in the treatment well at 7%. Bacteria and test agent, with or without S9 mixture, were added to a UV-sterilized, round-bottomed 96-well microplate. The treatment wells were sealed with AlumaSeal™ and incubated at 37°C and shaken at 200 rpm for 60 min.

3.5 Results

Eleven pure compounds, nitrosamines and nitramines, were assayed for chronic cytotoxicity and for genotoxicity both with and without S9 activation. Twelve complex mixtures of washwaters were also assayed over the course of this project.

3.5.1 CHO Chronic Cytotoxicity

Data from each compound or mixture were averaged and are plotted as a percentage of the negative control versus the concentration or concentration factor. The chronic cytotoxicity of the complex mixtures showed distinct differences between samples, but most of the nitrosamines and nitramines did not exhibit chronic cytotoxicity without metabolic activation. Exceptions to this pattern included a lower cell density at 12.5 mM in Nitrosomorpholine, 10 mM
Nitrosopiperazine, 10 mM Nitropiperazine, 1,4-Dinitrosopiperazine, and the most pronounced decline in 1,4-Dinitropiperazine. Evidently, these agents express a toxic effect without S9 activation contrary to predictions about the chemical classes as a whole. Individual concentration-response curves for N-Nitrosodiethanolamine (Figure 3.12), N-Nitrodiethanolamine (Figure 3.13), N-Nitromethylethanolamine (Figure 3.14), N-Nitrosodimethylamine (Figure 3.15), N-Nitrodimethylamine (Figure 3.16), N-Nitrosomorpholine (Figure 3.17), N-Nitromorpholine (Figure 3.18), 1-Nitrosopiperazine (Figure 3.19), 1-Nitropiperazine (Figure 3.20), 1,4-Dinitrosopiperazine (Figure 3.21), and 1,4-Dinitropiperazine (Figure 3.22).

All complex mixtures were toxic over the concentration range used. Their concentration-response curves are split between two graphs (Figure 3.23 and Figure 3.24) with an additional sample presented in Figure 3.25. A summary bar graph using the %C½ (Figure 3.26) illustrates a direct comparison between the different treatments. The disparities in toxicity between different trials show that the risks posed by the carbon capture by-products vary across experimental conditions. If the causal conditions can be identified, then the industrial process can be adapted to shift its by-product output to a more benign composition. The most cytotoxic samples were found to be A10-0075 > A10-0075 (preserved with sulfamic acid) >> A10-0029 > A10-0017. The remaining samples had more similar toxicities. It is important to note that when the chemical characteristics of the materials were compared, the priority nitrogenous materials did not provide the majority of the toxic response. In this way, the biological data was able to identify an important factor in safety that was not immediately obvious by only considering the concentrations of the priority contaminants.
3.5.2 Induction of Genomic DNA Damage by Priority Agents

The mean % tail was determined over a range of concentrations that did not result in significant acute cytotoxicity (over 70% viability was required). Due to the small sample sizes of the complex mixture material, only two were assayed for genotoxicity.

For the pure compounds, the nitroso- and nitro- analogs can be compared in terms of genotoxicity. Three samples were found to be genotoxic in CHO cells with S9 metabolic activation, and followed a pattern of NDMA > Nitrosomorpholine > 1,4-Dinitrosopiperazine. Their analogous nitramines did not induce genomic DNA damage. Nitrosodiethanolamine, Nitrodiethanolamine, Nitrosopiperazine, and Nitropiperazine were not genotoxic in CHO cells with or without metabolic activation. Individual concentration response curves with applicable cell viability are presented for Nitrosodiethanolamine (Figure 3.27), Nitrodiethanolamine (Figure 3.28), nitromonoethanolamine (Figure 3.29), NDMA (Figure 3.30), Nitrodimethylamine (Figure 3.31), Nitrosomorpholine (Figure 3.32), Nitromorpholine (Figure 3.33), 1,4-Dinitrosopiperazine (Figure 3.34), 1,4-Dinitropiperazine (Figure 3.35), Nitrosopiperazine (Figure 3.36), and Nitropiperazine (Figure 3.37). The two complex mixtures assayed for genotoxicity, A10-0054 (Figure 3.38) and A10-0171 (Figure 3.39) were negative over 500 or 1000 times concentration without S9B150 metabolic activation – again, limitations on sample size prevented the use of the larger volume S9 assay.

3.5.3 Mutagenicity Results in S. typhimurium

Although CHO cells are useful and informative as in vitro models, bacterial mutagenicity assays offer a different set of advantages. While a rapid assay in CHO can provide insight into whether or not an agent or mixture damages the genome, the S. typhimurium allows for an
analysis of mutation. Genotoxicity is necessary but not sufficient for the mutagenicity and the *S. typhimurium* model allows for the investigation of actual mutants from a sample. Strain YG7108 was also selected to be more sensitive that the CHO cells to these agents. Especially when using S9 metabolic activation, the simpler cells of *S. typhimurium* might be more able to glean differences between individual agents and complex mixtures.

With the *S. typhimurium* assay, nine of the priority compounds were found to be mutagenic while two were negative over the concentration ranges tested. Nitrosodiemethylamine, the model nitrosamine in most of the literature, was by far the most mutagenic and required metabolic activation for its mutagenic activity (Figure 3.40). Its analogous nitramine, nitrodimethylamine, was found to be less mutagenic but still active with metabolic activation but inactive without it (Figure 3.41). Nitromorpholine was positive only with S9 (Figure 3.42), as was its analogous nitramine, Nitromorpholine (Figure 3.43). For 1,4-Dinitrosopiperazine, again, the sample is positive in the presence of S9 but not without it (Figure 3.44). The 1,4-Dinitropiperazine is mutagenic both with and without S9, deviating from the activity patterns of the other nitroso- and nitramines and indicating a different mechanism of action for mutagenicity (Figure 3.45). Nitrosodiethanolamine was found to be mutagenic both with and without S9 at very high concentrations and exhibiting a higher level of mutagenicity with S9, perhaps indicating multiple pathways of mutagenicity (Figure 3.46). Nitrodiethanolamine, unlike its analogous nitrosamine, was only active in the absence of S9 (Figure 3.47). Nitromonoethanolamine, much like the 1,4-Dinitropiperazine, was equally mutagenic both with and without S9 activation (Figure 3.48). Both N-Nitrosopiperazine (Figure 3.49) and N-Nitropiperazine (Figure 3.50) were not active mutagens with or without S9. A summary slide, including the “mutagenic potency” method of comparison, is included as Table 3.3.
Cursory analysis of complex mixtures for mutagenicity provided valuable insight into process characteristics of carbon capture and biological endpoints. As previously stated, CHO toxicity was unable to determine a relationship between different individual agents and the overall toxicity of complex mixtures. Early *S. typhimurium* mutagenicity data, however, did present an important finding. From the first set of complex mixtures provided by the company (Figure 3.51 and Figure 3.52), the mutagenicity of the samples was very divergent. Though not all of the inputs were available, from the chemical information that was available suggested a relationship between NO$_X$ in the entering gas and the mutagenicity of the effluent was positive and is illustrated in (Figure 3.53). Samples A10-0054 and A10-0171 were further assayed, as they were in CHO. In *S. typhimurium*, A10-0054 was not mutagenic over the concentration range tested, but produced significant toxicity at higher concentrations in the absence of S9 (Figure 3.54). In A10-0171, the mixture was found to be mutagenic both with and without S9 metabolic activation, but with reduced mutagenicity in the presence of S9 (Figure 3.55). These profiles are unusual and indicate that the driving forces of the mixtures as a whole are not from agents that require metabolic activation, indicating that perhaps the focus of hazard mitigation in carbon capture should focus on direct acting mutagens rather than the nitrogenous promutagens of this study.

3.6 Discussion

This research progress considers both single-compound and complex mixture toxicity, examining both the traditional paradigm of risk assessment as measurements of single compounds as well as the toxicity associated with whole mixtures. The results of both types of experiments are enlightening.
First, the model organism and assay type are important considerations when assessing novel mixtures. Sensitivity is especially important to distinguish between unknown samples. Because of their sensitivity, YG7108 was able to provide much more specific response information than the CHO cells. Had the assay only been conducted in mammalian cells, the results would have been less sensitive as CHO cells were only able to detect a few differences.

Second, the complex mixtures told a very different story than the original project had expected. While NDMA and other nitrogenous materials are important topics of study because they are ubiquitous in the environment and are positive in a number of our assays, the complex mixture samples here showed that the overall toxicity, both cytotoxicity and mutagenicity, is not driven by these priority agents. The total quantifiable nitrogenous materials present in the samples could not account for the cytotoxicity and mutagenicity observed. The changes in cell viability in the *S. typhimurium* assays show that rather than enhance their toxicity and mutagenicity, the metabolic activation actually tempers these biological outcomes. The most important constituents were not the nitrosamines and nitramines – they were the unknowns that are direct-acting agents. In the same way, the comparative toxicology of complex mixtures was able to illustrate, with the *S. typhimurium* data, that a given input (NO$_X$) was very important to the ultimate toxicity of a sample, moreso than relative concentrations of the priority compounds.

Third, this study begins to elucidate a number of important features of the relationship between nitrosamines and their analogous nitramines, which have broad applications beyond the scope of this project. Most obviously, the relationships between Nitroso/nitrodimethylamine, Nitroso/nitromorpholine, 1,4-Nitroso/nitropiperazine, and 1-Nitroso/nitropiperazine show a relationship between the activity of the nitrosamine and nitramines through both CHO and *S. typhimurium*. In *S. typhimurium* +S9, NDMA > Nitromorpholine > 1,4-Nitrosopiperazine. In
the same way, Nitrodimethylamine > Nitromorpholine > 1,4-Nitropiperazine. In CHO, which were less sensitive than the *S. typhimurium*, showed the same pattern of genotoxicity, NDMA > Nitrosomorpholine > 1,4-Nitrosopiperazine, but no activity with the weaker nitramines. The 1-Nitrosopiperazine and 1-Nitropiperazine were negative across all assays, also suggesting a relationship based on structure.

Additionally, a number of results were surprising and diverged from expectations of activity. Nitrosodiethanolamine was mutagenic at very high concentrations both with and without S9, but became more mutagenic with metabolic activation. NDELA can be inferred to have multiple pathways of mutagenicity, one as a direct actor and a more efficient pathway that relies on metabolic activation. Nitromonoethanolamine and 1,4-Dinitropiperazine were active both with and without S9 with the same activity, indicating an activity that does not require metabolic activation and not characterized by the S9-dependant alkylation model of NDMA. Nitrodiethanolamine, perhaps most perplexingly, was only active without S9 and was found to be negative with the activation. Further investigation of the events resulting in mutagenicity would provide insight into the potential activity pathways of these important classes of compounds.

3.7 Conclusions

- The emerging focus on chemical mixtures in toxicology is uniquely equipped to handle a number of complicated environmental questions, including the optimization of emerging engineering technologies.
- Mixtures can allow for the assessment of constituents that are not specifically being sought out – the priority nitrosamines and nitramines only constitute a small fraction of the whole mixture.
• Nitrosamines and nitramines do, in some cases, share some relationship due to their structure, as the NDMA/NO\textsubscript{2}DMA, Nitrosomorpholine/Nitromorpholine, and 1,4-Dinitrosopiperazine/1,4-Dinitropiperazine illustrated.

• Not all nitramines follow the same mechanism of action, as 1,4-Dinitropiperazine was found to be mutagenic both with and without S9 metabolic activation, bucking the trend seen in most of the nitrosamines and other nitramines.

• Despite the emphasis on nitrosamine and nitramine toxicity in the project proposal for StatOil, the toxicological data from the samples indicate that other chemical constituents, not simply the nitrogenous materials, are responsible for the bulk of the toxic effects observed.

• In this case, biological information provided important feedback to technological development to direct technical solutions towards less environmentally-hazardous alternatives. Because the assays used in this research were able to identify product sets that were more toxic and the mutagenicity assay specifically pointed to the % NO\textsubscript{X} in air in the absorber column as related to enhanced mutagenicity, more information was available to the company about toxicology even beyond the few priority agents.
Table 3.1 Identified compounds from monoethanolamine reclaimer column from a CO₂ capture facility [4].

<table>
<thead>
<tr>
<th>peak</th>
<th>compound</th>
<th>method of identification</th>
<th>GC column</th>
<th>% of total area</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>GC-MS</td>
<td>GC-FTIR</td>
<td>LVHRMS</td>
</tr>
<tr>
<td>1</td>
<td>N-acetylenolamine (C₄H₆NO₂)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>2</td>
<td>N-glycylglycine (C₄H₈N₂O₃)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>3</td>
<td>N-(hydroxyethyl)-succinimide (C₆H₈NO₃)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>4</td>
<td>N-(2-hydroxyethyl)-lactamide (C₅H₁₁NO₂)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>5</td>
<td>1-(2-hydroxyethyl)-2-imidazolidinone (C₅H₁₀N₂O₂)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>6</td>
<td>N,N-diacetylenolamine (C₄H₁₁NO₃)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>7</td>
<td>ammonia (NH₃)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>8</td>
<td>acetic acid (C₂H₄O₂)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>9</td>
<td>propionic acid (C₃H₆O₂)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>10</td>
<td>n-butyric acid (C₄H₉O₂)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>11</td>
<td>monoethanolamine (C₅H₁₂NO)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>12</td>
<td>2,6-dimethyl-4-pyridinamine (C₇H₁₄N₂)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>13</td>
<td>2-imidazolocarboxaldehyde (C₄H₆N₂O)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>14</td>
<td>1-methyl-2-imidazolocarboxaldehyde (C₅H₁₀N₂O)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>15</td>
<td>2-oxazolidone (C₃H₇NO₂)</td>
<td>x</td>
<td>x</td>
<td>x</td>
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</table>

* Area percentage not calculated due to overlap with other peaks
Table 3.2 Priority Nitrosamines and Nitramines.

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>CAS #</th>
</tr>
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<tbody>
<tr>
<td><em>N</em>-Nitrosodiethanolamine</td>
<td>C₄H₁₀N₂O₃</td>
<td>1116-54-7</td>
</tr>
<tr>
<td><em>N</em>-Nitrodiethanolamine</td>
<td>C₄H₁₀N₂O₄</td>
<td>4185-47-1</td>
</tr>
<tr>
<td><em>N</em>-Nitromonoethanolamine</td>
<td>C₂H₆N₂O₃</td>
<td>unavailable</td>
</tr>
<tr>
<td><em>N</em>-Nitrosomorpholine</td>
<td>C₄H₈N₂O₂</td>
<td>59-89-2</td>
</tr>
<tr>
<td><em>N</em>-Nitromorpholine</td>
<td>C₄H₈N₂O₃</td>
<td>4164-32-3</td>
</tr>
<tr>
<td><em>N</em>-Nitrosodimethylamine</td>
<td>C₂H₆N₂O</td>
<td>62-75-9</td>
</tr>
<tr>
<td><em>N</em>-Nitrodimethylamine</td>
<td>C₂H₆N₂O₂</td>
<td>4164-28-7</td>
</tr>
<tr>
<td>1-Nitrosopiperazine</td>
<td>C₄H₉N₃O</td>
<td>5632-47-3</td>
</tr>
<tr>
<td>1-Nitropiperazine</td>
<td>C₄H₉N₃O₂</td>
<td>42499-41-2</td>
</tr>
<tr>
<td>1,4-Dinitrosopiperazine</td>
<td>C₄H₈N₄O₂</td>
<td>140-79-4</td>
</tr>
<tr>
<td>1,4-Dinitropiperazine</td>
<td>C₄H₈N₄O₃</td>
<td>4164-37-8</td>
</tr>
</tbody>
</table>
Table 3.3 Summary of *S. typhimurium* assay results.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS #</th>
<th>Conc. Range (µM)</th>
<th>S9</th>
<th>2x Mutat. Conc. (µM)</th>
<th>r²</th>
<th>Mutagenic Potency (rev/µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N</em>-Nitrosodimethylamine</td>
<td>62-75-9</td>
<td>50 – 5000</td>
<td>−S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td><em>N</em>-Nitrosodimethylamine</td>
<td>62-75-9</td>
<td>50 – 5000</td>
<td>+S9</td>
<td>4.5</td>
<td>0.98</td>
<td>21642</td>
</tr>
<tr>
<td><em>N</em>-Nitrodimethylamine</td>
<td>4164-28-7</td>
<td>50 – 7500</td>
<td>−S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td><em>N</em>-Nitrodimethylamine</td>
<td>4164-28-7</td>
<td>50 – 7500</td>
<td>+S9</td>
<td>279.6</td>
<td>0.99</td>
<td>1660</td>
</tr>
<tr>
<td><em>N</em>-Nitrodimethylamine</td>
<td>4164-28-7</td>
<td>50 – 7500</td>
<td>+S9</td>
<td>21.4</td>
<td>0.99</td>
<td>6724</td>
</tr>
<tr>
<td><em>N</em>-Nitrosomorpholine</td>
<td>59-89-2</td>
<td>100 – 7500</td>
<td>−S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td><em>N</em>-Nitrosomorpholine</td>
<td>59-89-2</td>
<td>100 – 7500</td>
<td>+S9</td>
<td>21.4</td>
<td>0.99</td>
<td>6724</td>
</tr>
<tr>
<td><em>N</em>-Nitromorpholine</td>
<td>4164-32-3</td>
<td>100 – 7500</td>
<td>−S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td><em>N</em>-Nitromorpholine</td>
<td>4164-32-3</td>
<td>100 – 7500</td>
<td>+S9</td>
<td>1177.2</td>
<td>0.99</td>
<td>272</td>
</tr>
<tr>
<td><em>N</em>-Nitroso-diethanolamine</td>
<td>1116-54-7</td>
<td>7500 – 30000</td>
<td>−S9</td>
<td>26838</td>
<td>0.95</td>
<td>10</td>
</tr>
<tr>
<td><em>N</em>-Nitroso-diethanolamine</td>
<td>1116-54-7</td>
<td>7500 – 30000</td>
<td>+S9</td>
<td>10304</td>
<td>0.95</td>
<td>22</td>
</tr>
<tr>
<td><em>N</em>-Nitrodiethanolamine</td>
<td>4185-47-1</td>
<td>10000 – 30000</td>
<td>−S9</td>
<td>25193</td>
<td>0.95</td>
<td>8</td>
</tr>
<tr>
<td><em>N</em>-Nitrodiethanolamine</td>
<td>4185-47-1</td>
<td>7500 – 30000</td>
<td>+S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td>1-Nitrosopiperazine</td>
<td>5632-47-3</td>
<td>100 – 7500</td>
<td>−S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td>1-Nitrosopiperazine</td>
<td>5632-47-3</td>
<td>100 – 7500</td>
<td>+S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td>1-Nitropiperazine</td>
<td>42499-41-2</td>
<td>500 – 10000</td>
<td>−S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td>1-Nitropiperazine</td>
<td>42499-41-2</td>
<td>500 – 7500</td>
<td>+S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td>1,4-Dinitrosopiperazine</td>
<td>140-79-4</td>
<td>100 – 7500</td>
<td>−S9</td>
<td>Neg</td>
<td>NA</td>
<td>Neg</td>
</tr>
<tr>
<td>1,4-Dinitrosopiperazine</td>
<td>140-79-4</td>
<td>100 – 7500</td>
<td>+S9</td>
<td>49.1</td>
<td>0.99</td>
<td>1263</td>
</tr>
<tr>
<td>1,4-Dinitropiperazine</td>
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<td>5000 – 20000</td>
<td>−S9</td>
<td>2118</td>
<td>0.99</td>
<td>157</td>
</tr>
<tr>
<td>1,4-Dinitropiperazine</td>
<td>4164-37-8</td>
<td>1000 – 20000</td>
<td>+S9</td>
<td>1099</td>
<td>0.99</td>
<td>191</td>
</tr>
<tr>
<td><em>N</em>-Nitromono-ethanolamine</td>
<td>Unavailable</td>
<td>5000 – 30000</td>
<td>−S9</td>
<td>1732</td>
<td>0.99</td>
<td>134</td>
</tr>
<tr>
<td><em>N</em>-Nitromono-ethanolamine</td>
<td>Unavailable</td>
<td>5000 – 30000</td>
<td>+S9</td>
<td>1366</td>
<td>0.99</td>
<td>133</td>
</tr>
</tbody>
</table>
Figure 3.1 Schematic of CO₂ Capture System [18].
Figure 3.2 Detail of the Absorber Column [18].
Figure 3.3 General chemistry of amine-based carbon capture [19].
Figure 3.4 A proposed solvent degradation pathway [4].
Figure 3.5 A proposed oxygen-centric degradation pathway [7-8]
**Figure 3.6** Structures of A) N-nitrosodimethylamine B) N-nitrodimethylamine C) N-nitrosodiethylamine D) N-nitrosodipropylamine E) N-nitrosodibutylamine F) N-nitrosomethylethylamine G) N-nitrosopyrrolidine H) N-nitrosopiperidine I) N-nitrosomorpholine J) N-nitromorpholine [18].
**Figure 3.7** Image of Chinese Hamster Ovary (CHO) Cells, AS52, Clone 11-4-8.
Figure 3.8 Summary of Plate Set-up for CHO Cytotoxicity.
Figure 3.9 Summary of SCGE Procedure.
Figure 3.10 *S. typhimurium* reverse mutation assay.
Figure 3.11 Reverse mutations of *S. typhimurium* increases as the concentration of the mutagen increases.
Nitrosodiethanolamine (mM)

10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}

Direct Chronic CHO Cell Cytotoxicity: Cell Density as the Percent of the Negative Control

Figure 3.12 Nitrosodiethanolamine shows no toxicity in the chronic cytotoxicity assay.
**Figure 3.13** A concentration-response curve for Nitrodiethanolamine after a 72-hour chronic cytotoxicity exposure.
Figure 3.14 A concentration-response curve for Nitromethylethanolamine after a 72-hour chronic cytotoxicity exposure.
Figure 3.15 Concentration-response curve for NDMA after a 72-hour chronic cytotoxicity exposure.
Figure 3.16 Concentration-response curve for Nitrodimethylamine after a 72-hour chronic cytotoxicity exposure.
Figure 3.17 Concentration-response curve for Nitrosomorpholine after a 72-hour chronic cytotoxicity exposure.
Figure 3.18 Concentration-response curve for Nitromorpholine after a 72-hour chronic cytotoxicity exposure.
Figure 3.19 Concentration-response curve for Nitrosopiperazine after a 72-hour chronic cytotoxicity exposure.
Figure 3.20 Concentration-response curve for N-Nitropiperazine after a 72-hour chronic cytotoxicity exposure.
Figure 3.21 Concentration-response curve for 1,4-Dinitrosopiperazine after a 72-hour chronic cytotoxicity exposure.
Figure 3.22 Concentration-response curve for 1,4-Dinitropiperazine after a 72-hour chronic cytotoxicity exposure.
Figure 3.23 Cytotoxicity concentration-response curves for washwater samples A10-0014, A10-0017, A10-0029, A10-0033, A10-0039 after a 72 h exposure.
Figure 3.24 Cytotoxicity concentration-response curves for washwater samples A10-0047, A10-0052, A10-0054, A10-0058, and A10-0058 Control.
Figure 3.25 Concentration-response curve for A10-0075 with and without Sulfamic Acid Preservative.
Figure 3.26 Summary of Cytotoxic Potency of Complex Mixtures.
Figure 3.27 Concentration-response curves for acute genomic DNA damage after 4 h exposure to Nitrosodiethanolamine.
Figure 3.28 Concentration-response curves for acute genomic DNA damage after 4 h exposure to Nitrodiethanolamine.
Figure 3.29 Concentration-response curves for acute genomic DNA damage after 4 h exposure to Nitromonoethanolamine.
Figure 3.30 Concentration-response curves for acute genomic DNA damage after 4 h exposure to NDMA.
Figure 3.31 Concentration-response curves for acute genomic DNA damage after 4 h exposure to N-Nitrodimethylamine.
Figure 3.32 Concentration-response curves for acute genomic DNA damage after 4 h exposure to N-Nitrosomorpholine.
Figure 3.33 Concentration-response curves for acute genomic DNA damage after 4 h exposure to N-Nitromorpholine.
Figure 3.34 Concentration-response curves for acute genomic DNA damage after 4 h exposure to 1,4-Dinitrosopiperazine.
Figure 3.35 Concentration-response curves for acute genomic DNA damage after 4 h exposure to 1,4-Dinitropiperazine.
Figure 3.36 Concentration-response curves for acute genomic DNA damage after 4 h exposure to 1-Nitrosopiperazine.
Figure 3.37 Concentration-response curves for acute genomic DNA damage after 4 h exposure to 1-Nitropiperazine.
Figure 3.38 Concentration-response curves for acute genomic DNA damage after 4 h exposure to A11-0054.
Figure 3.39 Concentration-response curves for acute genomic DNA damage after 4 h exposure to A11-0171.
Figure 3.40 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to N-Nitrosodimethylamine.
Figure 3.41 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to Nitrodimethylamine.
Figure 3.42 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to Nitrosomorpholine.
Figure 3.43 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to Nitromorpholine.
Figure 3.44 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to 1,4-Dinitrosopiperazine.
Figure 3.45 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to 1,4-Dinitropiperazine.
Figure 3.46 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to Nitrosodiethanolamine.
Figure 3.47 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to Nitrodiethanolamine.
Figure 3.48 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to Nitromonoethanolamine.
Figure 3.49 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to 1-Nitrosopiperazine.
Figure 3.50 Concentration-response curves for mutagenicity in S. typhimurium after 1 h exposure to 1-Nitropiperazine.
Figure 3.51 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to complex mixtures A10-0014, A10-0017, A10-0029, A10-0033, A10-0039 (no cytotoxicity).
Figure 3.52 Concentration-response curves for mutagenicity in S. typhimurium after 1 h exposure to complex mixtures A10-0047, A10-0052, A10-0054, A10-0058, A10-0058 Control (no cytotoxicity).
**Figure 3.53** Mutagenicity in *S. typhimurium* as a factor of %NOx in Air in Absorber.
Figure 3.54 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to complex mixture A10-0054.
Figure 3.55 Concentration-response curves for mutagenicity in *S. typhimurium* after 1 h exposure to complex mixture A10-0171.


CHAPTER 4

Conclusions

- Organisms experience chemical exposures as complex mixtures.
- Interactions between individual constituents, or the effects of individual constituents below the limits of detection, make the traditional single-compound approach to toxicology and regulation necessarily incomplete.
- The emerging focus on chemical mixtures in toxicology is uniquely equipped to handle a number of complicated environmental questions.
- Complex mixtures can help us to confirm hypotheses, such as the origins of highly toxic individual constituents in drinking water emerging from organic substrates rather than the expected inorganic iodide.
- Mixtures can also help us to assess constituents that are not being sought out – the change in toxicity due to the addition of iopamidol is greater than simply accounting for previously identified iodinated DBPs.
- Nitrosamines and nitramines do, in some cases, share some relationship due to their structure, as the NDMA/NO₂DMA, Nitrosomorpholine/Nitromorpholine, and 1,4-Dinitrosopiperazine/1,4-Dinitropiperazine illustrated.
- Not all nitrosamines follow the same mechanism of action, as 1,4-Dinitropiperazine was found to be mutagenic with and without S9 metabolic activation.
- Despite the emphasis on nitrosamine and nitramine toxicity in the project proposal for StatOil, the toxicological data from the samples indicate that other chemical constituents,
not simply the nitrogenous materials, are responsible for the bulk of the toxic effects observed.

- Biological information can provide important feedback to technological development to direct technical solutions towards less environmentally-hazardous alternatives.
APPENDIX A

Supporting Information for Chapter 2

A.1 ICM Methods

Water samples were filtered through glass fiber filters (Schleicher and Schuell, Dassel, Germany), acidified to pH 3 adding sulfuric acid (3.5 M H$_2$SO$_4$). One L was extracted via Bakerbond$^{TM}$ SDB-1 (3 mL, 200 mg, J.T. Baker). Prior to extraction, all samples were spiked with 10 µL of a surrogate solution, consisting of iohexol-d$_5$, iomeprol-d$_3$, iopamidol-d$_3$, diatrizoate-d$_6$, desmethoxyiopromide (DMI), and desdihydroxypropyliopromide (DDHPI) (20 ng/µL). DMI and DDHPI were kindly provided by Bayer Schering Pharma (Berlin, Germany), diatrizoate-d$_6$ was purchased from Campro Scientific (Berlin, Germany), and iohexol-d$_5$, iomeprol-d$_3$, and iopamidol-d$_3$ were purchased from Toronto Research Chemicals (North York, Canada). Analytes were eluted with $4 \times 2$ mL of methanol, evaporated to 100 µL using nitrogen gas, and reconstituted with 900 µL Milli-Q water. ICM were detected with liquid chromatography (LC)/electrospray ionization (ESI)/MS/MS in the positive ion mode. An Agilent 1200 Series LC system was coupled to a 4000 Q Trap$^{TM}$ MS system (Applied Biosystems/MDS Sciex, Darmstadt, Germany).

Chromatographic separation was achieved on two coupled Chromolith® Performance RP-18e columns (4.6 mm × 100 mm, Merck, Darmstadt, Germany) equipped with a Chromolith® RP-18e guard column (4.6 mm × 5 mm, Merck, Darmstadt, Germany). Sample aliquots of 50 µL were injected onto the LC/MS/MS system, and the analytes were eluted from the column using two mobile phases, 95% Milli-Q water, 5% methanol, and 0.5% formic acid (A), and 99.5% methanol and 0.5% formic acid (B). Further details can be found in Kormos et al. [1].
A.2 Iodo-THM and Iodo-Acid Methods

Water samples were extracted using a previously described method with minor modification [2]. The sample was first acidified to pH < 0.5 with 5 mL concentrated H\textsubscript{2}SO\textsubscript{4}. Then 30 g dried Na\textsubscript{2}SO\textsubscript{4} was added to the sample and shaken by hand until it completely dissolved. The sample was then spiked with 10 µL of 1,2-dibromopropane (25 µg/mL in methanol) internal standard prior to 3 mL of methyl tert-butyl ether (MTBE) being added. The samples were then placed on a wrist-action shaker for 30 min. The sample was allowed to settle and the MTBE extract was removed and dried with a plug of dried Na\textsubscript{2}SO\textsubscript{4}. The sample was then split, with 0.5 mL removed for methylation/derivation with diazomethane for analysis of iodo-acids, and the remaining sample was used to analyze for iodo-trihalomethanes (iodo-THMs).

Six iodo-THMs (dichloroiodomethane, bromochloroiodomethane, dibromoiodomethane, chlorodiodomethane, bromodiiodomethane, and iodoform) were measured in the extracts using gas chromatography (GC)/electron ionization (EI)-mass spectrometry (MS), carried out in selected ion monitoring (SIM) mode (m/z 127, representing iodine, and m/z 123 representing the internal standard, 1,2-dibromopropane). Analyses were carried out on either a Hewlett-Packard 6890 gas chromatograph equipped with a 5973 mass selective detector (MSD) or a Waters Micromass Autospec high resolution mass spectrometer (8 kV, 1000 resolution, and a source temperature of 200°C). GC conditions were as follows: 30-m Restek Rtx-5 or J&W Scientific/Agilent DB-5 column with a 0.25-mm ID and 0.5 or 0.25-µm film thickness. The injection port was controlled at 250°C, and GC/MS transfer lines at 280°C. Injections of 1.0 µL of the extracts were introduced via a split/splitless injector (in splitless mode). The GC temperature program consisted of an initial temperature of 35°C, which was held for 10 min,
followed by an increase at a rate of 3°C/min to 45°C, and increased again at a rate of 25°C/min to 280°C, which was held for 18 min.

Five iodo-acids (iodoacetic acid, bromoiodoacetic acid, (Z)-3-bromo-3-iodo-propenoic acid, (E)-3-bromo-3-iodo-propenoic acid, and (E)-2-iodo-3-methylbutenedioic acid) were measured in the extracts according to a previously published procedure using derivatization and analysis with GC/negative chemical ionization (NCI)-MS [2]. Diiodoacetic acid was also included in the experiments involving iodide. The derivatization procedure was modified slightly from the earlier published method [3] because the commercial reagent for generating diazomethane recently changed from 1-methyl-3-nitro-2-nitrosoguanidine (MNNG) to Diazald (Sigma-Aldrich). Briefly, this involved the generation of diazomethane by dissolving 0.367g Diazald (Sigma Aldrich, St. Louis, MO) in 1 mL carbitol in the inner part of a diazomethane generator (Sigma Aldrich), adding 2-3 mL of MTBE in the outer part of the reactor, closing the vessel and immersing in ice. The reaction was then initiated by adding 1.5 mL KOH (37%) to the inner vessel, and allowed to proceed for 1 h. The diazomethane formed collected in the MTBE, and 0.250 mL of this solution was added to the treated water extract (0.5 mL) and allowed to react for approx. 30 min, after which it was quenched with approximately 50 mg activated silica (for 30 min).

GC/NCI-MS analyses were carried out in SIM mode (on the m/z 127 ion, representing iodine) on a Shimadzu QP2010 Plus GC/mass spectrometer. The Shimadzu mass spectrometer was operated at a source temperature of 200°C and electron energy of 124 eV. Methane was used as the CI gas. Injections of 0.5 µL of the extract were introduced via a split/splitless injector (in splitless mode) onto a GC column (DB-5, 30-m x 0.25 mm id, 0.25 µm film thickness, J&W Scientific/Agilent). The GC temperature program consisted of an initial
temperature of 40°C, which was held for 1 min, followed by an increase at a rate of 12°C/min to 150°C, which was held for 5 min, followed by an increase at a rate of 25°C/min to 280°C, which was held for 14.8 min. The injection port temperature was controlled at 250°C, and GC/MS transfer lines at 280°C. Calibration standards of the synthesized iodo-acid methyl esters (synthesis reported in reference [2]) were measured with each set of samples analyzed quantitatively.

A.3 Fulvic and Humic Acid Experiments

Suwannee River fulvic and humic acids (International Humic Substances Society) were reacted individually in purified water at pH 7.5 using 5 µM iopamidol and 100 µM chlorine or monochloramine at increasing concentrations of fulvic or humic acid (1 to 20 mg/L). Reaction conditions were the same as carried out for experiments using real source waters. Samples were extracted at 24, 48, and 72 h and analyzed for iodo-THMs and iodo-acids using the methods described above.

A.4 Chinese Hamster Ovary (CHO) Cell Assays

A.4.1 Reagents

General reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St. Louis, MO). Media and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Logan, UT). The water organic extracts were received from U.S. EPA in ethyl acetate. The ethyl acetate was concentrated such that the organics from 1 L of water sample were dissolved in 10 µL ethyl acetate (10^5× concentration). The organic samples were solvent transferred to dimethyl sulfoxide (DMSO) and stored at −22°C in sterile glass vials.
Chinese hamster ovary (CHO) cells, line AS52, clone 11-4-8 were used in all biological assays. The CHO cells were maintained in Ham’s F12 medium containing 5% FBS at 37°C in a humidified atmosphere of 5% CO₂.

A.4.2 CHO Cell Chronic Cytotoxicity Assay

This assay measures the reduction in cell density as a function of the organic extract concentration over a period of approximately 3 cell divisions (72 h). Chronic cytotoxicity to CHO cells was measured using an assay we previously developed for the analysis of DBPs [4]. Flat-bottom, tissue culture 96-well microplates were employed; 4-8 replicate wells were prepared for each concentration of a specific organic extract. Eight wells were reserved for the blank control consisting of 200 µL of F12 medium + 5% fetal bovine serum (FBS). The negative control consisted of 8 wells containing 100 µL of a titered CHO cell suspension (3×10⁴ cells/mL) plus 100 µL F12 + FBS. The wells for the remaining columns contained 3,000 CHO cells, F12 + FBS and a known concentration of an organic extract for a total of 200 µL. To prevent cross-over contamination between wells due to volatilization of the organic extract, a sheet of sterile AlumnaSeal™ (RPI Corporation, Mt. Prospect, IL) was pressed over the wells before the microplate was covered. The microplate was placed on a rocking platform for 10 min to uniformly distribute the cells, and then placed in a tissue culture incubator for 72 h. After incubation, each well was gently aspirated, fixed in 100% methanol for 10 min, and stained for 10 min with a 1% crystal violet solution in 50% methanol. The plate was gently washed, and 50 µL of dimethyl sulfoxide/methanol (3:1 v/v) was added to each well for 10 min. The plate was analyzed in a BioRad microplate reader at 595 nm. The data were automatically recorded and transferred to an Excel spreadsheet on a microcomputer connected to the microplate reader. The
blank-corrected absorbancy value of the negative control (cells with medium only) was set at 100%. The absorbancy for each treatment group well was converted into a percentage of the negative control. For each organic extract concentration, 4-8 replicate wells were analyzed per experiment, and the experiments were repeated.

### A.4.3 Single Cell Gel Electrophoresis Assay

Single cell gel electrophoresis (SCGE) is a molecular genetic assay that quantitatively measures the level of genomic DNA damage induced in individual nuclei of treated cells [5]. The day before treatment, $4 \times 10^4$ CHO cells were added to each microplate well in 200 µL of F12 + 5% FBS and incubated. The next day, the cells were washed with Hank’s balanced salt solution (HBSS) and treated with a series of concentrations of a specific organic extract in F12 medium without FBS in a total volume of 25 µL for 4 h at 37°C, 5% CO$_2$. The wells were covered with sterile AlumnaSeal™. After incubation, the cells were washed 2× with HBSS and harvested with 50 µL of 0.01% trypsin + 53 µM EDTA. The trypsin was inactivated with 70 µL of F12 + FBS. Acute cytotoxicity was measured from a 10 µL aliquot of cell suspension mixed with 10 µL of 0.05% trypan blue vital dye in phosphate-buffered saline (PBS). SCGE data were not used if the acute cytotoxicity exceeded 30%. The remaining cell suspension from each well was embedded in a layer of low melting point agarose prepared with PBS upon clear microscope slides that were previously coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. The cellular membranes were removed by an overnight immersion in lysing solution at 4°C. The microgels were placed in an alkaline buffer (pH 13.5) in an electrophoresis tank, and the DNA was denatured for 20 min. The microgels were electrophoresed at 25 V, 300 mA (0.72 V/cm) for 40 min at 4°C. The microgels were
neutralized with Tris buffer (pH 7.5), rinsed in cold water, dehydrated in cold methanol, dried at 50°C, and stored at room temperature in a covered slide box. For analysis, the microgels were hydrated in cold water for 30 min and stained with 65 µL of ethidium bromide (20 µg/mL) for 3 min. The microgels were rinsed in cold water and analyzed with a Zeiss fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. For each experiment, 2 microgels were prepared per treatment group. Randomly chosen nuclei (25 per microgel) were analyzed using a charged coupled device camera. A computerized image analysis system (Komet version 3.1, Kinetic Imaging Ltd., Liverpool, UK) was employed to determine the SCGE % tail DNA value of the nuclei as indices of DNA damage. The digitalized data were automatically transferred to a computer based spreadsheet for subsequent statistical analysis. Within each experiment, a negative control, a positive control (3.8 mM ethylmethanesulfonate), and 9 concentrations of an organic extract were analyzed concurrently. The experiments were repeated 2-3 times for each organic extract.
### Table A.1 Unusual Plants from Iodo-DBP Occurrence Study\(^a\).

<table>
<thead>
<tr>
<th>Drinking Water Plant</th>
<th>Iodide (µg/L)</th>
<th>Sum iodo-acids (µg/L)</th>
<th>Sum iodo-THMs (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant 2</td>
<td>1.0</td>
<td>0.37</td>
<td>4.9</td>
</tr>
<tr>
<td>Plant 4</td>
<td>ND</td>
<td>0.10</td>
<td>1.2</td>
</tr>
<tr>
<td>Plant 11</td>
<td>1.5</td>
<td>0.21</td>
<td>2.3</td>
</tr>
<tr>
<td>Plant 15</td>
<td>ND</td>
<td>0.17</td>
<td>2.4</td>
</tr>
</tbody>
</table>

\(^a\)ND = Non-detect; detection limit = 0.13 µg/L
Table A.2 NOM and Source Water Characteristics for Athens-Clarke County (ACC) Raw Source Water$^a$.

<table>
<thead>
<tr>
<th>Source</th>
<th>TOC (mg/L-C)</th>
<th>SUVA$_{254}$ (L/m-mg)</th>
<th>[Br$^-$] (µM)</th>
<th>[I$^-$] (µM)</th>
<th>[NH$_3^-$]$_T$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>2.1</td>
<td>4.88</td>
<td>0.15</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$[NH$_3^-$]$_T$ = [NH$_4^+$] + [NH$_3$]; ND = Non-detect; detection limits = 0.5 µM and 1.0 µM for NH$_3$ and I$^-$, respectively.
Table A.3 Iodo-DBP Formation from Iopamidol in the Absence of NOM\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Iodo-DBPs (nM)</th>
<th>HOCl</th>
<th>pH</th>
<th>NH\textsubscript{2}Cl</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.5</td>
<td>7.5</td>
<td>8.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Cl\textsubscript{2} I</td>
<td>2.2</td>
<td>3.6</td>
<td>5.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Cl Br I</td>
<td>&lt;0.5</td>
<td>0.5</td>
<td>&lt;0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Br\textsubscript{2} I</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Cl I\textsubscript{2}</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Br I\textsubscript{2}</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>I\textsubscript{3}</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>IAA</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>BrIAA</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Z</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>E</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Diacid</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Iodo-DBP formation at 72 h of reaction mixtures containing iopamidol and aqueous chlorine or monochloramine in purified water. [Cl\textsubscript{2}]\textsubscript{T} or [NH\textsubscript{2}Cl] = 100 \textmu M, [Iopamidol] = 5 \textmu M, [Buffer] = 10 mM, temperature = 25 \degree C. Cl\textsubscript{2}I = dichloroiodomethane, ClBrI = bromochloroiodomethane, Br\textsubscript{2}I = dibromoiodomethane, ClI\textsubscript{2} = chlorodiiodomethane, BrI\textsubscript{2} = bromodiiodomethane, I\textsubscript{3} = iodoform, IAA = iodoacetic acid, BrIAA = bromoiodoacetic acid, Z = (Z)-3-bromo-3-iodopropenoic acid, E = (E)-3-bromo-3-iodopropenoic acid, diacid = (E)-2-iodo-3-methylbutenedioic acid.
Table A.4 Iodo-DBP Formation from Iopamidol in the Presence of NOM (Athens-Clarke County [ACC] Raw Source Water)\textsuperscript{a}.

| Iodo-DBPs (nM) | \(\text{HOCl} \) | | | \(\text{NH}_2\text{Cl} \) | | |
| | \(\text{pH} \) | 6.5 | 7.5 | 8.5 | \(\text{pH} \) | 6.5 | 7.5 | 9.0 |
| Cl\(_2\) I | 43.7 | 171.9 | 211.8 | 11.0 | 6.3 | 0.6 |
| Cl Br I | 0.6 | 1.4 | 1.3 | 0.8 | <0.5 | <0.5 |
| Br\(_2\) I | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| Cl I\(_2\) | 12.2 | 75.6 | 41.5 | 8.6 | 4.9 | <0.5 |
| Br I\(_2\) | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| I\(_3\) | <0.5 | 1.2 | <0.5 | <0.5 | <0.5 | <0.5 |
| IAA | 2.0 | 3.0 | 1.9 | 4.0 | 1.4 | 0.6 |
| BrIAA | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| Z | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| E | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| Diacid | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |

\textsuperscript{a} Iodo-DBP formation at 72 h of reaction mixtures containing iopamidol, aqueous chlorine or monochloramine, and natural source waters. \([\text{Cl}_2]\text{I} \) or \([\text{NH}_2\text{Cl}] \) = 100 \(\mu\text{M}\), \([\text{Iopamidol}] \) = 5 \(\mu\text{M}\), \([\text{Buffer}] \) = 10 mM, temperature = 25 °C, and total organic carbon \([\text{TOC}] \) = 2.1 mg/L. \(\text{Cl}_2\text{I} \) = dichloroiodomethane, \(\text{ClBrI} \) = bromochloroiodomethane, \(\text{Br}_2\text{I} \) = dibromoiiodomethane, \(\text{ClI}_2 \) = chlorodiiodomethane, \(\text{BrI}_2 \) = bromodiodomethane, \(\text{I}_3 \) = iodoform, \(\text{IAA} \) = iodoacetic acid, \(\text{BrIAA} \) = bromoiodoacetic acid, \(Z = (Z)-3\)-bromo-3-iodopropenoic acid, \(E = (E)-3\)-bromo-3-iodopropenoic acid, diacid = \((E)-2\)-iodo-3-methylbutenedioic acid.
Table A.5 Iodo-DBP Formation for Other ICM—Reaction with Chlorine in the Presence of NOM (in Athens-Clarke County [ACC] Raw Source Water)\(^a\).

<table>
<thead>
<tr>
<th>Iodo-DBPs (nM)</th>
<th>Iohexol</th>
<th>Iopromide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Cl(_2) I</td>
<td>4.3</td>
<td>5.2</td>
</tr>
<tr>
<td>Cl Br I</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Br(_2) I</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Cl I(_2)</td>
<td>0.7</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Br I(_2)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>I(_3)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>IAA</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>BrIAA</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Z</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>E</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Diacid</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

\(^a\) Iodo-DBP formation at 72 h of reaction mixtures containing iohexol or iopromide, aqueous chlorine, and natural source waters. [Cl\(_2\)]\(_T\) = 100 μM, [ICM] = 5 μM, [Buffer] = 10 mM, temperature = 25 °C, and total organic carbon [TOC] = 2.1 mg/L. Cl\(_2\)I = dichloroiodomethane, ClBrI = bromochloroiodomethane, Br\(_2\)I = dibromoiodomethane, ClI\(_2\) = chlorodiiodomethane, BrI\(_2\) = bromodiiodomethane, IAA = iodoacetic acid, BrIAA = bromoiodoacetic acid, Z = (Z)-3-bromo-3-iodopropenoic acid, E = (Z)-3-bromo-3-iodopropenoic acid, diacid = (E)-2-iodo-3-methylbutenedioic acid.
Table A.6 Iodo-DBP Formation for Other ICM—Reaction with Monochloramine in the Presence of NOM (in Athens-Clarke County [ACC] Raw Source Water)\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Iodo-DBPs (nM)</th>
<th>pH</th>
<th>Iohexol</th>
<th></th>
<th>Iopromide</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl2 I</td>
<td>6.5</td>
<td>6.4</td>
<td>7.5</td>
<td>4.7</td>
<td>8.5</td>
</tr>
<tr>
<td>Cl Br I</td>
<td>7.5</td>
<td>0.5</td>
<td>0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Br2 I</td>
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<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Cl I2</td>
<td>6.5</td>
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<td>7.5</td>
<td>3.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Br I2</td>
<td>6.5</td>
<td>&lt;0.5</td>
<td>7.5</td>
<td>&lt;0.5</td>
<td>8.5</td>
</tr>
<tr>
<td>I3</td>
<td>7.5</td>
<td>&lt;0.5</td>
<td>8.5</td>
<td>&lt;0.5</td>
<td>8.5</td>
</tr>
<tr>
<td>IAA</td>
<td>&lt;0.5</td>
<td>3.6</td>
<td>0.5</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>BrIAA</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Z</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>E</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Diacid</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Iodo-DBP formation at 72 h of reaction mixtures containing iohexol or iopromide, aqueous monochloramine, and natural source waters. $[\text{NH}_2\text{Cl}] = 100 \mu\text{M}$, $[\text{ICM}] = 5 \mu\text{M}$, $[\text{Buffer}] = 10 \text{ mM}$, temperature $= 25 ^\circ\text{C}$, and total organic carbon $[\text{TOC}] = 2.1 \text{ mg/L}$. Cl2I = dichloriodomethane, ClBrI = bromochloriodomethane, Br2I = dibromiodomethane, ClI2 = chlorodiiodomethane, BrI2 = bromodiiodomethane, IAA = iodoacetic acid, BrIAA = bromoiodoacetic acid, $Z = (Z)$-3-bromo-3-iodopropenoic acid, E = $(Z)$-3-bromo-3-iodopropenoic acid, diacid = $(E)$-2-iodo-3-methylbutenedioic acid.
Figure A.1 Iodate formation as a function of time and pH in reaction mixtures containing iopamidol and aqueous chlorine. $[\text{Cl}_2] \_f = 100 \, \mu\text{M}$, $[\text{iopamidol}] = 5 \, \mu\text{M}$, $[\text{Buffer}] = 10 \, \text{mM}$, and temperature $= 25^\circ \text{C}$. 
Figure A.2 Iodo-THM and iodo-acid formation at 72 h as a function of increasing humic acid (A) or fulvic acid (B) concentration in reaction mixtures containing iopamidol and aqueous chlorine. $[\text{Cl}_2]_T = 100 \mu\text{M}$, $[\text{iopamidol}] = 5 \mu\text{M}$, $\text{pH} = 7.5$, $[\text{Buffer}] = 10 \text{ mM}$, and temperature $= 25 \degree \text{C}$. 
Figure A.3 Pseudo first-order rate constant as a function of time and pH in reaction mixtures containing iopamidol and aqueous chlorine. \([\text{Cl}_2]_T = 100 \ \mu\text{M}, [\text{iopamidol}] = 5 \ \mu\text{M}, \ [\text{Buffer}] = 10 \ \text{mM}, \ \text{and temperature} = 25 \ ^\circ\text{C}.\)
Figure A.4 Proposed mechanism for formation of iodo-DBPs with chlorine and chloramine disinfection (adapted from [6, 7]). Estimated rate constant of $2 \times 10^{-3} \text{M}^{-1} \text{s}^{-1}$ for the $\text{NH}_2\text{Cl} + \text{HOI}$ reaction and $2 \times 10^{-3} \text{M}^{-1} \text{s}^{-1}$ if $\text{OI}^-$ is the reactive species.
Literature Cited


