Acknowledgements

Abstract

Since the 1970s when the first disinfection by-products (DBPs) were discovered in drinking water, over 600 of them have been reported in literature, however, the U.S. Environmental Protection Agency currently regulates only 11 of these species. Many toxicological studies have been performed to assess the health and exposure impacts of a subset of DBPs on various living systems including rodents, bacteria, and mammalian cells. Epidemiologists have found weak linkages between individual DBPs and incidences of bladder cancer, spontaneous abortions, and reproductive problems in humans. While these studies have provided valuable information on possible health endpoints associated with single chemical species, what is more relevant to public health are the exposure effects from DBP mixtures in drinking water which consumers come in contact with on a daily basis.

The challenge with the study of mixtures is that they are complex and difficult to study leading many toxicologists to avoid incorporating them into their research and, as a result, little is known about their applicability to toxicity assays. Our present understanding of DBPs is that the bromine- and iodine-containing species are more geno- and cytotoxic than their chlorine-containing counterparts, but for now we only have individual measures of toxicity for these compounds. In an effort to evaluate the exposure effects of mixtures of DBPs in real drinking waters, the study described in this report took two approaches. The first was to chemically analyze drinking waters that underwent different treatment scenarios as part of a pilot plant study, including UV irradiance. Individual concentrations of the most commonly occurring DBPs were measured, along with total organic halogen (TOX), total organic chlorine (TOCl), and total organic bromine (TOBr) content. The proportions of known and unknown TOX, TOCl, and TOBr in the water samples were also determined. The second approach aimed to tie together

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the chemical analysis of real drinking waters with their inherent toxicities. The first attempt at using a mixture of DBPs with the growth inhibition assay (GIA) involved spiking two very toxic DBPs, bromoacetic acid (BrAA) and iodoacetic acid (IAA), into the same matrix to evaluate whether the combination of the two caused a synergistic or antagonistic effect on the growth inhibition of human colon cells. Several preliminary experiments were also performed to evaluate the toxicity (by GIA) of water concentrated 10-fold by reverse osmosis from different disinfection scenarios.

Results from both approaches included but were not limited to the following findings. In the pilot plant study samples, the percentage of TOX in real drinking waters that was not accounted for by the measurement of 9 DBPs was between 40% and 80%. UV irradiation followed by post-disinfection treatments created higher TOBr concentrations than in samples that were not UV irradiated, while ozonation (O_3) contributed to formation of a greater proportion of unknown TOBr than TOCl in chloraminated waters. This suggests that in waters containing bromide, the use of UV or O_3 can potentially increase the toxicity of the water.

The combination of BrAA and IAA in the same matrix produced an apparent toxicity that was greater than the sum of the effects of the two individual species, leading to the postulate that studies of the toxicity (and by inference concentrations) of individual DBPs cannot accurately predict the potential impact of a mixture. This work demonstrates that current techniques for determining exposure effects of individual DBPs do not truly reflect the toxicity of the drinking water matrix. Thus, drinking water should be treated as a mixture of DBPs, not as individual compounds. Measuring TOX, TOCl, and TOBr content in addition to individual THM and HAA species chemically characterized mixtures, while the GIA evaluated their toxic potency. Applying both methods to the same types of waters would provide a better evaluation of the

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drinking water quality resulting from different source waters and treatment processes.

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I. Background

1. A. Drinking Water Treatment

Drinking water treatment is designed with several goals in mind, some of which are to inactivate harmful pathogens, prevent bacterial regrowth, remove suspended particles and toxic chemicals, and produce safe and palatable drinking water (Xie, 2004). The surface water entering a treatment plant contains natural organic matter (NOM) from decaying vegetation, timber, etc. whose age and source contributes to the overall characteristics of the water. NOM is quite reactive with chemical oxidants, producing by-products that have been shown to have potential human health implications (Boorman et al., 1999; Reif et al., 1996).

Conventional treatment processes for surface waters attempt to reduce the total organic carbon (TOC) load in the water mainly contributed by NOM using coagulation, flocculation, sedimentation, filtration, and disinfection (Figure 1.1). Some surface water treatment plants may use a process called pre-sedimentation, where the water settles in a basin to remove large suspended solids and then undergoes conventional treatment, if the source water contains high amounts of sediment (Logsdon et al., 1999). Prior to coagulation, treatment plants may adjust the pH of the water or pre-treat the water with chemical oxidants such as ozone or chlorine, for controlling seasonal taste and odor problems or enhancing coagulation (Xie, 2004; Logsdon et al., 1999). The downside to pre-oxidation is that depending on type and contact time, it may cause much higher concentrations of disinfectant by-products (DBPs) to form than if added further down the treatment train. As a result, many treatment plants prefer intermediate or post-disinfection instead of pre-oxidation to minimize the DBPs formed in finished drinking water and in order to meet DBP regulations. Treatment plants also utilize the CT concept for disinfecting the water to meet these regulations, where C is a target disinfectant concentration

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present in the water for a sufficient contact time, T, to successfully inactivate harmful pathogens (Symons, 1999). DBP formation is dependent on both C and T.

Coagulation neutralizes the negative charge on suspended particles through addition of alum, ferric chloride, or polymers (Xie, 2004). Once the target dose of coagulant is added, effective mixing of the coagulant and raw water is necessary for ensuring that all of the particles have been neutralized. The water undergoes flocculation to bind these previously suspended particles into large bundles, called "flocs," which are removed by gravity during sedimentation.

Figure 1.1 Conventional surface water treatment process with chloramination for disinfection.
Adapted from Logsdon et al., 1999.

Next, the water undergoes filtration, commonly with sand or anthracite, to further separate any remaining suspended particles from the water, which is then treated with a disinfectant, usually chlorine or chloramines in the United States, to destroy bacteria or inactivate harmful viruses in the water. CT is sometimes achieved by adding disinfectant before filtration. Doing so also prevents the filter from becoming biologically active. If chloramines are the desired disinfectant, a pre-formed chlorine and ammonia solution may be added prior to filtration, or chlorine would

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be added before filtration followed by addition of ammonia in the clearwell. Some water treatment plants treat the water with granular activated carbon (GAC) or powdered activated carbon (PAC) to remove synthetic or natural organic chemicals, such as pesticides or humic substances, and to improve the taste, odor, and color of the finished water.

Facilities using UV irradiance may follow the conventional treatment train until sedimentation, whereupon the water would be filtered and transported to a clearwell. At this point, most of the particulate matter has been removed and the water would be treated with UV thus optimizing UV transmittance and reducing costs associated with maintaining UV lamps (U.S. EPA, 2006a). The water is treated with chlorine or chloramines to provide a disinfectant residual and then distributed.

After treatment of drinking water, it is transported through a network of pipes to water towers, storage areas, or directly to consumers. The distance between the point of entry into the distribution system and the point at which the drinking water reaches a faucet varies from consumer to consumer. Within this distance, a variety of factors can affect the quality of the water. These include how long the water stays inside the pipes, the disinfectant residual, pH, temperature, DBP precursors, microbial intrusions, corrosion of the pipes, and bromide content in the water (Singer & Reckhow, 1999). In order to keep pathogens from growing in the distribution system, treatment plants add an excess amount of disinfectant at the plant or boost the disinfectant concentration during distribution to maintain a residual concentration in the finished water. Typically, free chlorine residuals range between 0.1 and 0.3 mg/L, while chloramine residuals range between 0.2 and 2.0 mg/L (Geldreich & LeChevallier, 1999). Current U.S. Environmental Protection Agency (EPA) regulations are set at a maximum residual disinfectant level (MRDL) of 4.0 mg/L for both free chlorine and chloramine residuals (U.S.

EPA, 2009a).

Although maintaining a disinfectant residual is necessary to prevent microbial regrowth, doing so affects DBP formation (Singer & Reckhow, 1999). As long as a disinfectant residual and DBP precursors are present in the finished drinking water, the disinfectant will continue to react with these precursors or perhaps with some of the less stable DBPs while the water remains in the pipes. The concentration of certain halogenated DBPs is heavily influenced by pH especially those affected by base-catalyzed hydrolysis mechanisms (Singer & Reckhow, 1999). Formation of bromine- or iodine-containing DBPs is affected by the concentration of bromide or iodide in the source water. Bromide is easily oxidized by aqueous chlorine to form hypobromous acid (with a log rate of formation of 3.47 equivalent to 2.95×10^3 $M^{-1}s^{-1}$ at 25°C; Wojtowicz, 1979 cited in Haas, 1999), which reacts with NOM to generate bromine-containing DBPs. Similarly, iodide forms hypoiodous acid in the presence of chlorine or chloramine; the rates of reaction are 4.3×10^8 $M^{-1}s^{-1}$ and $2.4 \times 10^{10} \times [H^+]$ $M^{-2}s^{-1}$, respectively (Bichsel & von Gunten, 1999). With chloramine, the resulting hypoiodous acid reacts with NOM to form iodine-containing DBPs, while with chlorine, iodate is formed (Bichsel & von Gunten, 1999). In general, the rate of DBP formation is directly proportional to temperature, which explains why DBP concentrations are so elevated during the summer months (Singer & Reckhow, 1999).

1. A.1. Chlorination

Chlorine gas (Cl_2), liquid sodium hypochlorite (NaOCl), or solid calcium hypochlorite ($Ca(OCl)_2$) are the most commonly used chemicals for chlorinating drinking water (AWWA, 2006). These chemicals quickly equilibrate in water to form hypochlorous acid (HOCl) and hypochlorite ion (OCl⁻), the sum of whose concentrations is known as the free chlorine residual.

When chlorine gas is added to the water, the following equilibria are established:

$$Cl_2 + H_2O \rightleftharpoons HOCl + HCl$$

$$HOCl \rightleftharpoons H^+ + OCl^-$$

Sodium hypochlorite and calcium hypochlorite both dissociate in water to form hypochlorite ions and hydroxide ions:

$$NaOCl + H_2O \rightleftharpoons HOCl + NaOH$$

$$Ca(OCl)_2 + 2H_2O \rightleftharpoons 2HOCl + Ca(OH)_2$$

The advantages of using chlorine as a disinfectant are that it is a strong oxidant, provides a persistent residual, is relatively inexpensive, easy to use, and has a long history of use (Singer & Reckhow, 1999). However, its use might contribute to taste and odor problems in the drinking water, perhaps associated with the DBPs that form. Chlorination produces high to moderate levels of trihalomethanes (THMs), high to low levels of haloacetic acids (HAAs), moderate levels of haloketones, haloaldehydes, halonitriles, and trace levels of halogenated furanones.

1. A.2. Chloramination

Another common disinfectant that is used in drinking water treatment is chloramines, which are created by combining ammonia with free chlorine. Chloramines are weaker oxidants and disinfectants than chlorine, but they do produce a persistent residual in the distribution system (Singer & Reckhow, 1999). Thus, they are somewhat effective at controlling microbial growth. When free chlorine reacts with ammonia and amino nitrogen compounds in water, monochloramine (NH_2Cl), dichloramine ($NHCl_2$), and trichloramine (NCl_2) can be formed. The chemical reactions for creating chloramines are as follows (Haas, 1999):

$$NH_4^+ + HOCl \rightleftharpoons NH_2Cl + H_2O + H^+$$

$$NH_2Cl + HOCl \rightleftharpoons NHCl_2 + H_2O$$

$$NHCl_2 + HOCl \rightleftharpoons NCl_2 + H_2O$$

The sum of monochloramine, dichloramine, trichloramine, and free chlorine concentrations is known as the total or combined chlorine residual in the water.

When chlorine reacts with ammonia in the water, its disinfection efficiency over time as measured by residual free chlorine follows an applied dose versus residual pattern known as the "breakpoint" curve (Haas, 1999; AWWA, 2006). As the chlorine dose added to the water is increased, the free chlorine residual increases in a near-linear manner while monochloramine is the predominant chloramine species. Once the chlorine to ammonia ratio exceeds 1:1, dichloramine is formed, the curve is no longer linear but instead flattens. Eventually, the curve drops due to dichloramine degradation and begins to rise when more chlorine is added. The lowest point on the curve, before the curve rises again, is known as the "breakpoint" and is the point in the treatment process when all ammonia compounds have become oxidized (AWWA, 2006). Since all source waters have different compositions, drinking water treatment plants must determine their own breakpoint curve.

Chloramination of drinking water can be practiced in two ways: a preformed solution of chloramines can be made prior to water disinfection or chlorine can be added first to the water, sometimes before filtration, and then ammonia is added before or in the clearwell. In the latter method, chlorine has time to react with the remaining NOM to form the usual DBPs. With the preformed chloramine solution, THMs are not produced, while moderate levels of HAAs and moderate to low levels of halogenated furanones are generally present (Singer & Reckhow, 1999). Despite this benefit, usage of chloramines can cause nitrification problems in water

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distribution systems.

A preformed solution of chloramines can be created in the following way. First the free chlorine (Cl_2) concentration of a NaOCl solution must be determined. To calculate the concentration of ammonium needed as nitrogen, a 4:1 ratio of Cl_2 to NH_4 as N is used. Hence, if the Cl_2 concentration is 45 mg/mL, then 11.25 mg/mL NH_4 as N is needed. An ammonium sulfate (($NH_4)_2SO_4$) solution in deionized water (DIW) can be used to provide the ammonia content. The mass of solid ($NH_4)_2SO_4$ needed to prepare this solution is calculated from multiplying the ammonium concentration as nitrogen (11.25mg/mL as N) by the molar ratio of ($NH_4)_2SO_4$ to nitrogen in ($NH_4)_2SO_4$ (132.13:28). Using the numbers that have been provided, the mass of ($NH_4)_2SO_4$ needed is about 53mg, and the concentration of ($NH_4)_2SO_4$ necessary would be 53mg/mL. In order to make a larger volume of the ($NH_4)_2SO_4$ solution, the calculated ($NH_4)_2SO_4$ solution should be multiplied by the desired volume to determine the mass of solid ($NH_4)_2SO_4$ that needs to be added to the desired volume of lab grade water (LGW). The ($NH_4)_2SO_4$ solution should be buffered at a pH ~8.30 using a buffer power pillow per 50mL of solution (Hach, Loveland, CO). Next, equal volumes of the Cl_2 and the ($NH_4)_2SO_4$ solutions should be measured and the ($NH_4)_2SO_4$ solution is added to the Cl_2 solution very slowly, to minimize the formation of dichloramine and maximize the formation of monochloramine. This preformed NH_2Cl solution should be measured for its total chlorine concentration and then added to the water according to the desired chlorine residual.

In November 2000, Washington, D.C.'s water supplier changed the disinfectant from chlorine to chloramines. While this switch in disinfectants lowered the levels of THMs and HAAs formed, lead was leaching out from the distribution pipes as a result of contact with NH_2Cl (Edwards & Dudi, 2004). Another disadvantage to using NH_2Cl as a disinfectant is that

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nitrosamines, referred to as nitrated or nitrosated DBPs, are formed along with the other common DBPs and have been found to be very toxic (Plewa et al., 2004). The use of chloramines as a disinfectant has been found to elevate levels of nitrifying bacteria in drinking water (Lipponen et al., 2002), which leads to biofilm buildup in the distribution pipes. Facilities may choose to flush the pipes for a period of time each year to remove the biofilm by switching from chloramination to chlorination.

1. A.3. Ozonation

Ozone gas ($O_{3(g)}$) was first used in the United States at New York City's Jerome Park Reservoir in 1906 for taste and odor control (Haas, 1999). It is a very strong oxidant and disinfectant and does not produce halogenated DBPs, except in bromide-containing waters (Singer & Reckhow, 1999). Ozone is very unstable and can quickly decompose in water to form the hydroxyl radical ($OH\bullet$) as a result of exposure to UV irradiance, hydrogen peroxide, low CT, or high pH (Reckhow, 1999). Consequently, a disadvantage to using ozone is that it does not produce a persistent residual like chlorine. As ozone is very unstable, it is generated on-site. The chemical reaction is as follows:

$$O_2 + energy \rightarrow O + O$$

$$O + O_2 \rightarrow O_3$$

where molecular oxygen (O_2) reacts with an electric current to produce elemental oxygen (O) and then elemental oxygen combines with molecular oxygen to produce ozone (O_3) (Singer & Reckhow, 1999). Once ozone has been produced, it is passed through a gas adsorption device to transfer the ozone into an aqueous solution. It then begins an "autocatalytic" decomposition process initiated through the reactions between molecular oxygen and hydroxide, ferrous iron, or

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other organics, producing hydroxyl radicals (Reckhow, 1999). Presence of bicarbonate ions (HCO_3^-), carbonate ions (CO_3^-), some compounds possessing alcohol groups, and other solutes can inhibit the decomposition process by consuming OH• (Staehelln & Hoigné, 1985; von Gunten, 2003). This process is considered autocatalytic because hydroxyl radicals "participate in their own formation" (Reckhow, 1999). Advanced oxidation processes (AOPs) using ozone and hydrogen peroxide (H_2O_2) or ultraviolet irradiation promote the decomposition of ozone by producing large amounts of very reactive hydroxyl radicals (Reckhow, 1999).

Since ozone is known to break down some components of NOM into biodegradeable organic materials, it is widely used during pre-treatment of waters to aid with coagulation and flocculation (Siddiqui et al., 1997; Reckhow, 1999). Other DBPs that form specifically through the reaction between ozone and NOM are aldehydes, aldo-keto acids, carboxylic acids, and organic peroxides (Weinberg & Glaze, 1996). After ozone treatment, waters are usually post-chlorinated or post-chloraminated. Shukairy & Summers (1996) showed that in most cases ozonated and post-chlorinated waters produced lower levels of THM4 (chloroform, bromodichloromethane, dibromochloromethane, and bromoform) and TOX compared to a biotreatment without the use of chemical oxidants. HAA6 (chloroacetic acid, dichloroacetic acid, trichloroacetic acid, bromoacetic acid, bromochloroacetic acid, and dibromoacetic acid) concentrations decreased the most using biotreatment with and without preozonation.

Although ozonation and post-chlorination can reduce the concentrations of THM4 and HAA, bromate (BrO_3^-) is produced in waters containing bromide (Reckhow, 1999). This reaction occurs when hypobromous acid (HOBr), produced from a reaction between the disinfectant and bromide, reacts with ozone or a hydroxyl radical to form bromate. Currently, the maximum contaminant level (MCL) for bromate is 0.010 mg/L (U.S. EPA, 2009a),

1. A.4. Ultraviolet Irradiation

In 1877, Downes and Blount discovered that sunlight, the natural form of ultraviolet (UV) light, had biocidal effects on bacteria (Haas, 1999; Malley, 1999). The earliest form of UV technology was a mercury-vapor lamp enclosed in a quartz sheath, which was developed in 1910. These types of lamps are still being used but are now designed to emit UV light within a certain wavelength range and at different intensities and pressures.

The irradiance of any UV lamp is reported in units of mW/cm^2. UV dosage is reported as $mW\text{-}s/cm^2$ or mJ/cm^2 (Malley, 1999). Low-pressure UV lamps emit UV light at a wavelength of 254 nm, which has about 85% of the germicidal effectiveness at the ideal 260 – 265 nm range. Medium-pressure UV lamps emit UV light at a higher intensity and at a broader wavelength range than that of low-pressure lamps.

UV irradiation, when used for water disinfection purposes, is not a chemical oxidant like chlorine or chloramines but it still has the ability to affect DBP formation. When a molecule is at its ground state its bonds become excited from a light source, in this case UV light. As a result, chemical and physical changes to NOM are possible in which the molecule becomes an effective electron donor or acceptor. The following equation developed by Max Planck in 1901 represents the energy produced with respect to the light frequency and wavelength.

$$E = hv = \frac{hc}{\lambda}$$

where:

    E = quantum of radiation represented in joules (J)
    h = Planck's constant, 6.63×10^{-34} J-s
    v = frequency, cycles/s
    c = speed of light, $\sim 3.0 \times 10^{17}$ nm/s
    λ = wavelength, nm

At least 167 kJ of energy is required to break many chemical bonds, which would cause

chemical and physical changes to a molecule. The Beer-Lambert law is also an important relationship when determining the effects of UV irradiation.

$$A = \log_{10} \frac{I_o}{I} = \varepsilon \cdot C$$

where:

A = absorbance
I = number of quanta of light transmitted through the absorbing system, in watts
I_o = number of quanta of light produced (incident light on the absorbing system) per unit time, in watts
ε = molar extinction coefficient, in volume/mole/length
l = distance from light source to absorbing system, in length
C = concentration of absorbing system, in mole/volume

A modified form of this law is used in the drinking water UV industry because drinking water matrices are complex and there is no single chemical concentration;

$$A = \alpha l$$

where α is the absorbance coefficient equivalent to 2.303(A/l). The other terms were defined previously.

More recently, drinking water treatment facilities have looked to using UV irradiation as a pre-treatment in order to help meet strict present and future DBP regulations and guidelines and because it can meet CT requirements without the addition of chemicals. Because UV irradiation does not leave a residual in the water, treatment plants may couple it with post-chlorination or post-chloramination (U.S. EPA, 2006a). After the filtration step in the treatment process, use of UV irradiation may reduce subsequent disinfectant demand so that lower doses of chlorine or chloramines can be used. However, there is little published research describing the effect of UV irradiation on DBP formation. One part of a study by Malley et al. (1995) looked at the effects on DBP formation of UV irradiance doses at 60 mJ/cm^2, 130 mJ/cm^2, and 200 mJ/cm^2 on surface water. Comparing pre- and post-exposure to UV irradiance at those doses, there was

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no noticeable difference in concentrations of chloroform, bromodichloromethane, dibromochloromethane, chloroacetic acid, dichloroacetic acid, and trichloroacetic acid between the two treatment types (Malley et al., 1995). At UV doses greater than 400 mJ/cm^2 on raw drinking water sources, low levels of non-regulated DBPs, such as aldehydes, resulted (Liu et al., 2002). At UV doses typically used for drinking water disinfection (generally less than 140 mJ/cm^2) no significant increases or decreases in concentrations were observed for these types of DBPs (Kashinkunti et al., 2003). Under current drinking water regulations, the MCL of nitrate and nitrite is 10 mg/L and 1 mg/L as N, respectively (U.S. EPA, 2009b). Mack & Bolton (1999) discovered that medium-pressure lamps could convert nitrate to nitrite at wavelengths below 225 nm; however, a later study discovered that using a medium-pressure lamp at biocidal doses produced levels of nitrite much lower than 1 ppm as N, when starting with initial levels of nitrate at 10 ppm as N (Sharpless & Linden, 2001). This suggests that levels of nitrate in drinking water would need to be much higher than 10 mg/L as N to generate nitrite levels that would exceed its MCL.

UV irradiation has the potential to form radicals, as strong oxidants could be involved in the formation of other groups of DBPs. Mopper and Zhou (1990) discovered that under sunlight, NOM undergoes photolysis and produces hydroxyl radicals, OH•, while breaking down NOM into lower molecular weight organics. Similarly, UV irradiance causes nitrate (NO_3^-) to undergo photolysis to produce hydroxyl radicals and NO_2•:

$$NO_3^- + h\nu + H^+ \rightarrow NO_2^- \cdot + OH\cdot$$

As seen in the Sharpless & Linden (2001) study, photolysis of NO_3^- to $NO_2^- \cdot$ occurs at 228 nm. Post-chlorination or post-chloramination of UV irradiated water can thus produce nitrated or nitrosated DBPs (N-DBPs), which have been found to be among the most genotoxic and

cytotoxic DBPs for mammalian cells (Plewa et al., 2004b). Examples of N-DBPs include chloropicrins, which form from chlorination of NO_2^- in the presence of phenols (Merlet et al., 1985), and nitrosodimethylamine (NDMA), which forms as a result of chlorination of NO_2^- in the presence of dimethylamine (Choi & Valentine, 2004). In waters that contain bromide, it is likely that bromine-containing N-DBPs will also be produced from UV irradiation and post-chlorination or post-chloramination.

While there are a number of drinking water treatment facilities that currently use UV irradiation in their treatment train, previous research indicates that a new group of DBPs, N-DBPs, can form; thus, the UV process still needs to be evaluated to identify and quantify N-DBPs and to determine the overall exposure and health impacts that such treated waters will have on consumers.

1. B. Disinfection By-products (DBPs)

The drinking water treatment process is able to remove significant amounts of NOM; however, residual amounts still remain after the filtration process. NOM in drinking water is measured by the concentration of total organic carbon (TOC) in the water and the degree of its aromaticity indicated by specific ultraviolet absorbance (SUVA), which is determined by the ratio of UV to dissolved organic carbon (DOC). A nationwide DBP occurrence study found that the median and maximum TOC concentrations in drinking waters at 12 treatment plants in the United States was 5.8mg C/L and 13mg C/L, respectively (Krasner et al., 2006). Chemical disinfectants combine with the NOM and other precursors, such as bromide, in the water to produce DBPs.

About 600 to 700 DBPs have been identified in the literature, which have formed as a result of treatment of natural waters with chlorine, chloramine, ozone, or chlorine dioxide, or combinations of these disinfectants (Richardson et al., 2007; Krasner et al., 2006). However, halogenated DBPs only comprise a fraction of the total organic halogen (TOX) concentration in the waters. The nationwide occurrence study, for example, found that only about 30% of the halogenated DBPs were accounted for in the TOX content while the remaining 70% were unidentified DBPs (Krasner et al., 2006). The predominant groups of drinking water DBPs are trihalomethanes (THMs) and haloacetic acids (HAAs), followed by haloacetonitriles (HANs), haloketones, cyanogen halides, oxyhalides, aldehydes, and aldoketoacids, to name a few (Krasner, 1999). Factors affecting DBP formation include pH, temperature, seasonal variability, CT, disinfectant residual, and bromide or iodide content.

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1. B.1. Trihalomethanes (THMs)

There are four regulated THM species, chloroform ($CHCl_3$), bromodichloromethane ($CHBrCl_2$), chlorodibromomethane ($CHBr_2Cl$), and bromoform ($CHBr_3$), which when summed are represented as THM4 or TTHM. First discovered and characterized by Rook (1974), they are very volatile, have a high halogen to carbon ratio, and form in drinking water as a result of chlorination (Singer & Reckhow, 1999). The most common THM found in drinking waters is chloroform, while in waters with higher bromide concentrations elevated levels of bromine-containing THMs are present. In the nationwide occurrence study, maximum and median levels of THM4 in drinking waters in the U.S. were found to be 164 µg/L and 31 µg/L, respectively (Krasner et al., 2006). Iodine-containing THMs also exist; however, their occurrence levels are significantly lower than that of the THM4. Median and maximum levels of 6 iodinated THMs in plants treating waters whose sources contain iodide were 0.4 µg/L and 19 µg/L, respectively (Krasner et al., 2006). Chloraminated drinking waters tend to produce lower levels of THM4 but may generate higher levels of iodinated species than chlorination. The current U.S. EPA MCL for THM4 is 80 µg/L (U.S. EPA, 2009a).

1. B.2. Haloacetic acids (HAAs)

The second most dominant DBP group is the HAAs. Nine chlorine- and bromine-containing HAAs can be identified and quantified: chloroacetic acid (ClAA), bromoacetic acid (BrAA), dichloroacetic acid (Cl_2AA), bromochloroacetic acid (BrClAA), trichloroacetic acid (Cl_3AA), dibromoacetic acid (Br_2AA), bromodichloroacetic acid ($BrCl_2AA$), dibromochloroacetic acid (Br_2ClAA), and tribromoacetic acid (Br_3AA). The sum of these 9 species is represented as HAA9. These DBPs are much less volatile than THMs and are very

soluble in water. Cl_2AA and Cl_3AA are the most common HAAs in waters that contain low levels of bromide and are treated with chlorine or chloramine (Singer & Reckhow, 1999). Again, like bromine-containing THMs, bromine-containing HAAs are more prevalent in waters with elevated bromide content. Iodine-containing HAAs, present in treated waters containing iodide, were identified but not quantified in the nationwide occurrence study (Krasner et al., 2006). This finding was significant as Plewa et al. (2004a) determined that iodoacetic acid (IAA) was 3.2 times more cytotoxic in mammalian cells than BrAA and 287.5 times more cytotoxic than ClAA. While there are 9 HAAs that can be identified and quantified, the U.S. EPA only regulates 5 of them: ClAA, Cl_2AA, Cl_3AA, BrAA, and Br_2AA, the sum of which is known as HAA5. The MCL for HAA5 is currently 60 μg/L (U.S. EPA, 2009a).

1. B.3. Unregulated & Emerging DBPs

The U.S. EPA only has MCLs for THM4, HAA5, bromate, and chlorite (a DBP from chlorine dioxide disinfection). However, these 11 DBPs are only a small handful of the 600 to 700 DBPs that have been identified (Richardson, 1998). A review by Richardson et al. (2007) states that there are 74 emerging, unregulated DBPs that occur at moderate levels in drinking waters and possess cytotoxic and genotoxic properties. These 74 DBPs are classified in the review as halonitromethanes (HNMs), iodo-acids and other unregulated halo-acids, iodo-THMs and other unregulated halomethanes, halofuranones, haloacetamides, haloacetonitriles (HANs), tribromopyrrole, N-nitrosodimethylamine (NDMA) and other nitrosamines, aldehydes, and chlorate (Richardson et al., 2007). The review did not include certain DBPs associated with ozonation such as aldo-keto acids, carboxylic acids, or peroxides (Weinberg & Glaze, 1996) nor the emerging and unregulated haloketones and cyanogen halides.

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The nationwide occurrence study was able to identify and quantify many of these groups of emerging DBPs (Krasner et al., 2006). The water samples used in this study were obtained from 12 different drinking water treatment plants in the U.S. Some waters were treated conventionally as described in Chapter 1 of this report, while others were treated with lime softening and post-filtration with or without post-membrane softening. Varying combinations of alternative approaches to water treatment were also used. Disinfectants included: a) ozone, chlorine, chloramine; b) chlorine, chloramines; c) chlorine, d) ozone, chlorine; or e) chlorine dioxide, chlorine, chloramines. The median levels of HNMs, iodinated THMs, haloacetamides, HANs, haloacetaldehydes, and haloketones were between 0.4 $\mu g/L$ and 4 $\mu g/L$, while the maximum occurrence concentrations for these DBP groups was between 7.4 $\mu g/L$ and 20 $\mu g/L$. Halogenated furanones had a median level of 310 ng/L and a maximum level of 2380 ng/L. Bull & Kopfler (1991 cited in Cohn et al., 1999) reported that higher levels of cyanogen chloride resulted from systems using chloramination (median of 2 $\mu g/L$) for disinfection rather than those using chlorination (median of 1 $\mu g/L$). Another study reported that cyanogen bromide concentrations ranged from 1.9 $\mu g/L$ to 26 $\mu g/L$ in waters at various treatment plants that had been spiked with bromide and then ozonated (Weinberg & Glaze, 1996). That same study quantified formaldehyde, acetaldehyde, glyoxal, and methyl glyoxal concentrations in the range 3.9 $\mu g/L$ and 65 $\mu g/L$, 1.8 $\mu g/L$ and 25 $\mu g/L$, 1 $\mu g/L$ and 15 $\mu g/L$, and 1 $\mu g/L$ and 24 $\mu g/L$, respectively (Weinberg & Glaze, 1996). Richardson et al. (2007) reports that NDMA has been found to be present at low ng/L levels in chloraminated or chlorinated drinking water.

1. B.4. Total Organic Halides (TOX)

TOX represents of all of the halogenated organic compounds present in treated drinking

water. Essentially, the TOX concentration is the sum of the total organic chloride (TOCl), total organic bromide (TOBr), and total organic iodide (TOI) concentrations in the water. The nationwide occurrence study found that median and maximum TOX concentrations among the waters that were sampled were 178 µg/L as Cl⁻ and 284 µg/L as Cl⁻, respectively (Krasner et al., 2006). Median and maximum TOCl concentrations were 161 µg/L as Cl⁻ and 206 µg/L as Cl⁻, respectively, while median and maximum TOBr concentrations were 79 µg/L as Br⁻ (35 µg/L as Cl⁻) and 229 µg/L as Br⁻ (102 µg/L as Cl⁻), respectively. TOI was not measured in this study. The study also determined that on a median basis, about 30% of the TOX was accounted for by summing the concentrations of the halogenated DBPs, meaning that 70% of the TOX content is composed of unknown halogenated DBPs.

TOX did not change significantly with varying concentrations of bromide in two types of natural waters with TOC values of 8.5 mg C/L and 5.3 mg C/L and bromide levels of 9 µg/L and 63 µg/L, respectively (Hua et al., 2006). However, with increasing iodide concentrations, TOX decreased significantly. High bromide concentrations resulted in a lower level of unknown TOX and unknown TOCl, but elevated levels of unknown TOBr.

In recent years, scientists have shown less interest in measuring TOX compared to other DBP concentrations in drinking water, a trend that has caused one of the TOX instrument manufacturers to stop producing these instruments. However, the TOX content of finished drinking waters is still an important measurement (i.e. organic bromine and iodine versus chlorine), as it can be used as a surrogate indicator for toxicity. In previous studies examining the mutagenicity, cytotoxicity, or genotoxicity of drinking water DBPs, only individual compounds of DBPs were used (Plewa et al., 2004a; Plewa et al., 2004b; Plewa et al., 2002; Giller et al., 1997). Though understanding the individual exposure effects of these DBPs is very helpful, the

doses that were used in the studies were at concentrations higher than those found in typical drinking waters. These studies focused on the harmful health effects of individual DBP compounds as opposed to the collective negative effects of mixtures of DBPs. It is important to remember that it is the mixture of DBPs to which drinking water consumers are exposed on a daily basis, rather than individual DBPs. Moreover, the studies also leave a particular question unanswered: Due to the fact that about 70% of TOX in treated drinking water has not yet been identified (Krasner et al., 2006), how much of the exposure effect can be attributed to the identified DBPs and how much to the unidentified ones? Because of the uncertainties surrounding this question, it is very important to perform exposure assays with real waters that contain mixtures of DBPs. Since TOX is a measurement of all of the halogenated compounds in drinking water, it captures the TOX concentration of a finished drinking water, which would be a good starting point at which to better understand and assess the water's overall toxic potency.

1. B.5. DBP Regulations

Despite the use of drinking water treatments such as aeration, flocculation, and granular activated carbon adsorption, the aesthetic complications and the presence of anthropogenic pollutants and pathogens in drinking water provoked the federal government to investigate the nation's drinking water supply in the late 1960s (U.S. EPA, 2000). As a result of these studies, Congress and the public became cognizant of the chemical contamination in drinking water supplies (U.S. EPA, 2000). This awareness grew and evolved into the passage of many federal environmental and health laws, one of which was the Safe Drinking Water Act (SDWA) of 1974, to protect the public against such contaminants in public drinking water systems (U.S. EPA, 2000; U.S. EPA, 2001).

At around the same time, Rook discovered that chloroform, a THM, was a DBP produced from the interaction between chlorine and NOM in water (Rook, 1974). Rook also identified $CHBrCl_2$, $CHBr_2Cl$, and $CHBr_3$, the three other THMs in the THM4 group, in this study. Two years later, in 1976, the U.S. National Cancer Institute (NCI) published data showing that chloroform was carcinogenic in rodents (NCI, 1976). These results propelled the EPA to consider regulating THMs, and in 1978, an MCL for total trihalomethanes (THM4) was established at 0.10mg/L and applied to water utilities serving 75,000 or more people (*Federal Register*, 1976). As more DBPs were discovered in public drinking water systems, Congress amended the SDWA in 1986 to "increase the rate at which U.S. EPA regulated contaminants" (SDWA, 1986). These amendments required the U.S. EPA to widely publicize enforceable MCLs and non-enforceable maximum contaminant level goals (MCLGs) for 85 contaminants within a period of 3 years. Unfortunately, these requirements were unable to be met and Congress revised the SDWA once more in 1996 (SDWA, 1996). This time, the amendments focused on: a) requiring the U.S. EPA to provide Congress, the scientific community, and the public with the best available information regarding the regulation of contaminants; b) increasing the U.S. EPA's authority to consider costs and risk reduction when setting standards; c) establishing a financial program to assist communities in complying with changes; and d) developing a program to increase the protection of source waters.

As a result of the 1996 SDWA amendments, the U.S. EPA issued a set of rules called the Stage 1 Disinfectants and Disinfectant Byproducts Rule (S1 DBPR) in 1998, which established maximum residual disinfectant level goals (MRDLGs) and maximum residual disinfectant levels (MRDLs) for the disinfectants chlorine, chloramines, and chlorine dioxide (U.S. EPA, 1998). Additionally, these rules established MCLGs and MCLs for THM4, HAA5, chlorite, and

bromate. When these rules were issued, the MCLs for THM4, HAA5, chlorite, and bromate were based on annual or monthly averages of 0.080 mg/L, 0.060 mg/L, 1.0 mg/L, and 0.010 mg/L, respectively. The compliance deadline for S1 DBPR for large surface water treatment systems was January 2002, while ground water and small surface water systems had a compliance deadline set for January 2004. The driving factors for constructing these rules were results from toxicology studies, which indicated that several DBPs were carcinogenic in laboratory animals (Smith et al., 1986), and epidemiology studies that suggested weak association between bladder cancer (Cantor et al., 1998) and reproductive and developmental effects (Reif et al., 1996).

In efforts to reduce any possible cancer and reproductive and developmental health risks associated with drinking water DBPs, the U.S. EPA issued Stage 2 Disinfection Byproducts Rule (S2 DBPR) in 2005 (U.S. EPA, 2005). With S2 DBPR, the U.S. EPA requires water distribution systems to observe S1 DBPR MCLs as locational running annual averages (LRAA) for THM4 and HAA5 at concentrations of 0.080 mg/L and 0.060 mg/L, respectively (U.S. EPA, 2006b). LRAA is the average of 4 quarterly sampling events at a particular sampling location in a distribution system (U.S. EPA, 2006b). The previous method of calculating MCL compliances was a running annual average (RAA), where for each quarter, all sampling events from all sampling locations were averaged in the distribution system (U.S. EPA, 2006b). An MCLG for chloroform was established at 0.07 mg/L, while MCLGs for Cl_3AA and ClAA were set at 0.02 mg/L and 0.07 mg/L, respectively (U.S. EPA, 2006b).

Systems that must comply with S2 DBPR are "community and nontransient noncommunity water systems that produce or deliver water that is treated with a primary or residual disinfectant," not including ultraviolet light (U.S. EPA, 2005). Community water systems (CWS) are public water systems that provide drinking water for "year-round residents of

a community, subdivision, or mobile home park that has at least 15 service connections or an average of at least 25 residents" (U.S. EPA, 2005). Nontransient noncommunity water systems (NTNCWS) serve at least 25 of the same people more than six months out of the year in places such as schools, businesses, or day care facilities, not areas of primary residence (U.S. EPA, 2005). To guide CWS and NTNCWS, the U.S. EPA developed staggered compliance deadlines based on the number of people and residents these water systems serve. Thus, the EPA will require that all water systems be monitored in October 2013 (U.S. EPA, 2005).

These MCLs and MCLGs were established in order to minimize exposure to DBPs that can pose health risks to consumers. With the S2 DBPR, the U.S. EPA attempts to protect public health by providing stricter regulations to which drinking water treatment facilities must comply.

1. B.6. Drinking Water as a Public Health Concern

One of the most well-known public health events involving drinking water contamination was Dr. John Snow's investigation of the Broad Street pump in 1854 in London, England. As this pump had a "reputation for particularly good water," people living longer distances from its location came to fetch water from it (Winterton, 1980). Water from this pump was also widely used in "public houses, dining rooms and coffee shops and sold in shops…to make fizzy drinks." Snow himself noted that in this area, there were "upwards of five hundred fatal attacks of cholera in ten days" (Snow, 1855). After several days of examining the water from the pump and surveying the occurrence of cholera among the people living and working in the area, Snow made the connection between incidences of cholera outbreaks with usage of the public water pump, located on Broad Street (Snow, 1855; Winterton, 1980). As a result, the handle of the pump was removed to taper the spread of the epidemic.

The Broad Street pump event is a reminder of the public health benefits from chemical disinfection of drinking water. About fifty years after this incident, in 1908, the ability of chlorine to inactivate harmful pathogens in water was demonstrated in two settings in the United States: treatment of very polluted water from Chicago's Union Stockyards with chlorine yielded water of a higher quality than city water, and the first permanent circulation of chlorinated water to Jersey City, resulting in a "dramatic decline in the local typhoid fever rate" (Water Quality and Health Council, 2009). Since the introduction of chemical disinfectants in drinking water in the United States, cholera incidences have been reduced by 90%, typhoid by 80%, and amoebic dysentery by 50% (Orme et al., 1990). Thus, disinfection of drinking water has been widely regarded as one of the most significant public health advances in the past century.

While drinking water disinfection is essential to improving quality of life, a different public health issue has emerged concerning the presence of DBPs in finished drinking water. Over 600 DBPs have been reported in literature (Richardson, 1998) and the unknown TOX content in typical finished drinking waters in the United States is about 70% (Krasner et al., 2006). These DBPs have been linked to incidences of bladder cancer (Cantor et al., 1998), pregnancy loss (Savitz et al., 2006), and adverse effects on the male reproductive system (Luben et al., 2007). Yet the U.S. EPA currently only regulates 11 of the DBPs that have been identified (U.S. EPA, 2009b), because they are the most commonly occurring DBPs, are easy to measure and are present at greater concentrations than the non-regulated DBPs.

The U.S. EPA should continue to regulate these DBPs, but perhaps also consider the value of TOX content of finished drinking waters. Measuring TOX in addition to currently regulated DBPs would provide information on the overall quality of water and potential exposure effects since TOX captures the concentration of halogenated DBPs present in the drinking water.

While the TOX value itself is not precise, it would be a better indicator than measuring only HAA5 and THM4 because TOX presents a value that best represents the mixture of DBPs that are present. Consumers are not exposed to only HAAs, THMs, bromate, or chorite, rather they are exposed to a mixture of these DBPs, the unregulated DBPs, and the DBPs that have yet to be identified.

The majority of toxicity studies concerning exposure effects of drinking water DBPs use individual DBP compounds (Melnick et al., 2006; DeAngelo et al., 1999; Daniel et al., 1992). Though this information is valuable, humans are exposed to mixtures of DBPs at low levels on a daily basis. Mixtures of DBPs have been traditionally thought to be too complex and expensive to work with (Teuschler et al., 2000). In addition, the general fear is that the results may be too difficult to interpret. However, mixtures are very important to understanding the exposure effects of drinking water DBPs and should not be ignored. Using TOX as a surrogate measure for toxicity in drinking water would be helpful for better understanding exposure effects of DBP mixtures. In the absence of a better measure of toxicity, it is therefore beneficial to use TOX to evaluate exposure effects because it is possible that the unknown TOX can contribute to the toxicity of drinking waters.

1. C. Health/Exposure Effects & Toxicity Studies

Since Rook (1974) discovered the presence of THMs in chlorinated drinking water, there have been numerous studies on the health/exposure effects and toxicity of drinking water DBPs. Some of the most common types of studies or assays are *in vivo* using animals, or the *in vitro* Ames mutagenicity and comet assays. Another assay that has not been widely used is the growth inhibition assay (GIA). These studies and assays have been instrumental in assessing the health effects of human exposure to drinking water DBPs. This section aims to summarize the basic components of each assay and evaluate their effectiveness in determining toxicity for DBP mixtures.

1. C.1. *In Vivo*/Animal Toxicity Studies

Animal toxicity studies have played a large role in establishing regulations for DBPs in drinking water (Boorman et al., 1999). Rodents are commonly used in *in vivo* toxicity studies because they share 99% of their genes with humans and, as a result, their physiological and pathological features are very similar to each other (Rosenthal & Brown, 2007). Additionally, the benefits of using mice and rats in such studies also include that their lifetimes are short and can be controlled within a laboratory setting and that they reproduce more quickly than many other mammals.

To my knowledge, there have not yet been any published drinking water DBP toxicity studies that accurately predicted human health endpoints. An explanation for this can perhaps be found in the animal toxicity studies where researchers aim to limit any possible environmental effects that may interfere with the exposure study itself. Humans, who are exposed to DBPs on a

daily basis, are also exposed to a number of other environmental or maybe even genetic factors making it more difficult to pinpoint the exact cause of a specific health endpoint.

The basic objective of animal toxicity studies is to expose a cohort of rodents to a DBP through gavage (force-feeding), oral doses, drinking water, inhalation, or intravenous injection. Several DBPs can be tested at one time, but in general, individual DBP compounds are assigned to different groups of rodents and the DBPs are not administered in mixtures. The daily dose levels for these DBPs are also much higher than the levels that are typically present in U.S. drinking waters. At the end of the study, the rodents are usually sacrificed and pathologically examined for any internal organ or tissue deviations, physical signs of cancer precursors, or carcinoma.

Animal toxicity studies with THMs have shown health endpoints in rodents to include full litter resorption (Bielmeier et al., 2001), congested and enlarged kidneys and livers (Chu et al., 1980), carcinoma in liver, kidney, and intestines (Dunnick & Melnick, 1993), precursors to colon cancer (aberrant crypt foci) (Geter et al., 2004), and kidney and liver damage (Lilly et al., 1997). Rodent health endpoints with HAAs include lung and liver damage and male reproductive abnormalities (Bhat et al., 1991), liver carcinoma (DeAngelo et al., 1999), and multi-site and liver neoplasms and leukemia (Melnick et al., 2006). Researchers discovered that HANs could cause full litter resorption and embryolethality (Smith et al., 1989), and induce oxidative stress (Abdel-Wahab, 2003).

While these studies have been significant in understanding possible toxicological endpoints from exposure to drinking water DBPs, it is difficult to extrapolate these results from rodents to humans. When drinking water is consumed, humans are exposed via ingestion, inhalation, and skin absorption to a mixture of DBPs that have formed in the water, as opposed

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to individual DBP compounds. A major downside to these *in vivo* studies is that the rodents were given doses of a singular DBP instead of a combination of DBPs, which is not a good representation of what humans are exposed to on a daily basis. Additionally, the negative health endpoints that resulted were from exposure to a DBP at a concentration significantly greater than the average concentration found in drinking waters.

1. C.2. *In Vitro* Studies

Although rodents are considered to be good mammalian models for toxicity studies, such studies are time-consuming and expensive. An ideal toxicological assay is one that can provide an endpoint in a shorter timeframe, that is less costly than studies using live animals, and that can be easily extrapolated to humans. These assays evaluate mutagenicity, genotoxicity, or cytotoxicity of a certain compound. Mutagenicity refers to a chemical's ability to induce mutations in an organism's DNA, genotoxicity describes a chemical's potential to cause any damage to a cell's genetic material, while cytotoxicity evaluates the ability of a chemical to cause cell death. Thus, to determine the exposure effects of DBPs in treated drinking water without animals, three relatively simple, *in vitro* assays have been used: the Ames mutagenicity assay, the comet assay, and the growth inhibition assay (GIA).

1. C.2.a. Mutagenicity: The Ames Assay

The Ames assay is a mutagenicity assay using different strains of the bacteria *Salmonella typhimurium*. The *S. typhimurium* strains have undergone a mutation that renders them unable to synthesize the amino acid histidine, preventing the bacteria from growing and forming colonies, (Mortelmans & Zeiger, 2000). Exposure to a mutagenic chemical can reverse this preexisting

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mutation, allowing the newly mutated bacteria to produce histidine, which can be detected from bacterial colony growth on agar plates. Bacterial colonies, called revertants, are then counted and compared to the number revertants that appeared on the control agar plates. Some chemicals are not carcinogenic until they are metabolized. Because *S. typhimurium* lack a metabolic mechanism, a rat liver homogenate, commonly called an S9 fraction, may be added to the agar plate. Currently, the Ames assay is used internationally as a way to determine the "mutagenic potential of new chemicals and drugs" because when a mutagenic response with the Ames assay results, there is a "high predictive value" for carcinogenesis in rodents.

Pegram et al. (1997) discovered that at $CHBrCl_2$ concentrations from 200 to 4800 mg/L mutated revertants resulted in a dose-dependent relationship, while exposure to chloroform ($CHCl_3$) at concentrations from 200 to 25,600 mg/L did not. This finding indicates that the bromine-containing THMs species are more mutagenic than chloroform. In another study, researchers found that of the 6 HAAs tested in the study ($ClAA$, Cl_2AA, Cl_3AA, $BrAA$, Br_2AA, and Br_3AA), all were mutagenic except for $ClAA$ (Giller et al., 1997). HAA concentrations that were used ranged from 0.3 to 20,000 µg/mL. The results from this study further show bromine-containing species are more mutagenic than their chlorine counterparts because $BrAA$ and Br_2AA induced mutagenicity at concentrations at least two orders of magnitude lower than Cl_2AA, Cl_3AA, and Br_3AA. Additionally, these HAAs were tested with and without the presence of S9 and the results indicate that metabolism (with S9) produces by-products that are less mutagenic than those that have not been metabolized (without S9). These results imply that these chemicals are potentially mutagenic in humans.

The haloacetonitriles (HANs), monochloroacetonitrile (ClAN), dichloroacetonitrile (Cl_2AN), trichloroacetonitrile (Cl_3AN), monobromoacetonitrile (BrAN), and dibromoacetonitrile

(Br$_2$AN) were used in an Ames assay study without S9 at concentrations ranging from 5.0×10^{-9} M to 0.91 M, equivalent to 9.9×10^{-4} µg/mL to 10,000 µg/mL (Muller-Pillet et al., 2000). Results showed that the lowest concentration to induce mutagenicity for ClAN, Cl$_2$AN, and Cl$_3$AN were 1.3×10^{-3} M (98 µg/mL), 9.1×10^{-3} M (1000 µg/mL), and 7.0×10^{-5} M (10 µg/mL), respectively. Values could not be determined for BrAN and Br$_2$AN because at the concentrations that were tested, the compounds were either non-toxic at the lower concentrations or completely toxic, killing all of the bacteria, at the higher concentrations.

A disadvantage to using the Ames assay as a means to evaluate toxicity of drinking water DBPs is that the biological system is bacteria, which makes the results from this assay difficult to extrapolate and apply to humans. Moreover, this assay is a very controlled experiment: results can vary depending on the presence of S9, so when is it appropriate to use S9? Also, the assay requires chemical doses at much higher concentrations that those that are typically found in drinking waters. Much like the animal toxicity studies, researchers only exposed the bacteria to individual DBPs, not mixtures.

1. C.2.b. Genotoxicity: The Comet Assay

The comet assay, also known as single-cell gel electrophoresis (SCGE), is a sensitive genotoxic assay that measures single and double-strand breaks in DNA (Collins et al., 2008). In this assay, cells are exposed to a potential genotoxic compound, treated with a dye, spread on SCGE microscope slides pre-treated with a layer of agarose gel, lysed, and then undergo electrophoresis to denature the DNA. During electrophoresis, the breaks in the DNA strand(s) are pulled towards the anode, creating an appearance of comets (Collins et al., 2008). After electrophoresis, the slides are treated for scoring the comets, which can be done visually using a

microscope or using an image analysis program in a computer. While scoring, researchers look for the length of the comet tail, the percentage of DNA in the head of the comet, and the percentage of DNA in the tail. Tail moment, calculated by the product of tail length percentage of tail DNA, or the percentage of DNA in the tail can be used to determine the amount of DNA damage.

Landi et al. (2003) used the comet assay to determine the genotoxicity of the 4 regulated THMs on various human lung epithelial cells. The experiments were performed at the concentrations listed in Table 1.1. Landi et al. (2003) used a statistical multiple-range test to check each dose against the control dose. Tail Extent Moment was determined for each dose and was used as the dependent variable, while the THM concentration and varying lung cell samples were used as the independent variable. The slope (Tail Extent Moment/μM) of the linear regression (r^2) of the data represents that THM's genotoxic potency. Thus, the rank in DNA damaging ability was as follows: $CHBrCl_2$ > $CHBr_3$ > $CHCl_3$, while $CHBr_2Cl$ could not be detected.

Table 1.1 THM concentrations converted from "μM" to "μg/L" with corresponding r^2 values and linear regression slopes (Landi et al., 2003).

THM	Concentrations (μM)	Concentrations (μg/L)	Linear Regression (r^2)	Slope*
$CHCl_3$	10 100 1000	1.19×10^3, 1.19×10^4, 1.19×10^5	0.41	0.004
$CHBr_2Cl$		849, 8.49×10^3, 8.49×10^4	ND	ND
$CHBrCl_2$		2.08×10^3, 2.08×10^4, 2.08×10^5	0.55	0.02
$CHBr_3$		2.53×10^3, 2.53×10^4, 2.53×10^5	0.48	0.006

* = Tail Extent Moment/μM
ND = Not Detected

The genotoxicity of 6 HAAs (BrAA, Br_2AA, Br_3AA, ClAA, Cl_2AA, Cl_3AA) were tested on Chinese hamster ovary (CHO) cells at the concentrations seen below in Table 1.2 (Plewa et al., 2002). Results from these samples were plotted using log-linear dose-response curves, dose

being the HAA concentration (mM) and response represented by the tail moment (µM).

Regression analysis was used to calculate the relative genotoxic potency for each HAA as the

concentration at the midpoint in the tail moment response curve. The r^2 value for each regression

analysis was also determined. The rank order for the potency of these HAAs was BrAA $>>$

ClAA $>$ Br$_2$AA $>$ Br$_3$AA. Cl$_2$AA and Cl$_3$AA were not genotoxic to the CHO cells at these

concentrations.

Table 1.2 HAA concentration ranges converted from "M" to "µg/L" with corresponding
genotoxic potencies and r^2 values (Plewa et al., 2002).

HAA	Concentration Range (M)	Concentration Range (µg/L)	Genotoxic Potency	r^2
BrAA	$2.5\times10^{-6} - 2.5\times10^{-5}$	347 - 3474	0.017 mM = 2360 µg/L	0.99
Br$_2$AA	$2.5\times10^{-4} - 5.00\times10^{-3}$	$5.45\times10^4 - 1.09\times10^6$	1.756 mM = 3.83×10^5 µg/L	0.95
Br$_3$AA	$2.5\times10^{-4} - 5.00\times10^{-3}$	$7.42\times10^4 - 1.48\times10^6$	2.456 mM = 7.29×10^5 µg/L	0.95
ClAA	$1.0\times10^{-4} - 1.0\times10^{-3}$	$9.45\times10^3 - 9.45\times10^4$	0.411 mM = 3.88×10^4 µg/L	0.99
Cl$_2$AA	$1.0\times10^{-3} - 2.5\times10^{-2}$	$1.29\times10^5 - 3.22\times10^6$	NS	NA
Cl$_3$AA	$1.0\times10^{-4} - 3.0\times10^{-3}$	$1.63\times10^4 - 4.90\times10^5$	NS	NA

NS = Not significantly different from control.
NA = Not applicable.

Five HANs were tested for genotoxic potential on HeLa S3 cells from a cervical cancer cell line

using the comet assay (Muller-Pillet et al., 2000). The HANs and the concentration range at

which each was tested can be seen in Table 1.3. For ClAN, Cl$_2$AN, and BrAN, the lowest

concentration before a toxic response was observed could not be detected. However, for Cl$_3$AN

and Br$_2$AN, these concentrations were 1.44×10^3 µg/L and 1.99×10^3 µg/L, respectively.

Table 1.3 HAN concentration ranges converted from "M" to "µg/L" and the lowest concentration before a toxic response was observed (Muller-Pillet et al., 2000).

HAN	Concentration Range (M)	Concentration Range (µg/L)	Lowest Concentration Before Toxic Response Was Observed (µg/L)
ClAN	$0 - 0.001$	$0 - 7.55 \times 10^4$	ND
Cl$_2$AN	$0 - 0.01$	$0 - 1.10$	ND
Cl$_3$AN	$0 - 1.00 \times 10^{-4}$	$0 - 1.44 \times 10^4$	1.44×10^3
BrAN	$0 - 1.00 \times 10^{-4}$	$0 - 1.20 \times 10^4$	ND
Br$_2$AN	$0 - 1.00 \times 10^{-4}$	$0 - 1.99 \times 10^4$	1.99×10^3

ND = Not detected.

An advantage to using the comet assay over the Ames assay is that results from the comet assay can be obtained more quickly than from the Ames assay (Muller-Pillet et al., 2000). The comet assay can be completed in one day, whereas the Ames assay requires about 4 days to run from start to finish (Muller-Pillet et al., 2000). Also, the studies cited in this section used mammalian cells, which indicates that the results from these comet assay studies are more applicable to humans than results from the Ames assay. However, the same issues arise again: the DBP concentrations used in the study were frequently several orders of magnitude higher than those that are typically observed in drinking water and that the DBPs were tested individually, not as a mixture.

1. C.2.c. Cytotoxicity: The Growth Inhibition Assay (GIA)

One of the objectives that was examined in this technical report is to compare the growth inhibitive effects of water that has undergone different types of disinfection and contains a mixture of DBPs. The growth inhibition assay (GIA) was based on the microplate cytotoxicity assay developed by Plewa et al. (2002). Dr. Anthony DeAngelo, a toxicologist at the U.S. EPA in Research Triangle Park, NC, modified the Plewa method to use normal human colon cells (NCM 460) instead of CHO cells. The basic premise of this method is to expose the cells to a chemical at varying concentrations in a 96-well microplate for a period of 3 days. Within the

same microplate, a control set of cells was also prepared in which the cells were only exposed to a cell culture media. After that, growth inhibition was determined by fixing the cells to the plate, staining them, and measuring the absorbance of the dye in each well. The greater the absorbance value, the less the cells experienced growth inhibition. The two research groups measure growth inhibition by comparing the experimental values to the control value and calculating the percentage of cell density as a percentage of the control. The control sample involved cell exposure to cell culture media. The procedure is explained in more detail in the methods section.

Table 1.4 shows the results from an experiment exposing 6 HAAs to CHO cells (Plewa et al., 2002). The concentration ranges are listed for each HAA and there is also a rank order for the HAA that was most cytotoxic (1) to the least cytotoxic (6) determined using an IC50 value, which represents half the maximal inhibitory concentration of the chemical.

Table 1.4 HAA results from Plewa et al. (2002).

HAA	Concentration Range (M)	Concentration Range (µg/L)	IC50 (M)	CHO Cytotoxicity Rank Order
BrAA	1×10^{-6} - 5×10^{-5}	$139 - 6.95\times10^{3}$	8.9×10^{-6}	1
Br$_2$AA	$1\times10^{-6} - 1.25\times10^{-3}$	$218 - 2.72\times10^{5}$	5.0×10^{-4}	3
Br$_3$AA	$1\times10^{-6} - 0.01$	$297 - 2.97\times10^{6}$	1.0×10^{-3}	4
ClAA	$1.0\times10^{-4} - 0.01$	$9.45\times10^{3} - 9.45\times10^{5}$	9.44×10^{-4}	2
Cl$_2$AA	$5\times10^{-4} - 0.043$	$6.45\times10^{4} - 5.54\times10^{6}$	0.0115	6
Cl$_3$AA	$1.0\times10^{-3} - 0.043$	$1.63\times10^{5} - 7.03\times10^{6}$	0.0175	5

Table 1.5 displays DeAngelo's results using the GIA with THM4 and HAA9. The potency of each HAA was ranked and compared with the rank order seen in Plewa et al. (2002).

Table 1.5 GIA results for THM4 and HAA9.

		Concentration Range (M)	Concentration Range (µg/L)	IC50 (M)	GIA Rank Order	Plewa et al. (2002) Rank Order
THM4	CHCl$_3$	1.00×10^{-9} – 3.00×10^{-3}	0.12 – 3.58×10^5	ND		
	CHBrCl$_2$		0.16 – 4.91×10^5	ND		
	CHBr$_2$Cl		0.21 – 6.25×10^5	~3.00×10^{-3}		
	CHBr$_3$		0.25 – 7.58×10^5	~3.00×10^{-3}		
HAA9	ClAA	1×10^{-6} – 1×10^{-3}	94.5 – 9.45×10^4	2.87×10^{-4}	5	2
	BrAA	1×10^{-6} – 1×10^{-4}	139 – 1.39×10^4	1.48×10^{-5}	1	1
	Cl$_2$AA	1×10^{-6} – 0.5×10^{-3}	129 – 6.45×10^4	3.41×10^{-3}	6	6
	BrClAA		173 – 8.67×10^4	5.00×10^{-4}	2	NA
	Cl$_3$AA		163 – 8.17×10^4	4.17×10^{-3}	4	5
	Br$_2$AA		218 – 1.09×10^5	4.70×10^{-4}	3	3
	BrCl$_2$AA		209 – 1.04×10^5	2.03×10^{-3}	9	NA
	Br$_2$ClAA		252 – 1.26×10^5	2.18×10^{-3}	8	NA
	Br$_3$AA		297 – 1.48×10^5	2.37×10^{-3}	7	4

The GIA is not widely used and has only been published by Plewa and the researchers in his lab. Some advantages to using the growth inhibition over the Ames assay or the comet assay are that a) the GIA uses mammalian cells so results are more applicable to exposure effects on humans, b) although the comet assay can also use mammalian cells, the GIA is overall more sensitive than the Ames and comet assays, and c) with the GIA, the control and the experimental samples are all on one microplate, so the conditions to which both are exposed are the same. As with the previous animal toxicity, Ames and comet assay studies, the recurring issues were that the DBP concentrations used were much higher than the concentrations that exist in typical finished waters and that no mixtures of DBPs in real waters were tested.

The main difference between the Plewa et al. (2002) and the DeAngelo method is that the former uses CHO cells and DeAngelo's method uses NCM 460 cells. The CHO cells were from a cell line, while the NCM 460 cells are from a mixed colon cell culture. Cells from a cell line are more likely to mutate over a long period of time; thus, the genetic material of the cells at the

time of use is probably different than when the cells were first selected to begin the line. On the other hand, the NCM 460 cells that are used in the GIA experiments originate from a cell culture and do not mutate over time because the cells begin as stem cells, then differentiate, and then die, similar to the behavior of actual cells in a live colon. To keep these cells alive, they are passaged, where a small amount of cells from a previous cell culture is removed and placed into another test tube containing fresh growth media. Because the behavior of the NCM 460 cells is very similar to a real human colon, exposure effects from the GIA can be better related to humans than results from the previously mentioned studies. Thus, the GIA was chosen for the experiments regarding exposure effects in this technical report.

1. D. Research Objectives

- To show that the THMs and HAAs account for only a small fraction of the TOX of chlorinated or chloraminated drinking waters, while the majority of organic halogenated by-products is composed of chemicals that have yet to be identified. In doing so, the importance of looking at drinking waters as DBP mixtures is emphasized since it is uncertain which part (accounted-for or unaccounted-for) of the TOX contributes more to the overall toxicity of the water.

- To develop a working method for the GIA with mixtures of DBPs in real waters.

- To examine the effects of DBP mixtures in RO-concentrated and disinfected waters on the GIA.

- To quantify the concentrations of a group of known and potentially carcinogenic DBPs present in these waters.

- To evaluate the effectiveness of the GIA in determining the toxicity of real waters.

- To develop a method for determining potential indicators of drinking water toxicity using TOX and the GIA.

2. Experimental Methods & Materials

2. A. Description of the Project

The project outlined in this technical report is organized into two parts. The first part is derived from a two-year American Water Works Association Research Foundation (AwwaRF) study involving researchers from the University of North Carolina at Chapel Hill (UNC-CH), Duke University, and Yale University. This project examined drinking water DBPs resulting from treatment of water with UV irradiance with and without subsequent addition of chlorine or chloramines. For a period during this AwwaRF research project, a partner water treatment facility from Contra Costa Water District (CCWD) in CA performed a series of pilot plant evaluations using ozone with and without peroxides on waters with elevated levels of DBP precursor materials. The waters were then treated with UV irradiance and post-chlorinated/chloraminated in the facilities at Duke University and brought to UNC-CH for DBP analysis. This report examines various aspects of halogen incorporation affected by these treatments.

Since bromide and/or iodide incorporation into DBPs can increase the potency of these chemicals, the second part of this report examines the use of a toxicity assay for evaluating changes in the potency of water that undergoes different treatments. The water samples were prepared at UNC-CH and brought to the DeAngelo Toxicology Lab at the U.S. Environmental Protection Agency (EPA) in Research Triangle Park (RTP), NC for analysis using the GIA.

2. A.1. Contra Costa Water District (CCWD)

Table 2.1 displays the phased treatment schemes and their codes. The dates for water collection, treatment at Duke University, quenching (removal) of free chlorine, and analysis at

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UNC-CH are also listed in the table. Results from Phases 3B Pre-NF and 3B Post-NF are not included in this report. Prior to ozone or ozone/peroxide treatment, the water was spiked at CCWD with bromide (Br⁻), chloride (Cl⁻), iodide (I⁻), and sulfate (SO₄²⁻). The spiked concentration of Br⁻ and I⁻ (the relevant DBP precursors) was 1 mg/L and 0.1 mg/L, respectively.

Table 2.1 Dates for actions.

Phase	CCWD Treatment		Duke Treatment	Quenched at UNC-CH	UNC-CH Extractions	
					HAA9	Volatiles
1A	6/2/2008	Ozone/GAC*	6/09/2008	6/10/2008	6/16/2008	6/12/2008
2A	6/3/2008	Ozone/peroxide/GAC	6/11/2008	6/12/2008	6/17/2008	6/13/2008
1B	6/9/2008	Ozone/GAC	6/22/2008	6/25/2008	7/02/2008	6/26/2008
2B	6/10/2008	Ozone/peroxide/GAC	6/27/2008	6/30/2008	7/03/2008	7/01/2008
3A – Pre-NF	6/30/2008	Pre-nanofiltration (NF)	7/11/2008	7/14/2008	7/17/2008	7/15/2008
3A – Post-NF	6/30/2008	Post-NF	7/21/2008	7/24/2008	8/01/2008	7/25/2008
3B – Pre-NF	7/7/2008	Pre-NF	7/27/2008	7/30/2008	8/01/2008	7/31/2008
3B – Post-NF	7/7/2008	Post-NF	8/11/2008	8/14/2008	NE	8/15/2008

NE = Not Extracted
*Waters were post-filtered by granular activated carbon (GAC).

Table 2.2 lists the abbreviations for the various water treatments that were performed at Duke University.

Table 2.2 Treatment scheme abbreviations.

No Treatment	CCWD waters did not undergo any treatment schemes at Duke.
NH₂Cl	1 mg/L as Cl₂ target chloramine residual
Cl₂	1 mg/L target free chlorine residual
LP400	Low pressure lamp at 400 mJ/cm²
MP100	Medium pressure lamp at 100 mJ/cm²
MP400	Medium pressure lamp at 400 mJ/cm²

2. A.1.a. Water Characterization

Table 2.3 and Table 2.4 display the summary of organic and inorganic parameters of the CCWD waters measured upon receipt at Duke and compared to those provided by CCWD, prior to UV/post-disinfection treatment at Duke. Organic parameters include analysis of UV absorption at a wavelength of 254 nm (UV_{254}), total organic carbon (TOC), and calculated specific ultra violet absorbance (SUVA), which is determined by the ratio of UV_{254} to DOC. DOC values were not provided by Duke or CCWD, so SUVA was calculated using TOC instead. Inorganic parameters (Table 2.4) include bromide (Br^-), nitrate (NO_3^-), and nitrite (NO_2^-).

Table 2.3 Summary of water quality by organic parameters comparing measurements at CCWD and Duke laboratories.

Phase	UV_{254} (/cm) by Duke	UV_{254} (/cm) by CCWD	TOC (mg C/L) by Duke	TOC (mg/L) by CCWD	SUVA (L/mg-m) by Duke	SUVA (L/mg-m) by CCWD
1A	0.0274	0.03	2.96	2.8	0.93	1.07
1B	0.0298	0.04	2.86	2.8	1.04	1.43
2A	0.0255	0.03	2.86	2.6	0.89	1.15
2B	0.0224	0.04	2.96	2.8	0.76	1.43
3A - Pre-NF	0.0161	0.02	NC	2.3	NC	0.87
3A - Post-NF	0.0016	0.0	NC	0.5	NC	0.0
3B - Pre-NF	0.0159	0.02	NC	2.3	NC	0.87
3B - Post-NF	0.00179	0.0	NC	0.5	NC	0.0

NC = Not calculated

Table 2.4 Summary of water quality by inorganic parameters comparing measurements at CCWD and UNC-CH laboratories after spiking event.

Phase	Br^- (mg/L) by CCWD	NO_3^- (mg/L as N) by UNC-CH	NO_3^- (mg/L as N) by CCWD	NO_2^- (mg/L as N) by UNC-CH
1A	0.4	0.71	<2.0	0.51
1B	0.5	0.76	<2.0	0.37
2A	0.4	0.65	<2.0	0.40
2B	0.6	0.50	<2.0	0.26
3A-pre-NF	0.4	2.28	<2.0	3.07
3A-post-NF	0.3	2.24	<2.0	1.68
3B-pre-NF	0.5	2.12	<2.0	2.38
3B-post-NF	0.3	2.07	<2.0	1.58

2. A.1.b. NO_3^- and NO_2^- Analysis

NO_3^- was measured with a Hach DR/890 Colorimeter (Hach, Loveland, CO) using the nitrate mid-range (0 – 5.0 mg/L as N) cadmium reduction method (#8171). NO_2^- was also measured with a Hach DR/890 Colorimeter, but with the nitrite high range (0 – 150 mg/L NO_2^-) ferrous sulfate method (#8153).

2. A.1.c. UV Irradiation at Duke University

250mL or 500mL aliquots of water from CCWD were exposed to either low-pressure-Hg (LP) UV, medium-pressure-Hg (MP) UV, or no UV. UV fluences that were used were 100 mJ/cm^2 or 400 mJ/cm^2. During irradiation, the sample was stirred continuously while in a 500 mL irradiation vessel using a magnetic stir bar and a stir plate. Temperature was not controlled because the samples were post-disinfected.

The low pressure (LP) UV lamp set-up (monochromatic UV source) contains 4 LP Hg UV lamps (General Electric #G15T8) emitting primarily 254 nm wavelengths. Calculating the UV fluence ($mJ\ cm^{-2}$) applied to an aqueous solution required a measurement of the UV irradiance ($mW\ cm^{-2}$) incident to the solution surface. A radiometer (IL 1700, SED 240/W, International Light, Peabody, MA), with a UV detector calibrated to 254 nm, was used to measure the incident irradiance. This step was done by first placing the irradiation vessel containing the water sample on top of the stir plate and measuring the distance from the bottom of the vessel to the water's surface. Next, the irradiation vessel was removed and the radiometer was placed on top of the stir plate to achieve the same distance. Wooden blocks were placed in between the radiometer and the stir plate to ensure that the distance from the top of the stir plate to the top of the radiometer was congruent. Average UV fluence applied to the entire solution

volume was calculated by multiplying the incident irradiance by factors accounting for the divergence of the collimated beam, reflection at the water surface, variation in the irradiance over the surface of the solution, and photon absorption with depth in the water column (Bolton & Linden, 2003). The medium pressure (MP) UV lamp set-up (polychromatic UV source) consisted of a Hanovia Co. (Union, NJ) 1kW MP lamp in a housing manufactured by Calgon Carbon Corporation (Pittsburg, PA). Like LP UV fluence calculations, MP UV fluence is also a function of the incident irradiance measured with a calibrated radiometer and corrected for similar factors. The MP UV fluence is weighted to the UV absorbance spectra of DNA (Linden & Darby, 1997) for the calculation of the germicidal irradiance.

2. A.1.d. Disinfectant Dosing at Duke

Chlorination (Cl_2) doses were selected from demand tests undertaken to calculate the dose required for a 1 mg/L free chlorine residual after 24 hours contact time, for phase 1A and 2A samples, and 72 hours contact time, for all other phases. Free chlorine was obtained from a NaOCl stock solution. During reaction, the samples were contained in 500 mL or 1 L glass amber bottles, headspace-free, with plastic caps and PTFE-lined septa, and were kept in a dark cabinet at room temperature (~20°C). Chloramination (NH_2Cl) dosing was performed by adjusting the sample pH to 8.5, addition of free chlorine at 2 mg/L, and holding for 13 minutes before the addition of ammonium chloride to convert chlorine to monochloramine. This is the chloramination scheme used at the CCWD pilot plant. Contact times were as described for chlorination.

The residual disinfectant concentrations are presented in Table 2.5 and were measured using the Hach DPD methods for free chlorine (Cl_2 samples) and total chlorine (NH_2Cl samples)

with a Hach Pocket Colorimeter Test Kit (Hach Company, Loveland, CO).

Table 2.5 Disinfectant Residuals (mg/L as Cl_2).

Sample	Phase 1A		1B		2A		2B	
	free	combined	free	combined	free	combined	free	combined
No Treatment	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NH_2Cl	0.0	1.05	0.0	1.67	0.0	2.18	0.0	2.13
Cl_2	0.03	0.03	0.24	0.24	1.11	1.11	0.11	0.11
LP400 NH_2Cl	0.0	1.54	0.0	1.74	0.0	1.89	0.0	2.44
MP400 NH_2Cl	0.0	1.27	0.0	1.66	0.0	1.75	0.0	2.61
MP400 Cl_2	0.02	0.02	0.06	0.06	1.3	1.3	0.22	0.22
MP100 NH_2Cl	0.0	1.27	0.0	1.6	0.0	1.99	0.0	2.54
LP400 Cl_2	0.17	0.17	0.21	0.21	1.2	1.2	0.23	0.23

Sample	Phase 3A Pre-NF		3A Post-NF		3B Pre-NF		3B Post-NF	
	free	combined	free	combined	free	combined	free	combined
No Treatment	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NH_2Cl	0.0	2.74	0.0	0.83	0.0	2.17	0.0	0.57
Cl_2	1.19	1.19	2.87	2.87	0.02	0.02	1.67	1.67
LP400 NH_2Cl	0.0	2.55	0.0	0.67	0.0	1.53	0.0	0.32
MP400 NH_2Cl	0.0	2.76	0.0	0.61	0.0	1.59	0.0	0.42
MP400 Cl_2	1.08	1.08	3.03	3.03	1.53	1.53	1.72	1.72
MP100 NH_2Cl	0.0	2.08	0.0	0.49	0.0	1.56	0.0	1.29
LP400 Cl_2	0.95	0.95	3.0	3.0	1.4	1.4	1.7	1.7

2. A.1.e. Laboratory Grade Water (LGW) Preparation

Laboratory grade water (LGW) was prepared in the lab from a Pure Water Solutions (Hillsborough, NC) system that pre-filters inlet 7 MΩ deionized water to 1 μm, removes residual disinfectants, and reduces TOC to less than 0.2mg C/L using an activated carbon resin. Additionally, it removes ions to 18 MΩ with a mixed bed ion-exchange resins.

2. A.1.f. HAA9 Extraction and Analysis at UNC-CH

After the prescribed disinfection dose and water contact time, the Cl_2- and NH_2Cl-treated water was transferred to 40 mL vials containing about 8 grains of ammonium sulfate $((NH_4)_2SO_4$, granular, Mallinckrodt, Paris, KY) to quench residual disinfectant. The glass vials, filled headspace-free, were then capped with open-top plastic caps and PTFE-lined silicone septa. After mixing the vial contents, the samples were then stored in a refrigerator at 4°C prior to extraction, which took place within one week of quenching.

20 mL aliquots of each sample were extracted for HAA9 using a liquid-liquid extraction method with methyl *tert*-butyl ether (>99.99%, OmniSolv MtBE, EMD Chemicals, Darmstadt, Germany), derivitization with diazomethane, and analysis by gas chromatography (GC) on a Hewlett Packard 5890 Gas Chromatograph with an autosampler/HP 7673 injector, Varian FactorFour DB-1 Column: 30 m × 0.25 mm internal diameter (id) × 1 μm film thickness, and an Electron Capture Detector (ECD). The data system for the computer is the Hewlett Packard ChemStation (Rev. A.06.03 [509]) software. The chromatographic peaks had to be manually integrated due to inconsistent baselines. A detailed description of this procedure is presented as an SOP for this method in the Appendix.

2. A.1.g. Halogenated Volatiles Extraction and Analysis at UNC-CH

The disinfected (Cl_2- and NH_2Cl-treated) water was transferred to 60 mL glass vials containing 1.4 mg of L-ascorbic acid (Certified ACS grade, Sigma, St. Louis, MO) to quench residual disinfectant. The samples were collected in 60 mL glass vials, filled headspace-free, and capped with open-top plastic caps and PTFE-lined silicone septa. The samples were then stored in a refrigerator at 4°C prior to extraction, which took place the day after quenching.

The samples were extracted for halogenated volatiles using a liquid-liquid extraction method with MtBE (>99.99%, OmniSolv MtBE, EMD Chemicals, Darmstadt, Germany) and analyzed by GC on a Hewlett Packard GC5890 Series II with an autosampler/autotower injector, HP-1 (Agilent Technologies, Palo Alto, CA) capillary column 30 m × 0.25 mm id × 1 µm film thickness, and a Hewlett-Packard Model ECD. The analytes included in this extraction method are as follows: THM4, bromochloroacetonitrile, dibromoacetonitrile, 1,1-dichloro-2-propanone, 1,1,1-trichloroacetone, trichloroacetonitrile, trichloromethane, and chloral hydrate. Hewlett Packard ChemStation (Rev. A.06.03 [509]) software was used for automated chromatographic peak analysis/integration. Only the THM4 data were used for this report. A more detailed account of this method is presented as an SOP in the Appendix.

2. A.1.h. Total Organic Halogen (TOX) Analysis

The treated water was quenched for residual disinfectant using a solution of 40 mg/mL sodium sulfite (Na_2SO_3, anhydrous, Certified ACS grade, EM Science, Gibbstown, NJ) in LGW per 250 mL glass amber bottle. The bottle was filled headspace-free, and capped with open-top plastic caps and PTFE-lined silicone septa. The samples were then stored in a refrigerator at 4°C prior to TOX analysis, which took place within two weeks of quenching.

Immediately before TOX analysis, the samples were brought to room temperature and pre-treated with 50 drops of concentrated sulfuric acid (H_2SO_4, Certified ACS Plus, Fisher Scientific, Fair Lawn, NJ) using a glass Pasteur pipette. Next, 100 mL of each sample was adsorbed onto two glass-packed carbon columns (2 mm ID, CPI International, Santa Rosa, CA) that were connected in series using an AD-2000 Adsorption Module Model #890-161 (Tekmar Dohrman, Cincinnati, OH). The second carbon column was used to catch any residual organic

halogen (breakthrough) that could not be adsorbed onto the first carbon column. Both carbon columns were flushed with 2 mL of a 5,000 mg NO_3^-/L potassium nitrate (KNO_3, Certified ACS Grade, Fisher Scientific, Fair Lawn, NJ) solution in LGW to remove inorganic halides. The carbon packed in the column was removed and analyzed for TOX using a DX-2000 Organic Halide Analyzer Model #890-162 (Tekmar Dohrman, Cincinnati, OH) via combustion at 850°C to volatilize the organic halogens, which were then measured using a coloumetric titration. Before and after each set of sample analysis that were analyzed at once, a nitrate blank was prepared to determine the level of background TOX from the reagents, carbon, and carrier gases.

Quality control for TOX was carried out by running combustion checks of granulated activated carbon (GAC) powder and 2 µL of a 500 ng/µL 2,4,6-trichlorophenol solution injected directly into the boat. The coloumetric cell was also monitored by injecting 5 µL of a 200 ng Cl/µL sodium chloride (NaCl) solution directly into the cell and running checks to test the cell's recovery. Acceptance criteria for both tests were percentage recoveries between 90% and 110%. In order to check the overall performance of both the TOX adsorption module and analyzer, 50 mL of LGW was spiked with dichloroacetic acid (DCAA) to give a final DCAA concentration of 100 µg/L in the water, was adsorbed onto the carbon columns, and analyzed for TOX. Percentage recovery of DCAA was calculated to be over 100%. Based on this result, it was decided that recovery tests with other HAAs or THMs were not necessary. For more details on these procedures, please refer to the SOP located in the appendix.

2. A.1.i. Total Organic Chloride (TOCl) & Total Organic Bromide (TOBr) Analysis

The water samples used for this analysis were from the same bottles that were used in TOX analysis. When the samples were ready for TOCl/TOBr analysis, they had already

undergone quenching and pre-treatment with concentrated H_2SO_4 (Certified ACS Plus, Fisher Scientific, Fair Lawn, NJ) as described in the TOX analysis section.

50 mL of each sample was adsorbed onto two glass-packed carbon columns as described for TOX. The difference in this method is that prior to sample combustion, the acid scrubber vial containing 80% H_2SO_4 for TOX analysis was removed and replaced with a scrubber vial containing 7 mL of LGW. Organic halogens are vaporized into their hydrogen halides during combustion and collected as halides in this scrubber vial over a period of 220 seconds. To prevent the vapors from condensing on the glass combustion tube that is exposed to the air in the lab, heating tape maintained at 100°C was attached to the exposed area. The contents of this scrubber vial were transferred to a 20 mL glass vial with an open top plastic cap with a PTFE-lined silicone septa. 3 mL of LGW was used to rinse the scrubber vial for residual sample and the volume was transferred into the corresponding 20 mL glass vial. Samples were stored in a refrigerator at 4°C prior to analysis by ion chromatography (IC).

Quality control for measuring TOCl/TOBr was undertaken by performing the same cell and combustion checks as discussed in the previous section on TOX analysis (section 2. A.1.h). In addition, matrix and scrubber spikes were performed on samples selected at random. For the matrix spikes, pentachloroacetone (85%, Aldrich, St. Louis, MO) and (±)1,2-dibromopropane (95%, Aldrich, St. Louis, MO) were spiked into the selected water samples to give a desired concentration and then the samples were analyzed accordingly. Spiking concentrations were determined by assuming that half of the TOX value is chloride and the other half is bromide. The theoretical chloride and bromide values were doubled because the matrix spike concentrations should be about two times the expected value. The acceptance criterion for the matrix-spiked samples was a percentage recovery greater than 60%. The scrubber spikes used 5 mL of the 10

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mL TOCl/TOBr samples collected from the TOX analyzer, selected at random. The samples are spiked with 10 μL of an inorganic chloride and bromide calibration standard at 100 mg/L, to give a 200 ppm spike concentration. Then the spiked sample was analyzed by IC and percentage recovery was calculated. The acceptance criterion for scrubber-spiked samples was a percentage recovery greater than 65%. Details on these procedures are in the SOP in the Appendix.

A calibration curve for Cl⁻ and Br⁻ was prepared prior to each day of sample analysis using the IC. All of the samples, including nitrate blanks, were analyzed using a Dionex Ion Chromatograph (Dionex Corporation, Sunnyvale, CA) with a conductivity detector, AS-14 analytical column and guard column (Dionex Corporation, Sunnyvale, CA), AMMS III-4 mm ion suppressor (Dionex Corporation, Sunnyvale, CA), and 250 μL sample volume loop. The SOP is located in the Appendix.

2. B. Growth Inhibition Assay (GIA)

The methods and procedures in this section are grouped into two experiment sets. The objective of the first set was to use the GIA to determine the growth inhibitive effects of water samples that underwent different types of treatment processes producing a mixture of DBPs in the matrix. These water samples were prepared from University Lake (Carrboro, NC) that was concentrated by a 10-fold factor using a reverse osmosis (RO) membrane. A Shimadzu TOC-V_{CPH}/TNM-1 (Shimadzu Corporation, Columbia, MD) was used to measure a TOC concentration of 65 mg C/L. This water then underwent disinfection treatment.

The second set of experiments involved determining the combined growth inhibitive effects on M3:10 Culture Media of BrAA and IAA in a chlorinated RO concentrate. A question we wanted to answer was: does the combination of the two chemicals cause a synergistic or

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antagonistic effect on the growth inhibition of the cells? The preparation of this experiment is described in the following sections.

2. B.1. GIA Experiment Set 1 Methods

2. B.1.a. Sample Preparation at UNC-CH

Table 2.6 displays the samples that were used in this experiment set, their descriptions, and their respective dose volumes. A 59.9 mg/L as Cl_2 NaOCl (4-6%, Fisher Scientific, Fair Lawn, NJ) was used to chlorinate the samples at a 5:1 ratio of free chlorine to TOC concentration. A 1000 mg/L Br^- stock solution was prepared from granular sodium bromide (NaBr, Certified ACS grade, Fisher Scientific, Fair Lawn, NJ) in LGW and stored at 4°C. A 1000 mg/L I^- stock was prepared from granular sodium iodide (KI, EM Science, Gibbstown, NJ) in LGW and stored at 4°C. The samples listed in Table 2.6 were all prepared using RO concentrated water except for samples "LGW Q" and "BrAA." After the samples were prepared, they were placed in a dark cabinet for 5 days contact time with the disinfectant. After this time, the samples were measured for free chlorine residual using a Hach Pocket Colorimeter Test Kit (Hach Company, Loveland, CO) and the free chlorine DPD method. L-ascorbic acid (Certified ACS grade, Sigma, St. Louis, MO) was used as the quenching agent to remove free chlorine.

Table 2.6 GIA Experiment Set 1 samples, descriptions, and their respective dose volumes.

Sample	Description	Sample Total Volume (mL)	Cl_2 Dose Volume (μL)	Br Dose Volume (mL)	I Dose Volume (mL)	Ascorbic Acid Added (mg)	[Ascorbic Acid] (mg/L)
C	325mg/L Cl_2	126	685	NA	NA	42	333
SC B	10mg/L Br + 325mg/L Cl_2	126	685	1.26	NA	42	333
SC I	10mg/L I + 325mg/L Cl_2	126	685	NA	1.26	37	294
Pre-CB	Pre-formed 10mg/L Br + 325mg/L Cl_2	126	685	1.26	NA	46	365
Pre-CI	Pre-formed 10mg/L I + 325mg/L Cl_2	126	685	NA	1.26	45	357
LGW Q	145mg/L Cl_2 + ~23mg ascorbic acid in LGW	40	97	NA	NA	23	183
40C BrAA	40% of original C solution + BrAA	--	--	--	--	--	--
BrAA	Positive control	--	--	--	--	--	--

NOTE: NA = not applicable

Prior to sample preparation, the RO concentrated water was adjusted to a neutral pH (~7) using a 10 N solution of sodium hydroxide (NaOH, Fisher Scientific, Fair Lawn, NJ) and checked with pH indicator strips (EM Science, Gibbstown, NJ). The samples were prepared according to Table 2.6. After 5 days of contact time, the free Cl_2 residual was measured using a Hach Pocket Colorimeter Test Kit (Hach Company, Loveland, CO) using the DPD method. A 1:10 dilution of each sample was made in LGW. The same dilution factor of RO water was prepared in LGW for the blank. The concentration of free chlorine in the original sample was determined and then the appropriate amount of ascorbic acid needed to quench the free chlorine was calculated using this equation:

$$wt\ ascorbic\ acid\ (mg) = \frac{wt\ of\ Cl_2\ in\ solution\ (mg) \cdot 176.12\frac{g}{mol}}{70.906\frac{g}{mol}}$$

The quenching agent reacted with the dosed waters for about 5 minutes after which the free chlorine residual was measured again with Hach Pocket Colorimeter Test Kit using the same 1:10 dilution factor for the samples and blank. Once no residual was detected, each sample was poured into a 40 mL glass amber vial, which was filled headspace-free, and capped with open-

top plastic caps lined with PTFE silicone septa. The samples were stored at 4°C until samples were ready to be pipetted onto the NCM 460 cells in the GIA.

The LGW Q sample was prepared differently than the other samples. The purpose of this sample was to determine the growth inhibitive effects of a simulated residual free chlorine concentration that had been quenched with ascorbic acid in a water containing little to no organic carbon. The contact time between the disinfectant and the water was minimal. A 40 mL amber vial with was filled with LGW 75% full. The water was dosed with 97 µL of the 59.9 mg/mL as Cl_2 NaOCl stock solution so that the final concentration in 40 mL vial was 145 mg/L Cl_2. About 23 mg of ascorbic acid was added to the sample and allowed to react for 5 minutes. 10 mL of this sample was directly measured for free chlorine (DPD method) using the Hach Pocket Colorimeter Test Kit to be 0.00 mg/L Cl_2. This sample was stored at 4°C until ready to for use in the GIA.

2. B.1.b. GIA at EPA in RTP, NC

This section details the procedures for each day of the GIA, which took place at the DeAngelo Toxicology Lab at the U.S. EPA in RTP, NC. All procedures were performed under a biological safety hood.

On the first day of the GIA, two 10 mL, 25 cm^2 cell culture flasks (tissue culture treated, Corning, Sigma, St. Louis, MO) containing NCM460 (normal human colon cells), passage 7 on 04/21/09, were obtained from the incubator set at 37°C and monitored for constant CO_2 and O_2 gas concentration levels. The cells were grown in M3:10 Culture Media (INCELL Corporation, San Antonio, TX). This media was discarded and the cells were rinsed with 6mL of phosphate buffered saline (PBS) solution, (Ca^{2+} & Mg^{2+}-free, Gibco, Carlsbad, CA). The PBS was then

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discarded and 6 mL of Trypsin-EDTA (Gibco, Carlsbad, CA) was added to each flask in order to pull the cells off of the bottom of the flask. This flask was then placed in the incubator at 37°C for about 15 minutes. After 15 minutes, 1 mL of M3:10 Culture Media was added to each flask to inhibit Trypsin-EDTA reaction. The cells and the media were pipetted together to further break apart the cells clumps. The cells and media from both flasks were transferred to a 50 mL sterile culture tube (Fisher Scientific, Fair Lawn, NJ) and placed in a centrifuge at 800 rpm at 4°C for 5 minutes. The supernatant, the layer of media above the cell pellet, was discarded. 10 mL of M3:10 Culture Media was added to this tube to break up the cell pellet using rapid pipetting.

The cells were counted using a Coulter Particle Counter (Beckman Coulter, Fullerton, CA). A 1:100 dilution of the cell suspension in Isoton solution (Beckman Coulter, Fullerton, CA) was made: 200 μL of the cell suspension was pipetted into 20 mL of Isoton solution. This cell solution was counted twice at volumes of 0.5 mL each. Prior to cell counting, a background cell count was performed 3 times using just the isoton solution. The results from cell quantification can be found in Table 2.7.

Table 2.7 Cell quantification.

Run #	Sample Volume (mL)	# of Cells
Background 1	0.5	31
Background 2	0.5	20
Background 3	0.5	35
	Background Average	28.7
Sample 1	0.5	3854
Sample 2	0.5	4248
Sum Total	1	8102

The cell counts for the 1st and 2nd samples runs were added together to get the number of cells in

1 mL. Taking into account the 1:100 dilution factor, the original concentration of cells is equal to:

$$\frac{8102\ cells}{mL} \cdot \frac{100}{1} = 8.10 \times 10^5 \frac{cells}{mL}$$

Next, the cells were examined for their viability, which is basically determining the percentage of live cells in the cell suspension. 0.2 mL of the cell suspension was added to 0.8 mL of trypan blue, a dye (0.4%, Sigma, St. Louis, MO). This mixture was pipetted on a hemocytometer (Hausser Scientific, Horsham, PA) so that the cells could be counted on a microscope (Nikon Instruments Inc., Melville, NY) for viability. Cells that are dyed blue are dead while white cells are live. The results for cell viability are displayed in Table 2.8.

Table 2.8 Cell viability determination.

Live Cells	103
Dead Cells	6
Total	109
% Viable Cells	94.5
Viable Cells/mL	7.65×10^5

The volume of cell suspension to provide roughly 1×10^6 cells for a 96-well microplate was determined in the following way:

$$\left(\frac{7.65 \times 10^5\ viable\ cells}{mL}\right) 10mL = 7.65 \times 10^6\ viable\ cells$$

$$\frac{7.65 \times 10^6\ viable\ cells}{8\ plates} = 9.57 \times 10^5 \frac{viable\ cells}{plate}$$

$$\frac{10mL\ cell\ suspension}{8\ plates} = \frac{1.25mL\ cell\ suspension}{plate}$$

Each plate requires 20 mL of a cell suspension and M3:10 Culture Media, so 18.75 mL of M3:10 Culture Media was pipetted to bring the 1.25 mL cell suspension solution to 20 mL. This was

done for each of the 8 plates. Next, the 20 mL cell suspension solution was pipetted to a 96-well microplate (tissue culture treated, Molecular Devices, Sunnyvale, CA) at aliquots of 200 μL per well. The first column of wells for each microplate was reserved for a background control, so it was left empty. Once the microplates were filled with the cell solution, they were placed in the incubator at 37°C maintaining constant CO_2 and O_2 gas concentrations overnight.

On the second day of the assay, the chlorinated water samples were measured for free chlorine residual using the DPD method with the Hach Pocket Colorimeter Test Kit (Hach, Loveland, CO), quenched with the appropriate amount of L-ascorbic acid (Certified ACS grade, Sigma, St. Louis, MO) at UNC-CH, and transported to the EPA in a cooler with ice. Instead of using a liquid growth media such as the M3:10 Culture Media, a dry media was used to keep the TOC concentration of the water close to the original concentration. The dry media, Minimal Essential Medium (MEM, GIBCO, Carlsbad, CA) was prepared in the following way: to 18 mL of a sample, 190 mg of MEM, 44 mg of $NaHCO_3$ (Aldrich, St. Louis, MO), 2 mL of 10x SMX growth factor concentrate (INCELL Corporation, San Antonio, TX), 200 μL of antibiotic Pen/Strep L-Glut (GIBCO, Carlsbad, CA), and 88 μL of 250 μg/mL fungizone (Amphotericin B, Gibco, Carlsbad, CA) was added. The contents were mixed thoroughly using a vortex and then adjusted to a pH ~7.4. Next, 2 mL of fetal bovine serum (FBS, INCELL Corporation, San Antonio, TX) was added to each sample. Preparation using the MEM media was only used for samples containing the RO concentrated water (C, SC B, SC I, Pre-CB, and Pre-CI) and the LGW Q sample. The matrix for the 40C BrAA sample was prepared by making a 40% dilution of sample C with M3:10 Culture Media and 2 μg/mL fungizone (Amphotericin B, Gibco, Carlsbad, CA). Sample BrAA used a matrix containing M3:10 Culture Media (INCELL Corporation, San Antonio, TX) and 2 μg/mL fungizone (Amphotericin B, Gibco, Carlsbad, CA).

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All of the 8 samples were filtered using a sterile, disposable 0.22 μm pore size vacuum filter unit (Nalgene, Thermo Fisher Scientific, Rochester, NY).

Table 2.9 shows the series of dilutions that were prepared for each of the samples C, SC B, SC I, Pre-CB, Pre-CI, and LGW Q and the final TOC concentration per dilution of the sample.

Table 2.9 Dilutions of samples prepared for each microplate in GIA Experiment Set 1.

% of Total Sample Volume	Volume of Sample Needed (mL)	Volume of M3:10 media + 2μg/mL Fungizone Needed (mL)	Total Volume of Diluted Sample per Well (mL)	Final TOC (mg C/L)
100	3	0	0	58
80	2.4	0.6	0.2	46
60	1.8	1.2	0.2	35
50	1.5	1.5	0.2	29
40	1.2	1.8	0.2	23
20	0.6	2.4	0.2	12
10	0.3	2.7	0.2	6
5	0.15	2.85	0.2	3
1	0.03	3	0.2	0.6
0.5	0.015	3	0.2	0.3
Control	0	3	0.2	0
Bkg	Bkg	3	0.2	0

NOTE: "Bkg" = Background

A 2 M BrAA stock solution was prepared and diluted to a 2×10^{-3} M BrAA working solution, which was used to spike BrAA into the 40C BrAA and BrAA (positive control) samples. Table 2.10 shows the concentrations of BrAA that were used in each column of each well plate for the different filtered water samples + media.

Table 2.10 Dilutions of BrAA prepared for samples 40C BrAA and BrAA in GIA Experiment Set 1

[BAA] (M)	[BrAA] (mg/L)	Volume of 2×10^{-3} M BrAA solution (μL)	Volume of M3:10 media + 2μg/mL fungizone (mL)	Total Volume (mL)	Volume of Sample per Well (mL)
Bkg	Bkg	0	0	0	0
Control	Control	0	0	2 (M3:10 only)	0.2
1.00×10^{-6}	0.14	1	2	2	0.2
5.00×10^{-6}	0.70	5	2	2	0.2
8.00×10^{-6}	1.11	8	1.99	2	0.2
1.00×10^{-5}	1.39	10	1.99	2	0.2
2.00×10^{-5}	2.78	20	1.98	2	0.2
3.00×10^{-5}	4.17	30	1.97	2	0.2
4.00×10^{-5}	5.56	40	1.96	2	0.2
5.00×10^{-5}	6.95	50	1.95	2	0.2
6.00×10^{-5}	8.34	60	1.94	2	0.2
1.00×10^{-4}	13.9	100	1.90	2	0.2

NOTE: "Bkg" = Background

When the dilutions for all 8 samples were ready to be dosed onto the cells, the microplates were removed from the incubator and the culture media in each well was removed by inverting the plate over sterile gauze. Each dilution was pipetted to each column on the microplate in order of increasing concentration. Table 2.11 shows how the dilutions are arranged on the 96-well microplates for samples C, SC B, SC I, Pre-CB, and Pre-CI. The top line designates the TOC concentration of the sample in each well below. "0.2" represents the volume of sample in mL per well.

Table 2.12 shows how the dilutions are arranged on the microplate for the LGW Q sample. The top row indicates the percentage at which the original solution was diluted and hence the concentration of the sample in that column. Table 2.13 shows how the dilutions are arranged on the microplate for the 40C BrAA and BrAA samples. No sample was added to the blank or control columns, but M3:10 Culture Media and 2 μg/mL fungizone were added instead at a volume of 0.2 mL per well. Once all of the 8 microplates were dosed appropriately, the

plates were each covered with Alumna-Seal foil (RPI Corp., Mt. Prospect, Illinois) and placed in

the incubator at 37°C for 72 hours at constant CO_2 and O_2 gas concentrations.

Table 2.11 Arrangement of dilutions for samples C, SC B, SC I, Pre-CB, and Pre-CI on a 96-well microplate.

		TOC Concentrations in Each Well (mg C/L)									
Blank	Control	0.3	0.6	3	6	12	23	29	35	46	58
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Table 2.12 Arrangement of dilutions for sample LGW Q.

		Percentage of Sample C in Each Well									
Blank	Control	0.5%	1%	5%	10%	20%	40%	50%	60%	80%	100%
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Table 2.13 Arrangement of dilutions for samples 40C BrAA and BrAA.

		BrAA Concentration in Each Well (M)									
Blank	Control	1.00×10^{-6}	5.00×10^{-6}	8.00×10^{-6}	1.00×10^{-5}	2.00×10^{-5}	3.00×10^{-5}	4.00×10^{-5}	5.00×10^{-5}	6.00×10^{-5}	1.00×10^{-4}
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

On the sixth day of the GIA experiment, the plates were removed from the incubator and

the sample doses were discarded by inverting the plates over sterile gauze. The wells were washed with 200 μL PBS. Then cells in the wells were fixed with 100 μL of 100% methanol (Fisher Scientific, Fair Lawn, NJ) for 20 minutes. The methanol was removed and 100 μL of 1% crystal violet (Gibco, Carlsbad, CA) in 100% methanol was added to each well. After 20 minutes, the plates were rinsed under cold running water until no color in the rinse water was seen when the plates were inverted and tapped over blotting paper. The plates were tapped to remove excess water. 50 μL of dimethyl sulfoxide (DMSO, 99+%, Certified ACS Grade, Sigma Aldrich, St. Louis, MO) was added to each well. The plates were covered in foil and placed on a rotating shaker for 30 minutes. DMSO pulls the crystal violet dye off the cells that were still alive after the dosing. The plates were placed into a Wallac Victor 1420 Multilabel Counter (Perkin Elmer Wallac Inc., Gaithersburg, MD) and read at an absorbance of 600 nm.

2. B.1.c. HAA9 Extraction at UNC-CH

Samples C, SC B, SC I, Pre-CB, and Pre-CI were also extracted for HAA9 within one week of quenching. In order to ensure that the samples fell within the detection range of the GC/ECD, each sample was diluted 1:10 and 1:2 in duplicate before extraction. The original, undiluted samples were also extracted, except in the case for sample C. The instrumentation and procedure for this method was the same as described in the CCWD methods section and in the SOP (Appendix). The only major difference was that there were two additional calibration points for ClAA at concentrations of 600 μg/L and 1200 μg/L. The chromatographic peaks were manually integrated due to inconsistent baselines and tabulated in an Excel spreadsheet.

2. B.2. GIA Experiment Set 2 Methods

Sample C was prepared again for this experiment set in the same manner as it was prepared in GIA Experiment Set 1. The samples tested in this experiment set were sample C, 40C BrAA, 40C IAA, 40C BrAA & IAA, and 40C BrAA & IAA (0.5x). All samples were run in duplicate except for sample C. As in the previous experiment set, "40C" indicates that the matrix for each sample was a 40% solution of sample C.

For 40C BrAA and 40C IAA, the series of dilutions that were made for each sample were prepared at the same concentrations displayed in Table 2.13 for each species. For the 40C BrAA & IAA sample, BrAA and IAA were spiked into the same matrix each at the concentrations displayed in Table 2.13.

The 40C BrAA & IAA (0.5x) sample was prepared differently. Working solutions of 1.0×10^{-3} M BrAA and 1.0×10^{-3} M IAA were used to prepare the series of BrAA and IAA mixture solutions. In order to prepare a series of dilutions that contained a mixture of BrAA and IAA, each chemical was spiked at half of the concentration that was used in the 40C BrAA sample into the same container. The dilution concentrations that were made for this sample were 5.0×10^{-7} M, 2.5×10^{-6} M, 4.0×10^{-6} M, 5.0×10^{-6} M, 1.0×10^{-5} M, 1.5×10^{-5} M, 2.0×10^{-5} M, 2.5×10^{-5} M, 3.0×10^{-5} M, and 5.0×10^{-5} M of each BrAA and IAA. The dilutions were arranged in order of increasing concentration for each column on the microplate.

The GIA experiment was prepared in the same way as described in the previous experiment set: the cells were plated onto the 96-well microplates and held overnight in an incubator, a series of dilutions was prepared for each sample, the cells were dosed and held for 3 days in the incubator, and then the absorbance of the cells was read at 600 nm using a Wallac Victor 1420 Multilabel Counter (Perkin Elmer Wallac Inc., Gaithersburg, MD). Results were

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calculated and graphed using Excel.

2. C. Experiments with Chloraminated Samples

The experiment described in this section was designed to see whether or not the products obtained from quenching the disinfectant caused growth inhibitive effects on these cells. The results would indicate whether or not appropriate quenching techniques were available for viable use of the GIA.

In order to effectively perform this experiment, pre-formed solutions of chlorine and chloramine with added ascorbic acid at near stoichiometric doses to quench the disinfectant were prepared and then added to the RO-concentrated water (TOC = 65 mg C/L). These samples were named "W1" and "W2," respectively.

Sample W1 was prepared in the following way:

1. 2 mL of a 23 mg/mL Cl_2 solution of NaOCl was placed into a glass vial along with 132 mg of ascorbic acid. This quenching process was allowed to react for about 5 minutes under a ventilated hood. The ascorbic acid mass was determined using the equation on page 56.

2. 100 mL of this quenched solution was placed in 10 mL of LGW and measured for free chlorine using the DPD method with the Hach Pocket Colorimeter Test Kit.

3. If the reading was 0.00 mg/L Cl_2, then 145 μL of the quenched solution was placed in a 40 mL glass amber vial, after which the vial was filled headspace-free with the RO water and inverted 3 times to mix the contents. This sample contains 325 mg/L Cl_2 and 932 mg/L ascorbic acid.

4. To ensure that the quenching reaction was successful, the solution described in #3 was diluted 1:100 and 1:10 and the dilutions were measured for free chlorine using the DPD method with the Hach Kit. Both dilutions resulted in 0.00 mg/L Cl_2 free chlorine readings.

Sample W2 was prepared similarly; however, a chloramine solution was prepared first. The chloramine solution was prepared using a preformed method, which was described in section 1. A.2 of this report. A 1:10,000 dilution of that chloramine solution was measured for total chlorine using the DPD method with the Hach Kit three times, resulting in an average total chlorine concentration of 5067 mg/L as Cl_2. Average free chlorine concentration in the original chloramine solution was determined to be 200 mg/L Cl_2, which is about 4% of the total chlorine concentration of this solution indicating that the chloramine solution could be used for this experiment. Sample W2 was prepared by reacting 4 mL of the preformed chloramine solution with 65 mg of ascorbic acid. After 5 minutes, this solution's total chlorine concentration was measured with a 1:100 dilution in LGW using the Hach Kit. The resulting reading indicated that there was still 1 mg/L as Cl_2 of residual disinfectant in the original, so an additional 20 mg of L-ascorbic acid was added to that solution. After another 5 minutes, a 1:100 dilution in LGW of this solution was measured again for total chlorine using the Hach Kit. The reading indicated that there was 0.00 mg/L as Cl_2 in the sample. 2.6 mL of this quenched NH_2Cl sample was added to 40 mL of RO water in a glass amber vial, headspace free. Total chlorine was measured again on a 1:10 dilution of the sample using the DPD/Hach Kit method and the resulting reading was 0.00 mg/L as Cl_2. W2 contained 325 mg/L as Cl_2 of NH_2Cl and 140 mg/L of ascorbic acid.

Possible products of quenching chlorine and chloramine residuals can be seen in Figure 2.1 and Figure 2.2. These figures are only intended for visualizing where on the ascorbic acid

molecule the residual disinfectants may react. As seen in Figure 2.1, the reaction between ascorbic acid and chlorine is a redox reaction. Chlorine appears as hypochlorous acid (HOCl) in the presence of water. One mole of ascorbic acid reacts with one mole of HOCl to form dehydroascorbic acid, Cl⁻, H⁺, and one mole of water.

Figure 2.1 Product of quenching chlorine residual with ascorbic acid.

In Figure 2.2, the reaction between ascorbic acid and chloramine is also a redox reaction. In the presence of water and at the pH of the reaction, chloramine is in the form of monochloramine (NH_2Cl). One mole of ascorbic acid reacts with one mole of NH_2Cl to form dehydroascorbic acid, ammonium and Cl⁻ ions.
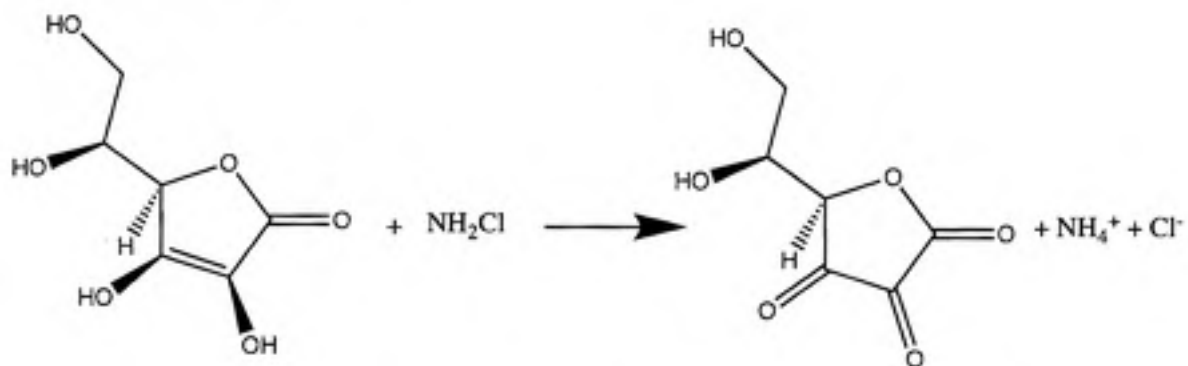

Figure 2.2 Possible product of quenching residual chloramine with ascorbic acid.

The colon cells were exposed to the following samples: W1 (control), W1 & BAA, W2 (control), W2 & BAA, and BAA. These samples are described below:

1) W1 (control): RO concentrate water with pre-quenched solution of 325 mg/L Cl_2 (from NaOCl) and 932 mg/L ascorbic acid.

2) W1 & BrAA: Sample W1 spiked with 1.00×10^{-6} M BrAA. This sample was chosen to determine whether or not the resulting cell density value would occur at around 100% at the lowest BrAA concentration that is used in the BrAA positive control. If so, then the cell density was not affected by the disinfectant type and there would be no false positive bias for cell growth inhibition.

3) W2 (control): RO concentrate water with pre-quenched solution of 325 mg/L as Cl_2 (from NH_2Cl) and 140 mg/L ascorbic acid.

4) W2 & BrAA: Sample W2 spiked with 1.00×10^{-6} M BrAA. This sample was chosen for the same reason as that stated in #2.

5) BrAA: 1.00×10^{-6} M BrAA in M3:10 cell growth media, for the purposes of being a positive control.

These samples were run according to the GIA procedure described in GIA Experiment Set 1. All samples were placed on one 96-well microplate with a column for background (bkg) and a column for control. The samples were arranged on the microplate according to Table 2.14. Each well contained 0.2 mL (200 μL) of sample except for the last column, which remained empty.

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Table 2.14 Arrangement of samples on the microplate.

Bkg	Control	W1	W1 & BrAA	W1 & BrAA	W2	W2 & BrAA	W2 & BrAA	BrAA	BrAA	BrAA	Empty
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0
0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0

Results from this experiment were tabulated using Excel.

3. Results

3. A. Contra Costa Water District Study

Water samples from the Contra Costa Water District (CCWD) pilot plant study were collected during different water treatment phases from June 2008 through July 2008 and then sent to UNC-Chapel Hill to be analyzed for THM4, HAA9, halogenated volatiles, TOX, and TOCl/TOBr. These procedures were described in the Methods section.

Data acquired from the TOX and TOCl/TOBr methods were analyzed using a series of calculations, as seen in the columns in Table 3.1. These calculations were performed for all of the water samples that were collected from Phases 1A, 1B, 2A, 2B, 3A Pre-Nano Filtration, and 3A Post-Nano Filtration. The water quality and various treatment schemes are described in Table 2.1 through Table 2.5 in the Methods section.

3. A.1. Calculations for Data Analysis

Organic Chlorine in HAA9 + THM4 (as µg Cl/L): Data analyzed from the HAA and THM extractions were used in performing this calculation. For each of the nine HAA and four THM species, the analyte concentration was converted from µg/L to µg Cl/L by multiplying the analyte concentration (µg/L) by the ratio of total amount of Cl (Mr(Cl)) in the compound to the compound's molecular weight. A sample calculation for the organic chlorine content of 10 µg/L $CHCl_3$ can be found below:

$$Organic\ chlorine\ content\ of\ CHCl_3 = \left(\left[CHCl_3 \right] \left(\frac{\mu g}{L} \right) \right) \left(\frac{Mr(Cl)}{Mr(CHCl_3)} \right) = \left(10 \frac{\mu g}{L} \right) \left(\frac{106.4}{119.4} \right) = 8.9 \frac{\mu g\ Cl}{L}$$

Next, these organic chlorine values from each of the individual HAA and THM species were

summed together for a specific same treatment method and phase type to give the value presented in Table 3.1. Data from other volatile compounds, such as HANs, were not included in this calculation because they represented ≤5% of the organic chlorine in HAA9 and THM4.

Organic Bromine in HAA9 + THM4 (as µg Br/L): This was calculated similarly to organic chlorine except that the analyte concentrations were converted from µg/L to µg Br/L by multiplying the analyte concentration by the ratio of total amount of Br in the compound (Mr(Br)) to the compound's molecular weight. A sample calculation for 10.9 µg/L BrCl$_2$CH can be found below:

$$Organic\ bromine\ content\ of\ BrCl_2CH = \left([BrCl_2CH] \left(\frac{\mu g}{L} \right) \right) \left(\frac{Mr(Br)}{Mr(BrCl_2CH)} \right) = \left(10.9 \frac{\mu g}{L} \right) \left(\frac{79.9}{163.8} \right) = 5.32 \frac{\mu g\ Br}{L}$$

The sum of the individual species is presented in column 3 of Table 3.1. Again, for the same reasons described for organic chlorine, the data from other volatile compounds, such as HANs, were not included in this calculation.

Organic halogen in HAA9 + THM4 (as µg Cl/L): This calculation represents the sum of the organic chlorine and organic bromine in an analyzed species. THM and HAA species are first converted into units of "µg Cl/L" by using the equation below, where "cmpd" represents a particular HAA or THM species:

$$[cmpd] \left(\frac{\mu g\ Cl}{L} \right) = [cmpd] \left(\frac{\mu g}{L} \right) \times \frac{Mr(Cl)}{Mr(cmpd)} + [cmpd] \left(\frac{\mu g}{L} \right) \times \frac{Mr(Br)}{Mr(cmpd)} \times \frac{Mr(Cl)}{Mr(Br)}$$

The values for each species are then summed, giving the value shown in column 4 of Table 3.1.

IC Measured TOCl (µg Cl/L) & IC Measured TOBr (µg Br/L): These values, presented in columns 5 and 6 of Table 3.1, were obtained from the IC analysis of samples processed on the

TOX analyzer for TOCl and TOBr. Concentrations for Cl⁻ and Br⁻ were determined by plugging the chromatographic peak area for Cl⁻ or Br⁻ into the respective regressed linear equation from a Cl⁻ or Br⁻ calibration curve generated from standards in the calibration range 0 μg Cl⁻ and Br⁻/L – 5000 μg Cl⁻ and Br⁻/L measured on the IC. Since the aqueous samples for IC analysis were collected from the TOX analyzer effluent gas in a 10 mL volume of LGW from an original sample volume of 25 mL or 50 mL, the concentrations obtained from the calibration curve were then multiplied by a concentration factor (either 1:2.5 or 1:5) to determine the value of TOCl or TOBr in the original sample.

TOCl + TOBr (μg Cl/L): The values for "IC Measured TOBr" are converted into units of μg Cl/L by multiplying by the molar ratio of chloride to bromide (35.45/79.9) and are presented in column 7 of Table 3.1. Within the same treatment type and phase, these values are added to the "IC Measured TOCl" values to arrive at the value for TOCl + TOBr.

TOX (μg as Cl/L): These values are initially obtained in units of "μg Cl" after the water samples were adsorbed onto two carbon columns using the TOX analyzer. The TOX of a water sample in units of "μg as Cl/L," is obtained as the sum of the readings from both columns from which the values of two blanks were subtracted. This net value was divided by the volume of water sample in liters adsorbed onto the carbon column to obtain the concentration presented in column 8 of Table 3.1.

% THM4 in TOX: After the THM4 concentrations were converted into "μg as Cl/L," they were divided by the TOX concentration, which was obtained using the TOX calculation above, and then multiplied by 100. This value is presented in column 9 of Table 3.1 and represents the proportion of each analytically measured TOX contributed to the organic halogen content of the measured THM4.

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% HAA9 in TOX: This value is presented in column 10 of Table 3.1 and was calculated in a manner similar to that described for THM4 but instead used the organic chlorine values from the HAA9 species.

% Unknown TOX (from TOX): This value, presented in column 11 of Table 3.1, was calculated by subtracting the HAA9 + THM4 organic halogen value (column 4) from "TOX" (column 8), dividing by "TOX," and multiplying by 100. This calculation determines the percentage of TOX found in the water samples unaccounted for by the presence of HAA9 and THM4. Percentages that were 50% or less were highlighted because the expected percentage of unknown TOX is around 60% (Krasner et al., 2006).

% Unknown Organic Halogen (from IC): In column 12 of Table 3.1, this value was calculated by subtracting the value in column 5 from the value in column 6, dividing by the value in column 6 and multiplying by 100. This calculation represents the proportion of organic Cl and Br not accounted for from the analyzed THM4 and HAA9. Although one might predict similarities between this value and that in column 11, the measurements are carried out using different techniques in which recovery is also different. Moreover, TOX also includes organic iodide, which was not measured by the IC technique.

% Unknown TOCl: Calculated in column 13 by subtracting the value in column 1 from the value in column 3, dividing by the value in column 3 and multiplying by 100. This calculation represents percentage of TOCl found in the water samples not accounted for in the analyzed THM4 and HAA9.

% Unknown TOBr: Calculated in column 14 by subtracting the value in column 2 from the value in column 4, dividing by the value in column 4, and multiplying by 100. This calculation represents percentage of unknown TOBr found in the water samples not accounted for in the

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analyzed THM4 and HAA9.

Quality control for the TOX adsoption module and the analyzer was carried out as described in section 2. A.1.h of this report. Quality control for TOCl and TOBr analysis was carried out as described in section 2. A.1.i of this report.

In Table 3.1, the values labeled "not defined" (nd) were calculated to be negative. These outcomes could have been due to the fact that the measurements in this table were performed using a combination of multiple techniques and the recovery for each technique is different and cumulative. Moreover, the organic iodine content of the samples was not taken into account using IC analysis. The combination of these factors is likely to have caused many indefinable (nd) values. Values for percentage unknown TOX and unknown organic halogen that are less than 40% and values for percentage of THM4 and HAA9 in TOX that are greater than 100% were flagged as they also failed to meet experimental validation criteria.

Table 3.1 TOX, TOCl, and TOBr data from CCWD samples.

	Organic Cl HAA9+THM4 (as µg Cl/L)	Organic Br HAA9+THM4 (as µg Br/L)	TOCl (µg Cl/L)	TOBr (µg Br/L)	Organic Halogen in HAA9+THM4 (as µg Cl/L)	TOCl + TOBr (as µg Cl/L)	TOX (as µg Cl/L)	%THM4 in TOX	%HAA9 in TOX	% Unknown TOX (from TOX)	% Unknown Organic Halogen (from IC)	% Unknown TOCl	% Unknown TOBr
Phase 1A													
no treatment	13.0	16.3	NM	NM	20.2	NA	53.4	25	13	62	NA	NA	NA
NH₂Cl	20.5	50.5	57.0	221	42.9	155	196	16	5	78	72	64	77
LP400 NH₂Cl	22.1	55.0	63.4	200	46.5	152	206	17	6	77	69	65	73
MP100 NH₂Cl	21.6	56.8	67.6	114	46.8	118	203	18	5	77	60	68	50
MP400 NH₂Cl	22.8	58.5	58.1	230	48.7	160	566	7	2	91	70	61	75
Cl₂	36.2	200	38.7	135	125	98.8	153	71	11	18	nd	6	nd
LP400 Cl₂	28.0	96.1	35.0	204	70.6	125	177	30	10	60	44	20	53
MP400 Cl₂	41.3	215	52.9	119	137	133	214	56	7	36	nd	22	nd
Phase 1B													
no treatment	16.0	15.9	NM	NM	24.2	NA	NM	NA	NA	NA	NA	NA	NA
NH₂Cl	24.6	83.4	37.7	252	63.7	150	129	41	9	50	57	35	67
LP400 NH₂Cl	25.9	85.4	78.7	283	66.0	204	251	21	5	74	68	67	70
MP100 NH₂Cl	25.4	85.4	51.1	234	65.5	155	240	22	5	73	58	50	63
MP400 NH₂Cl	26.0	91.3	44.0	237	68.8	149	220	25	6	69	54	41	61
Cl₂	60.7	222	56.3	191	165	141	191	74	12	14	nd	nd	nd
LP400 Cl₂	69.3	242	77.3	276	183	200	296	53	9	38	8	10	12
MP400 Cl₂	62.4	238	56.3	233	174	160	323	46	8	46	nd	nd	nd

NM = Not measured

NA = Not applicable

nd = not defined

Shaded cells are values less than 40% unknown TOX or organic halogen.

Table 3.1 (Continued from previous page.)

	Organic Cl HAA9+THM4 (as µg Cl/L)	Organic Br HAA9+THM4 (as µg Br/L)	TOCl (µg Cl/L)	TOBr (µg Br/L)	Organic Halogen in HAA9+THM4 (as µg Cl/L)	TOCl + TOBr (as µg Cl/L)	TOX (as µg Cl/L)	%THM4 in TOX	%HAA9 in TOX	% Unknown TOX (from TOX)	% Unknown Organic Halogen (from IC)	% Unknown TOCl	% Unknown TOBr
Phase 2A													
no treatment	8.89	13.3	NM	NM	15.3	NA	41	21	5	63	NA	NA	NA
NH₂Cl	21.0	50.5	65.0	164	45.8	138	234	15	1	80	67	68	69
LP400 NH₂Cl	21.6	54.7	51.4	190	48.6	156	266	14	1	82	69	58	71
MP100 NH₂Cl	22.5	52.9	87.4	322	48.8	230	83	45	3	41	79	74	84
MP400 NH₂Cl	22.4	49.7	46.7	196	47.1	134	127	28	2	63	65	52	75
Cl₂	73.1	229	79.8	172	196	156	300	58	1	35	nd	8	nd
LP400 Cl₂	80.4	252	105	203	216	194	307	63	1	30	nd	23	nd
MP400 Cl₂	81.6	254	112	214	218	206	200	98	2	nd	nd	27	nd
Phase 2B													
no treatment	10.2	13.5	NM	NM	16.0	NA	15	64	14	nd	NA	NA	NA
NH₂Cl	19.0	88.9	36.0	225	63.5	136	238	22	1	73	53	47	61
LP400 NH₂Cl	19.8	92.4	37.5	252	66.0	149	276	19	1	76	56	47	63
MP100 NH₂Cl	18.8	87.4	51.5	257	62.5	166	285	18	1	78	62	64	66
MP400 NH₂Cl	20.5	92.3	40.2	251	66.8	152	272	20	1	75	56	49	63
Cl₂	73.3	356	48.4	246	261	158	321	73	2	19	nd	nd	nd
LP400 Cl₂	70.6	396	32.3	288	278	160	281	91	2	1	nd	nd	nd
MP400 Cl₂	90.1	445	72.7	268	326	191	340	89	2	4	nd	nd	nd

NM = Not measured

NA = Not applicable

nd = not defined

Shaded cells are values less than 40% unknown TOX or organic halogen.

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Table 3.1 (Continued from previous two pages.)

	Organic Cl HAA9+THM4 (as µg Cl/L)	Organic Br HAA9+THM4 (as µg Br/L)	TOCl (µg Cl/L)	TOBr (µg Br/L)	Organic Halogen in HAA9+THM4 (as µg Cl/L)	TOCl + TOBr (as µg Cl/L)	TOX (as µg Cl/L)	%THM4 in TOX	%HAA9 in TOX	% Unknown TOX (from TOX)	% Unknown Organic Halogen (from IC)	% Unknown TOCl	% Unknown TOBr
Phase 3A Pre-NF													
no treatment	18.4	17.6	NM	NM	26.2	NA	38	56	13	31	NA	NA	NA
NH₂Cl	27.3	59.1	62.4	168	61.6	137	254	21	4	76	55	56	65
LP400 NH₂Cl	28.2	57.1	43.1	173	65.2	120	237	24	4	72	46	35	67
MP100 NH₂Cl	29.5	61.8	47.6	165	68.8	121	234	26	4	71	43	38	63
MP400 NH₂Cl	11.4	21.8	50.1	177	24.2	129	127	15	4	81	81	77	88
Cl₂	167	219	156	224	364	255	379	91	5	4	nd	nd	2
LP400 Cl₂	225	238	202	233	447	305	337	127	6	nd	nd	nd	nd
MP400 Cl₂	250	253	152	176	499	230	351	138	4	nd	nd	nd	nd
Phase 3A Post-NF													
no treatment	13.1	14.9	NM	NM	21.4	NA	5	233	222	nd	NA	NA	NA
NH₂Cl	10.4	25.7	23.4	138	22.4	84.5	nd	NA	NA	NA	73	56	81
LP400 NH₂Cl	14.7	15.7	30.7	151	22.3	97.7	50.0	24	21	55	77	52	90
MP100 NH₂Cl	13.2	16.7	16.1	177	21.2	94.7	3.34	338	296	nd	78	18	91
MP400 NH₂Cl	25.6	16.1	40.4	138	33.3	102	47.8	20	49	30	67	37	88
Cl₂	209	375	114	162	404	186	204	187	11	nd	nd	nd	nd
LP400 Cl₂	206	360	162	162	395	234	191	200	6	nd	nd	nd	nd
MP400 Cl₂	205	344	113	146	388	178	208	178	9	nd	nd	nd	nd

NM = Not measured

NA = Not applicable

nd = not defined

Cells shaded in pink are values less than 40% unknown TOX or organic halogen.

Cells shaded in light blue were flagged for values greater than 100% THM4 or HAA9 in TOX.

3. A.2. Chlorine vs. Chloramine Trends

Throughout all phases of the CCWD study, the fraction of TOX accounted for by THM4 was consistently larger than that accounted for by HAA9. The general trend was that the chloraminated samples had higher percentages of unknown TOX and TOCl and TOBr than that of chlorinated samples. Hence, THM4 and HAA9 concentrations account for more of the TOX in chlorinated samples than in chloraminated samples.

An exception to this trend was seen in the results for Phase 3A Post-NF (nano-filtered). Since these samples passed through a nano-filter before disinfection, the DBP precursors were removed and the concentrations of THM4 and HAA9 in the water somewhat decreased, compared to the amounts in Phase 3A Pre-NF prior to nano-filtration. Values for TOX were also significantly lower in Phase 3A Post-NF than in Phase 3A Pre-NF for this same reason.

A common occurrence among the chlorinated samples in all of the phases was that the percentages of unknown organic halogens (calculated from IC analysis results), unknown TOCl, and unknown TOBr frequently had "nd" values, while the chloraminated samples had percentages that were consistently in the positive range. Possible explanations for these results are that a) there is generally more unknown TOX among chloraminated waters than among chlorinated waters or b) the THM4 and HAA9 extraction techniques were more precise than the TOX analysis techniques (by TOX analyzer and IC) in determining TOX for the chlorinated samples.

In Phases 2A, 2B, and 3A Pre-NF samples, the organic halogen values (measured using the IC) and TOX were higher in the "Cl_2 only" samples than in the "NH_2Cl" only samples. There was no TOX value measured using the TOX analyzer for the "NH_2Cl only" sample in Phase 3A Post-NF, but through analysis with the IC, the "Cl_2 only" sample had a higher TOX value than

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the "NH$_2$Cl only" sample. However, Phases 1A and 1B did not have this trend, which may be explained by the differences in water treatment among the phases: the water in Phases 1A and 1B were only treated with ozone, while the waters in Phases 2A and 2B were treated with ozone and peroxides and Phases 3A Pre-NF and 3A Post-NF were not treated with ozone or peroxides.

Another trend seen in all of the phases was that the percentage of unknown TOBr in the chloraminated samples was generally greater than the percentage of unknown TOCl. This trend may be attributed to the fact that the CCWD water treatment facility spiked a 1 mg/L of bromide and 1 mg/L of iodide concentration into the water for each phase of the entire treatment process, which is further discussed in Chapter 4. TOI and iodinated DBPs were not included in this analysis, however.

3. A.3. UV Treatment vs. No UV Treatment Trends

Overall, it appears that the chloraminated water samples increased slightly in the percentage of TOX accounted for by THM4 and HAA9 when treated with UV prior to disinfection. Exceptions for this trend can be seen in Phase 3A – NF, where the "Cl$_2$ only" sample had a greater percentage of known TOX than samples treated with UV and then chlorine. In some phases (1A, 1B, and 2B), TOX analysis indicates that chloramine treatment following UV irradiance (UV/NH$_2$Cl) increases TOX concentration; whereas, in the other phases (2A, 3A Pre- and Post-NF) this is not always the case. One reason why this result was seen may be because when Phase 2A TOX analysis was taking place using the TOX analyzer, the instrument was having some technical trouble with the coulometric cell. This problem was resolved by removing air bubbles out of the electrodes and changing the 80% H$_2$SO$_4$ solution in the scrubber vial. Samples from Phase 2A analysis occurred right after those from Phase 1A, so this problem

was resolved before TOX analysis of the other phases. A possible explanation for why UV/NH$_2$Cl samples in Phase 3A Pre-NF did not have greater TOX concentrations than the "NH$_2$Cl only" sample may be that Phase 3A Post-NF had uncharacteristic TOX concentrations since the nano-filter was able to remove most of the dissolved organic carbon prior to UV/NH$_2$Cl treatment. Thus, fewer DBPs could have formed.

In Phases 1A and 1B, the TOX concentrations for non-UV treated chloraminated and chlorinated samples were lower than their UV-treated counterparts. In Phase 2B, this trend persisted with the chloraminated samples but not for the chlorinated ones, while in phases 2A, 3A Pre-NF, and 3A Post-NF this trend was not exhibited. The results for TOCl + TOBr show that in Phases 1B, 2A, and 2B, their sum TOX for non-UV treated chloraminated and chlorinated samples were lower than for their UV-treated counterparts. The chloraminated samples in Phase 1A did not follow this trend; however, the chlorinated samples did. All of the samples in Phase 3A Pre-NF and the chlorinated samples in Phase 3A Post-NF did not follow this trend, while the chloraminated samples in Phase 3A Post-NF did.

The percentage of unknown TOX also does not follow a clear trend throughout all the phases of treatment. Chloraminated and chlorinated samples in Phases 1A & 1B and chloraminated samples in 2B results indicate that UV treatment and post-disinfection increases the percentage of unknown TOX but the other phases do not always support this trend.

There was no general trend in the percentages of unknown organic halogen as measured by TOCl +TOBr between non-UV and UV treated samples in any of the phases. However, the calculation for the chlorinated samples in Phases 2B, 3A Pre-NF, and 3A Post-NF suggest that most of the measured TOX, TOCl, and TOBr was accounted for by THM4 and HAA9. Certain samples in Phases 1A, 1B, and 2A also showed a similar effect.

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3. A.4. Low Pressure Lamp (LP) vs. Medium Pressure Lamp (MP) Trends

In Phase 1A, when compared with the LP400 UV pre-treatment, the chloraminated water sample UV pre-treated with MP100 did not change the organic halogen accounted for by HAA9 and THM4 combined in the sample; however, MP400 showed a slight increase in this value. The trend for TOCl + TOBr is inconsistent among the three UV treatment processes: compared to LP400 treatment on chloraminated waters MP100 lowered this value, while MP400 slightly increased it. The results from TOX analysis indicate that, compared with LP400, MP100 pre-treatment of chloraminated water did not affect TOX, while the MP400 treatment more than doubled its concentration. The percentage of TOX accounted for by THM4 and HAA9 remained unchanged in the LP400 and MP100 treatments prior to chloramination, but dropped significantly in the MP400 sample. The amount of unaccounted organic halogen increased by only 1% when comparing the LP400 NH_2Cl sample with the MP400 NH_2Cl sample, while the MP100 NH_2Cl sample had 9% less than the LP400 NH_2Cl sample. The percentage of unknown TOCl was not affected significantly by any of the UV treatments: the LP400 sample contained 65% unknown TOCl, while the MP100 and MP400 samples contained 68% and 61%, respectively. However, the MP100 had 50% of unknown TOBr, while the LP400 and MP400 treatments had 73% and 75% of unknown TOBr, respectively.

For the chlorinated samples in Phase 1A, results indicate that more known TOX, TOCl, and TOBr accounted for by THM4 and HAA9, and TOX was created when the sample was UV treated with MP400 than with the LP400 treatment. Again, comparing MP400 with LP400, the percentage of unknown TOX and organic halogen was lower in the MP400 sample than in the LP400 sample. The amount of unknown TOBr was not definable (and probably negligible) with use of MP400 treatment, while the LP400 treatment had an unknown TOBr percentage of 53%.

The percentage of unknown TOCl remained about the same for both UV treatments: 20% for LP400 and 22% for MP400.

In Phase 1B samples, the minor fluctuations of organic halogen in HAA9 and THM4 among the different UV treatments followed by chloramination are negligible. The MP100 and MP400 UV treatments generated a lower TOCl + TOBr concentration in the chloraminated samples than in the LP400 treatment, a trend also observed in the values of TOX and the percentages of unknown TOX, unknown TOCl, and unknown TOBr.

TOX accounted for by HAA9 + THM4 and TOCl + TOBr was lower in the MP400 Cl_2 sample than in the LP400 Cl_2 sample in Phase 1B. However, the TOX was greater under MP400 Cl_2 conditions than under LP400 Cl_2 conditions. This trend was also found in the "% Unknown" values. However, the percentage of unknown TOX, TOCl, and TOBr indicate that MP400 Cl_2 increased the proportion of TOX accounted for by THM4 and HAA9.

In Phase 2A, the MP400 UV treatment prior to chloramination slightly lowered the proportion of TOX from HAA9 and THM4 when compared to the MP100 and LP400 NH_2Cl treatments. The known TOX in the MP100 treatment was only slightly higher than the LP400 treatment. This trend was not seen in the known TOX measured using the IC. Instead, the IC method indicated that the MP100 NH_2Cl treatment created a greater concentration of TOCl + TOBr than that of the LP400 and MP400 NH_2Cl treatments, while the treatment producing the smallest concentration of TOCl + TOBr was the MP400 NH_2Cl. This trend was not consistent in the TOX values and instead, the MP100 NH_2Cl treatment produced the lowest concentration of TOX. As stated earlier, it should be noted that in this section, the TOX analyzer was having some technical problems, so these TOX values may not be correct. In spite of this instrument malfunction, the percentage of unknown TOX (using the TOX analyzer's results) was highest for

the LP400 NH$_2$Cl sample at 75% and lowest for the MP100 NH$_2$Cl sample, at 21%. Percentages of unknown TOX, TOCl, and TOBr were highest for MP100 NH$_2$Cl, lowest for TOX and TOCl in the MP400 NH$_2$Cl sample, and lowest for unknown TOBr in the LP400 NH$_2$Cl sample.

With the chlorinated samples in Phase 2A, the organic halogen concentration in THM4 and HAA9 was greatest for the MP400 Cl$_2$ sample (218 µg Cl/L). TOCl + TOBr results support this trend (206 µg Cl/L versus 194 µg Cl/L from LP400 treatment). However, it is possible that these values are not statistically significant because organic iodine was not factored into the TOX values. This trend was not consistent in the TOX results, which showed that LP400 treatment produced a greater concentration (307 µg Cl/L) than the MP400 treatment (200 µg Cl/L). Again, this result may have been due to the problems the instrument was experiencing at the time. The LP400 treatment produced 30% unknown TOX, while the percentage of unknown TOX for MP400 treatment was not distinguishable from 0 (nd). TOCl + TOBr concentrations for both LP400 and MP400 treatments were also not defined. The percentage of unknown TOCl for these two treatments was about the same: MP400 produced 27% unknown TOCl, while LP400 produced 23%. The percentage of unknown TOBr for these treatments were indistinguishable from 0, as well.

In Phase 2B, the trends were virtually indistinguishable. Organic halogen in THM4 and HAA9 was greatest for the MP400 NH$_2$Cl sample (66.8 µg as Cl/L), while the LP400 sample (66.0 µg as Cl/L) was slightly lower than the MP400. The MP100 sample had the lowest concentration of known TOX (62.5 µg as Cl/L). TOCl + TOBr values were 166 µg as Cl/L for the MP100 NH$_2$Cl sample, 152 µg as Cl/L for MP400 NH$_2$Cl, and 149 µg as Cl/L for the LP400 NH$_2$Cl sample. MP100 NH$_2$Cl had a TOX concentration of 285 µg as Cl/L, while LP400 NH$_2$Cl had a TOX concentration of 276 µg as Cl/L, and MP400 NH$_2$Cl was 272 µg as Cl/L. The

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percentages of unknown TOX (from TOX) were 78%, 76%, and 75% for MP100 NH_2Cl, LP400 NH_2Cl, and M400 NH_2Cl, respectively. The percentages of unknown organic halogen, TOCl, and TOBr also fell around the same range: for MP100 NH_2Cl the percentages of unknown TOX, TOCl, and TOBr were 62%, 64%, and 66%, respectively, while the other samples had relatively the same values.

The chlorinated samples in Phase 2B had slightly higher value of organic halogen in HAA9 and THM4 in the MP400 sample (326 µg as Cl/L) than in the LP400 sample (278 µg as Cl/L). This trend was consistent with the TOCl + TOBr values. The percentage of unknown TOX was very low in both treatment types; as LP400 contained only 1% unknown TOX and MP400 contained 4%. The percentages of unknown TOCl, and TOBr were all indistinguishable from 0 providing comparable results to that of unknown organic halogen.

In Phase 3A Pre-NF, among all of the UV treatments prior to chloramination, MP400 produced the lowest concentration (24.2 µg Cl/L) of organic halogen accounted for from HAA9 + THM4, while MP100 produced the highest (68.8 µg as Cl/L), and LP400 was just slightly lower (66.0 µg as Cl/L). Among the TOCl + TOBr values, the MP and LP treatments with post-chloramination samples were indistinguishable: MP400 produced 129 µg as Cl/L, LP400 produced 120 µg as Cl/L, and MP100 produced 121 µg as Cl/L. For the TOX values, LP400, MP100, and MP400 treatment with post-chloramination resulted in 237 µg as Cl/L, 234 µg as Cl/L, and 127 µg as Cl/L, respectively. The percentage of unknown TOX for these samples reflected this trend as well: MP400 with 81%, while LP400 and MP100 were within 2% of each other, 72% and 41%, respectively. The percentages of unknown TOCl, and TOBr determined from the IC were similar for both LP400 and MP100 treatments; however, for MP400, the values were significantly higher, which is consistent with the trends shown by the percentages unknown

TOX and organic halogen. An explanation for this trend could be that the Pre-NF waters were not treated with ozone or peroxides like the other phases, and that they were also spiked with bromide and iodide.

Phase 3A Pre-NF's chlorinated samples showed higher organic halogen accounted for in HAA9 + THM4 after MP400 treatment (499 μg Cl/L) than after the LP400 treatment (447 μg Cl/L). TOCl + TOBr values showed that LP400 created a higher concentration (305 μg Cl/L) than the MP400 treatment (230 μg Cl/L). The difference in TOX between MP400 and LP400 treatments were trivial as they resulted in concentrations of 351 μg Cl/L and 337 μg Cl/L, respectively. Because the organic halogen values in THM4 and HAA9 were so high in this phase, instrument analysis may have been compromised, the linear range of detection may have been exceeded, and data interpretation may not be possible.

For Phase 3A Post-NF, the organic halogen accounted for in HAA9 + THM4 for the UV treated and chloraminated samples were lower than in the other phases. In this phase, the MP400 treatment created the highest among all of the UV treatments (33.3 μg Cl/L). LP400 and MP100 treatments produced similar known TOX concentrations, within 1% of each other, at 22.3 μg Cl/L and 21.2 μg Cl/L, respectively. Though these values were much lower than in the previous phases, the TOCl + TOBr values were still quite high, perhaps indicating that the nanofilter did not completely remove all precursors for these types of compounds. Following the trend for HAA9 + THM4, MP400 produced the most TOX (102 μg Cl/L), LP400 produced 97.7 μg Cl/L of TOX, and MP100 produced 94.7 μg Cl/L; however, these values are so close to each other that this trend is virtually trivial. The TOX concentrations, however, were much lower than in the previous phases. LP400 and MP400 treatments produced similar TOX concentrations at 50.0 μg Cl/L and 47.8 μg Cl/L, respectively, while MP100 produced the least amount of TOX (3.34 μg

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Cl/L). The percentage of unknown TOX was lower than its anticipated value between 60% and 80% for all of these samples. LP400 treatment created the highest percentage of unknown TOX (55%) compared with the other UV treatments for chloraminated waters. The percentage for MP100 NH_2Cl was "nd," indicating that perhaps the TOX analyzer was not effective in reading this sample, while MP400 NH_2Cl contained 30%. Percentages of unknown TOBr were highest with the MP100 treatment at 91% and lowest with the MP400 treatment at 88%. The percentages of unknown TOCl in the UV treated and chloraminated samples were 52% for LP400 NH_2Cl, 37% for MP400 NH_2Cl, and 18% for MP100 NH_2Cl.

In Phase 3A Post-NF, the water samples treated with UV and chlorine produced much higher concentrations of organic halogen in HAA9 + THM4 and TOX than in the UV treated and chloraminated waters. Perhaps these results are an indication that nanofilters are more effective for removing chloramination by-product precursors than those for chlorinated DBPs. LP400 and chlorination created slightly more known TOX (395 µg as Cl/L) than MP400 plus chlorination (388 µg as Cl/L), but this difference is indistinguishable. Though, this trend was consistent with the TOCl + TOBr, where LP400 Cl_2 produced 234 µg Cl/L and MP400 produced 178 µg Cl/L. This trend was not consistent for TOX; instead, MP400 Cl_2 created slightly more TOX (208 µg Cl/L) than LP400 Cl_2 (191 µg Cl/L). The percentages of unknown TOX, organic halogen, TOCl, and TOBr were all not definable (nd), perhaps as a result of the high concentrations of THM4 + HAA9.

3. B. Growth Inhibition Assay (GIA)

The results presented in this section are grouped into two experiment sets. The objective of the first set was to use the growth inhibition assay (GIA) to compare the growth inhibitive

effects of water that had undergone different types of disinfection, while the second set was performed to determine the combined growth inhibitive effects of BrAA and iodoacetic acid (IAA) in the same matrix also using the GIA.

In the first set, surface water from OWASA (Carrboro, NC) was concentrated by reverse osmosis (RO) to achieve a TOC concentration of 65 mg C/L, equivalent to a concentration factor of 10. The samples were prepared according to the procedure on page 55 in the Methods section. Samples C, SC B, SC I, Pre-CB, Pre-CI, LGW Q, 40C BrAA, and BrAA (positive control) were prepared for this set and their preparation is summarized in Table 2.6. The series of dilutions were prepared as percentages of the original solution, which were converted to TOC concentrations that had been adjusted for addition of culture media during the sample preparation process of the GIA.

A 40% solution of sample C was selected because in preliminary trial runs this solution did not inhibit the growth of the assay cells. Sample C (undiluted) was run again for this experiment set to ensure that a 40% dilution would not inhibit cell growth. TOC of the sample at this dilution was 23 mg C/L. Figure 3.1 shows the results for varying TOC concentrations of sample C. At a TOC concentration of 23 mg C/L (equivalent to the 40% C solution), the percentage of cell density was 110%±10, indicating that the dilution at that TOC concentration did not inhibit the growth of the assay cells.

Pre-forming a reaction mixture of chlorine with bromide or iodide prior to addition of NOM was carried out to generate higher levels of bromine- or iodine-containing DBPs with a potentially higher inhibitive effect on colon cell growth. Samples C, SC B, SC I, Pre-CB, and Pre-CI were also extracted for HAA9 within a week of quenching free Cl_2 to quantify their concentrations in the samples. Details for this procedure can be found in section 2. B.1.c of this

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report.

The objective of the second experiment set was to determine the combined growth inhibitive effects of BrAA and IAA together using the GIA. These compounds were selected because they have been found to be among the most cytotoxic of the HAA species (Plewa et al., 2002). A fresh sample C was prepared in the same way as described in the Methods section and was used to prepare another 40% matrix solution with a TOC concentration of 23 mg C/L in M3:10 Culture Media. The samples that were prepared for this experiment set were C, 40C BrAA, 40C IAA, and 40C BrAA & IAA and are also described in the Methods section (2. B.2).

3. B.1. Treated Water Concentrates

The colon cells were exposed to serial dilutions of each sample (C, SC B, SC I, Pre-CB, Pre-CI, and LGW Q) with a control (prepared MEM culture media). For all of the samples except LGW Q, the final TOC concentrations after each serial dilution was 0 mg C/L (control), 0.3 mg C/L, 0.6 mg C/L, 3 mg C/L, 6 mg C/L, 12 mg C/L, 23 mg C/L, 29 mg C/L, 35 mg C/L, 46 mg C/L, and 58 mg C/L. For the LGW Q sample, the serial dilutions are represented by the percentage of original sample in the dilution: 0%, 0.5%, 1%, 5%, 10%, 20%, 40%, 50%, 60% 80%, 100%. These dilutions were prepared as described in the Methods section. Figure 3.1 through Figure 3.6 show the effect on cell density of these solutions with the first data point for the control (shown at 0.01 mg C/L, or 0% for LGW Q) representing the average percentage of cell density to which no sample C was added. The results for these samples are displayed in Table 3.2 through Table 3.7. An IC50 value, which represents half the maximal inhibitory concentration of the sample, could not be calculated from these assay results. Instead, an IC90 for each sample (except LGW Q) was determined by interpolating the TOC concentration at 90%

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cell density from the dose-response curve. Because the graph uses a logarithmic scale, this data point could not be graphed at "0 mg C/L" along the x-axis, so it was plotted at 0.01 mg C/L. The values printed on the graph at each data point represent the average percentage of cell density obtained for that dilution of the sample. Standard deviations for each data point were also calculated for an $n = 8$, represented by the error bars.

After 3 days holding time in the incubator, the microplates were analyzed for cell density using the Wallac Plate Reader, as described in the Methods section. The Wallac Plate Reader reads the absorbance of each well on the microplate at a wavelength of 600 nm. To determine the average percentage of cell density per dilution of the sample the calculations were performed in the following manner. First, the absorbances obtained for the background column were averaged. Each absorbance under the control column was subtracted by the average background absorbance to obtain a "corrected control" value, and all 8 corrected control values were averaged. A sample calculation for determining corrected control can be found below using 0.414 as the absorbance from a control well and 0.084 from a background well.

Corrected control = Control – Average background $= 0.414 - 0.086 = 0.328$

Next, each corrected control of the 8 values was divided by the average corrected control and multiplied by 100 to obtain a "% of control," also representing percentage of cell density.

$$\% \text{ of Control} = \left(\frac{Corrected\ control}{Average\ corrected\ control} \right) \cdot 100 = \left(\frac{0.328}{0.361} \right) \cdot 100 = 91\%$$

These % control values were averaged and a standard deviation was calculated. The raw absorbance values in the remaining columns were treated the same way: a corrected value was obtained by subtracting the average background value from each raw absorbance value under

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that column, the corrected absorbance values were averaged, each corrected absorbance value was divided by the average corrected control and then multiplied by 100 to get a "% of control" value. These "% of control" values, or "% cell density," were averaged and used to calculate a standard deviation (n = 8) and then graphed logarithmically with respect to the BrAA concentration to which the cells were exposed. These graphs are referred to as dose-response curves.

The raw data obtained for samples BrAA and 40C BrAA (Figure 3.7 – Figure 3.8) were analyzed in the same manner as described above. The concentrations of BrAA that were used were 0 (control), 1.00×10^{-6}, 5.00×10^{-6}, 8.00×10^{-6}, 1.00×10^{-5}, 2.00×10^{-5}, 3.00×10^{-5}, 4.00×10^{-5}, 5.00×10^{-5}, 6.00×10^{-5}, and 1.00×10^{-4} M. If the average % cell density was calculated to be a negative value, that data point was graphed as "0" and no error bars were set for that value. An IC50 value, which represents half the maximal inhibitory concentration of the sample, was determined by extrapolating the concentration at 50% cell density from the dose-response curve. The results for these two graphs are displayed in Table 3.8 and Table 3.9. Because the graph uses a logarithmic scale, the first data point could not be graphed at a concentration of "0 M" along the x-axis, so it was plotted at 1.00×10^{-7} M BrAA. The numbers next to each data point represent the average percentage of cell density obtained for that dilution of the sample. Standard deviations for each data point were also calculated (n = 8) and are represented by the error bars.
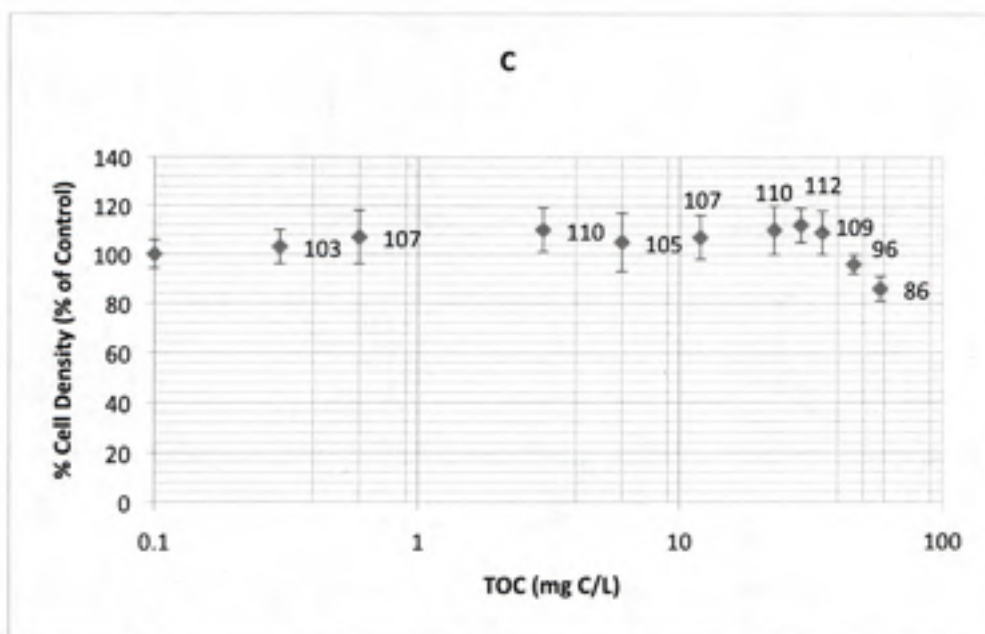
Figure 3.1 Sample C. RO concentrated water was dosed with 325 mg/L Cl_2 and was quenched for free Cl_2 after 5 days holding time with 340 mg/L ascorbic acid. The error bars represent the standard deviation for each data point. An IC90 value was deterined to be at a TOC concentration of 54 mg C/L.

Table 3.2 Results from sample C assay.

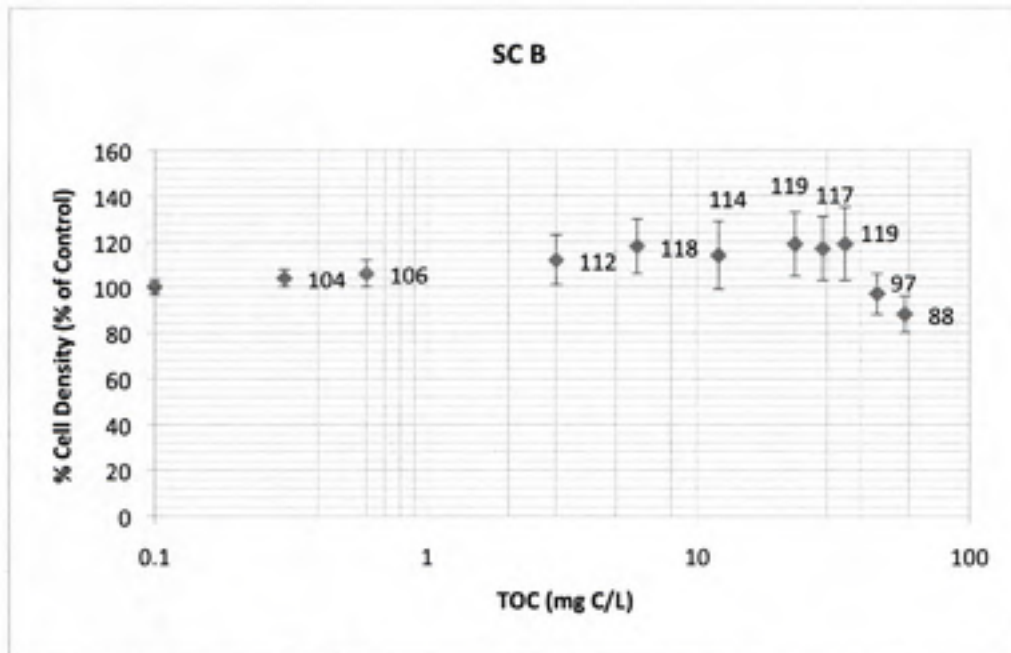| [TOC] (mg C/L)	% Cell Density ± Standard Deviation
0 (control)	100%±6
0.3	103%±7
0.6	107%±11
3	110%±9
6	105%±12
12	107%±9
23	110%±10
29	112%±7
35	109%±9
46	96%±4
58	86%±5

Figure 3.2 Sample SC B. RO concentrated water was spiked with 10 mg/L Br⁻, dosed with 325 mg/L Cl₂, and was quenched for free Cl₂ after 5 days holding time with 340 mg/L ascorbic acid. The error bars represent the standard deviation for each data point. An IC90 value was determined to be at a TOC concentration of 54 mg C/L.

Table 3.3 Results from sample SC B assay.

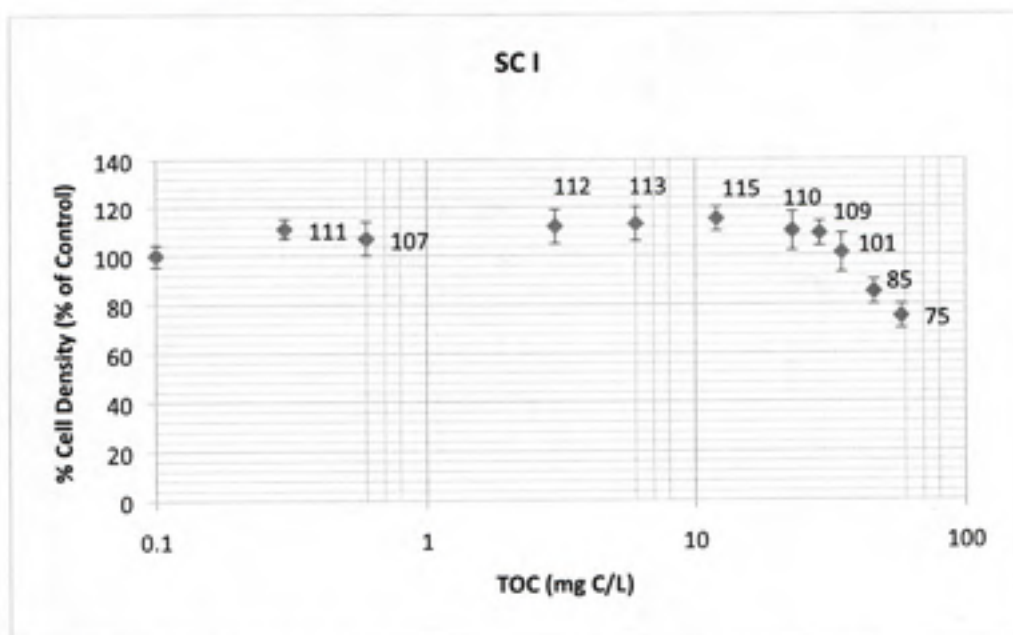| [TOC] (mg C/L)	% Cell Density ± Standard Deviation
0 (control)	100%±3
0.3	104%±4
0.6	112%±11
3	118%±12
6	114%±15
12	119%±14
23	117%±14
29	119%±16
35	109%±9
46	97%±9
58	88%±8

Figure 3.3 Sample SC I. RO concentrated water was spiked with 10 mg/L I⁻, dosed with 325 mg/L Cl_2, and was quenched for free Cl_2 after 5 days holding time with 300 mg/L ascorbic acid. The error bars represent the standard deviation for each data point. An IC90 value was determined to be at a TOC concentration of 42 mg C/L.

Table 3.4 Results from sample SC I assay.

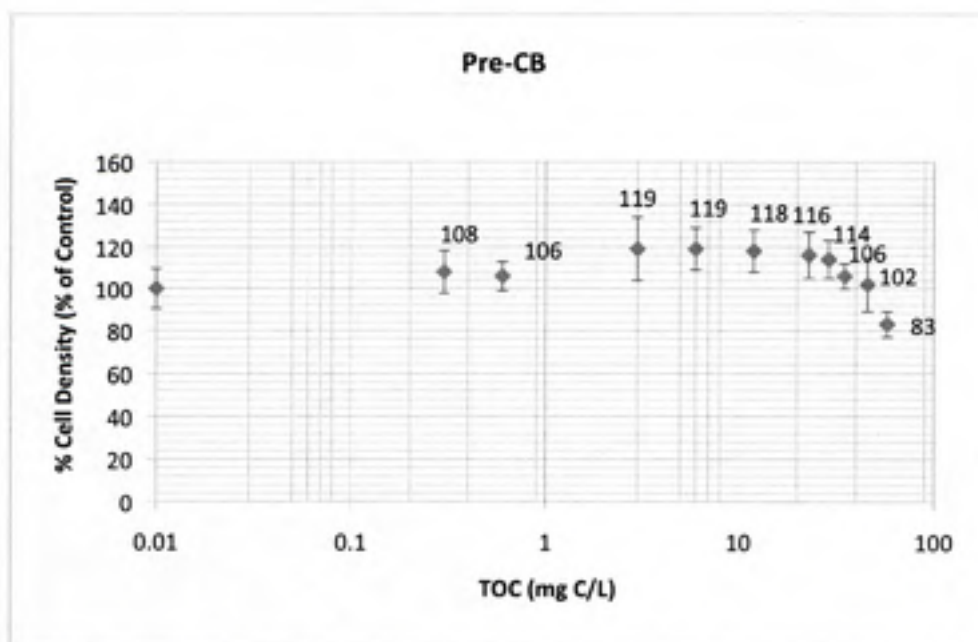| [TOC] (mg C/L)	% Cell Density ± Standard Deviation
0 (control)	100%±4
0.3	111%±4
0.6	107%±7
3	112%±7
6	113%±7
12	115%±5
23	110%±8
29	109%±5
35	101%±8
46	85%±5
58	75%±5

Pre-CB

Figure 3.4 Sample Pre-CB: A pre-formed solution of Cl_2 (325 mg/L) and Br^- (10 mg/L) in the volume of the final sample, was prepared and the added to the RO concentrated water. After 5 days holding time, free Cl_2 was quenched with 370 mg/L ascorbic acid. The error bars represent the standard deviation for each data point. An IC90 value was determined to be at a TOC concentration of 52 mg C/L.

Table 3.5 Results from sample Pre-CB.

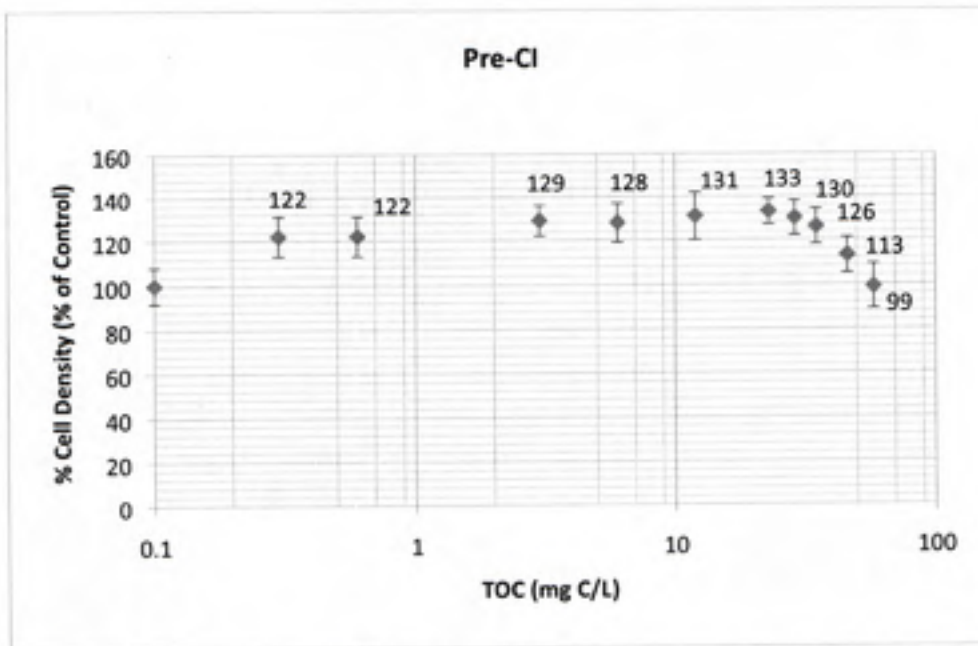| [TOC] (mg C/L)	% Cell Density ± Standard Deviation
0 (control)	100%±10
0.3	108%±10
0.6	106%±7
3	119%±15
6	119%±10
12	118%±10
23	116%±11
29	114%±9
35	106%±6
46	102%±13
58	83%±6

Figure 3.5 Sample Pre-CI: A pre-formed solution of Cl_2 and I^-, to give concentrations of 325 mg/L Cl_2 and 10 mg/L I^- in the volume of the final sample, was prepared and the added to the RO concentrated water. After 5 days holding time, free Cl_2 was quenched with 370 mg/L ascorbic acid. An IC90 value could not be determined for this sample.

Table 3.6 Results from sample Pre-CI.

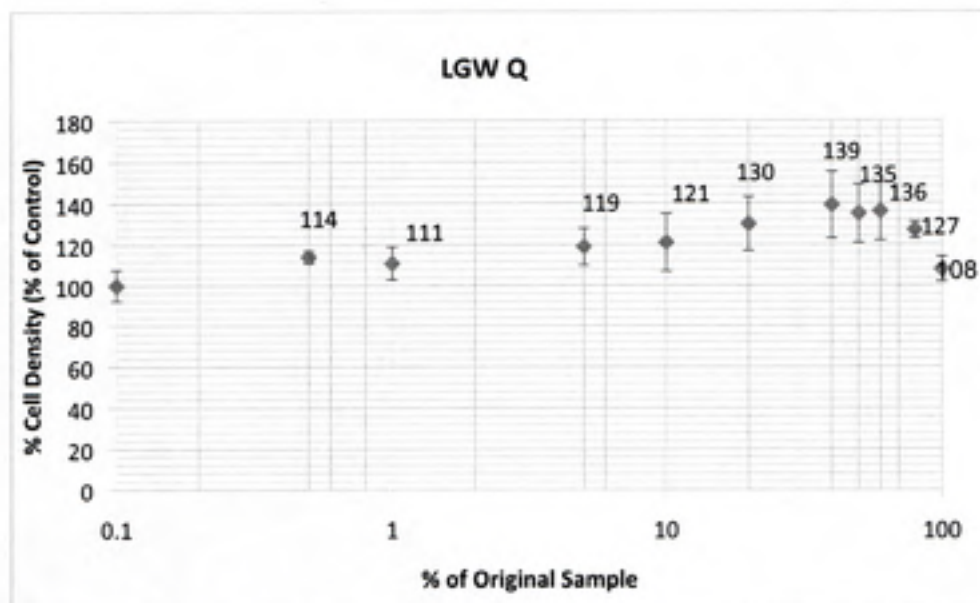| [TOC] (mg C/L)	% Cell Density ± Standard Deviation
0 (control)	100%±8
0.3	122%±9
0.6	122%±9
3	129%±7
6	128%±9
12	131%±11
23	133%±6
29	130%±8
35	126%±8
46	113%±8
58	99%±10

Figure 3.6 Sample LGW Q. This sample was prepared to determine the growth inhibitive effect of simulated chlorine residual quenched with ascorbic acid.

Table 3.7 Results for sample LGW Q.

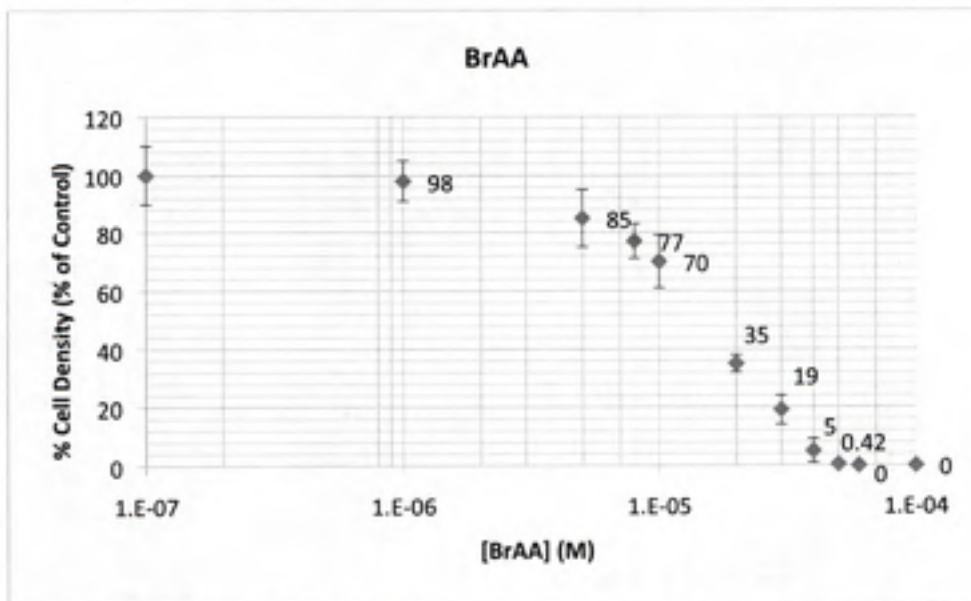| % of Original Sample	% Cell Density ± Standard Deviation
0 (control)	100%±8
0.5	114%±3
1	111%±8
5	119%±9
10	121%±14
20	130%±13
40	139%±16
50	135%±14
60	136%±14
80	127%±4
100	108%±6

Figure 3.7 Sample BrAA. This sample was prepared for the purposes of being a positive control. Varying concentrations of BrAA were spiked into M3:10 cell growth media. The error bars represent the standard deviation for each data point. The IC50 value for this sample is about 1.6×10^{-5} M.

Table 3.8 Results for sample BrAA.

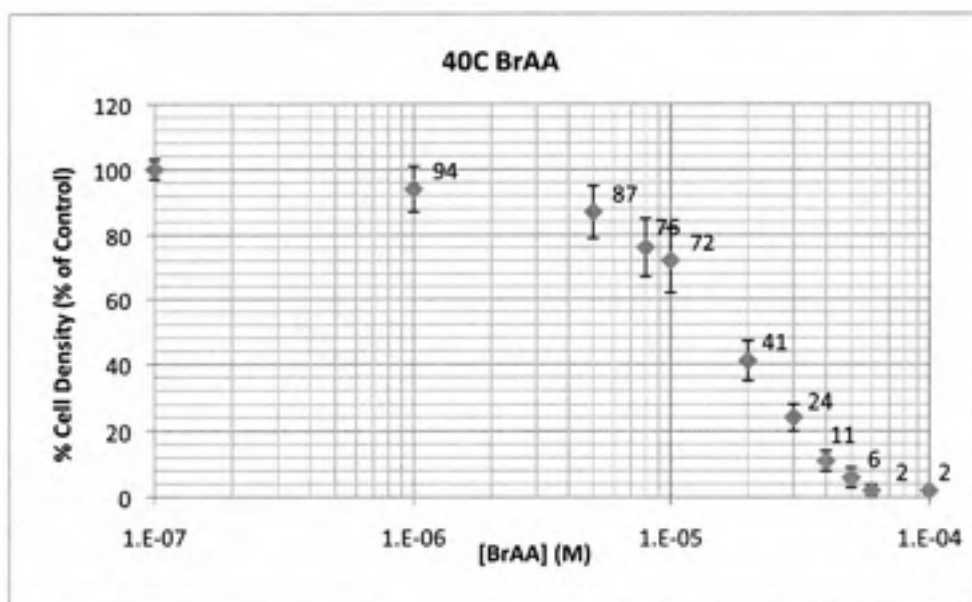| [BAA] (M)	% Cell Density ± Standard Deviation
0 (control)	100%±10
1.00×10^{-6}	98%±7
5.00×10^{-6}	85%±10
8.00×10^{-6}	77%±6
1.00×10^{-5}	70%±9
2.00×10^{-5}	35%±3
3.00×10^{-5}	19%±5
4.00×10^{-5}	5%±4
5.00×10^{-5}	0%±0
6.00×10^{-5}	0%±0
1.00×10^{-4}	0%±0

Figure 3.8 Sample 40C BrAA. Sample C was diluted using M3:10 growth media for the cells to produce a 40% solution. BrAA was spiked into this 40% solution of sample C at varying concentrations to determine the effect of the chlorinated matrix on the positive BrAA control curve. The error bars represent the standard deviation for each data point. The IC50 value for this sample is about 1.7×10^{-5} M.

Table 3.9 Results for sample 40C BrAA.

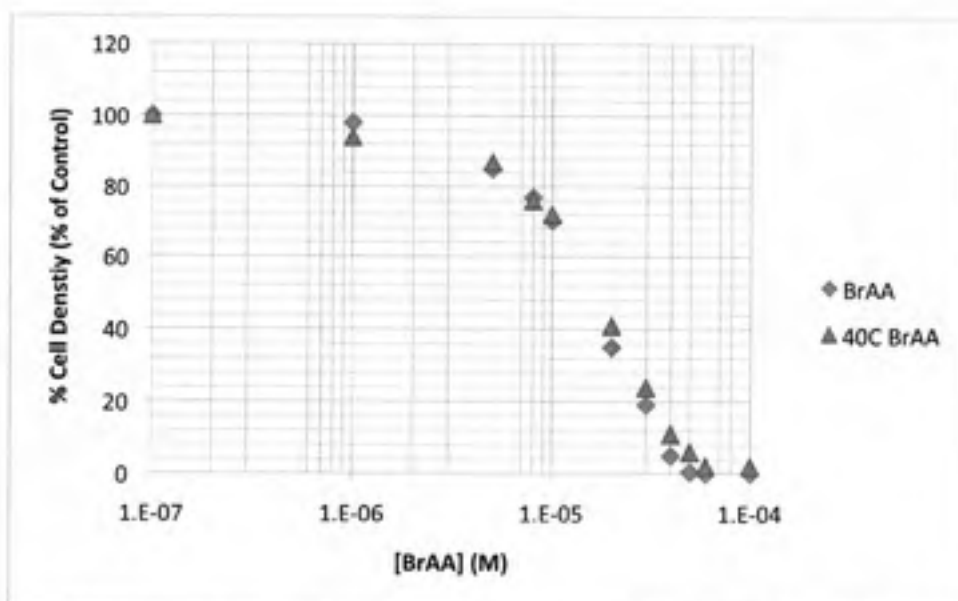| [BAA] (M)	% Cell Density ± Standard Deviation
0 (control)	100%±3
1.00×10^{-6}	94%±7
5.00×10^{-6}	87%±8
8.00×10^{-6}	76%±9
1.00×10^{-5}	72%±10
2.00×10^{-5}	41%±6
3.00×10^{-5}	24%±4
4.00×10^{-5}	11%±3
5.00×10^{-5}	6%±3
6.00×10^{-5}	2%±2
1.00×10^{-4}	2%±1

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Figure 3.9 Results from Sample BrAA and Sample 40C BrAA graphed together. IC50 values for BrAA and 40C BrAA are 1.6×10^{-5} M and 1.7×10^{-5} M, respectively.

The samples graphed in Figure 3.1 – Figure 3.5 were relatively non-growth inhibitive to the colon cells and LGW Q was not growth inhibitive to the cells either (Figure 3.6); thus, IC50 values for these samples could not be extrapolated from the dose-response curves. Instead, IC90 values were calculated for samples C, SC B, SC I, and Pre-CB: it is interesting to note that for all of these samples, the percentage of cell density increased prior to decreasing. While these samples did not inhibit cell growth very much, there was still a certain percentage of cell density lost between the least concentrated dose and the most concentrated dose. This percentage can be calculated by subtracting the percentage of cell density at the most concentrated dose (100% of sample) from the least concentrated dose (0.5% solution of the sample). For samples C, SC B, SC I, Pre-CB, and Pre-CI the percentage of cell density lost was 17%, 16%, 36%, 25%, and 23%, respectively. Based on these findings, the samples can be ranked in the following way, from most to least growth inhibitive: SC I > Pre-CB > Pre-CI > C ~ SC B.

In Figure 3.7 and Figure 3.8, IC50 values could be determined using the dose-response curves that were generated for each sample. The BrAA sample, the positive control, produced an IC50 at around 1.6×10^{-5} M, while the 40C BrAA sample produced an IC50 at around 1.7×10^{-5} M. Because the 40C BrAA sample contained DBPs that were produced in the C sample, it was initially supposed that the IC50 for this sample would be lower than the IC50 for the BrAA sample. Instead the results show that the IC50 value for both samples occurred at around the same concentration; thus, indicating that the 40% C solution did not increase the growth inhibitive effect of the matrix.

3. B.1.a. HAA9 Extraction

The objectives for this part of the experiment were a) to identify and quantify the HAA species that were present in samples C, SC B, SC I, Pre-CB, and Pre-CI by liquid-liquid extraction and b) to apply these results to those from the GIA, and evaluate the effect of an HAA mixture on the assay cells. The results for this part of the experiment can be seen in Table 3.10. In Table 3.10, the asterisks indicate that the chemical did not create a peak response in the chromatogram(s) for that sample. "NA" denotes that an RPD or RSD could not be calculated because the raw data were either below detection limit or did not create peak responses in the chromatograms. RPD values are represented in **bold** type. Additionally, a "<" sign indicates that the chromatogram produced a peak response for that chemical; however, it was below the response for the lowest quantifiable concentration.

Table 3.10 HAA9 data for samples C, SC B, SC I, Pre-CB, and Pre-CI. Percentages in parentheses represent either a RSD (for 3 values) or a RPD (for 2 values).

	[ClAA]	[BrAA]	[Cl₂AA]	[BrClAA]	[Cl₃AA]	[Br₂AA]	[BrCl₂AA]	[Br₂ClAA]	[Br₃AA]
C, Average (µg/L)	179 (4%)	8.78 (30%)	2752 (17%)	68.8 (13%)	1896 (48%)	<2* (NA)	131 (3%)	12.3 (22%)	<4* (NA)
C (M)	1.90E-06	6.32E-08	2.13E-05	3.97E-07	1.16E-05	1.22E-08	6.31E-07	4.89E-08	2.45E-08
SC B, Average (µg/L)	132 (7%)	67.4 (4%)	1322 (18%)	975 (12%)	1044 (23%)	539 (19%)	1256 (4%)	1163 (2%)	283 (22%)
SC B (M)	1.40E-06	4.85E-07	1.02E-05	5.62E-06	6.39E-06	2.48E-06	6.07E-06	4.63E-06	9.56E-07
SC I, Average (µg/L)	132 (7%)	5.62 (2%)	1775 (1%)	44.3 (5%)	1057 (7%)	<2* (NA)	176 (14%)	<2 (NA)	<4* (NA)
SC I (M)	1.40E-06	4.05E-08	1.38E-05	2.55E-07	6.47E-06	1.22E-08	8.50E-07	7.96E-09	2.45E-08
Pre-CB, Average (µg/L)	136 (0.3%)	72.7 (5%)	1616 (17%)	1082 (18%)	1302 (1%)	662 (7%)	1632 (6%)	1583 (16%)	445 (17%)
Pre-CB (M)	1.44E-06	5.23E-07	1.25E-05	6.24E-06	7.97E-06	3.04E-06	7.89E-06	6.30E-06	1.51E-06
Pre-CI, Average (µg/L)	147 (9%)	<4 (NA)	1678 (5%)	36.7 (16%)	1356 (30%)	<2* (NA)	121 (7%)	<2 (NA)	<4* (NA)
Pre-CI (M)	1.56E-06	2.88E-08	1.30E-05	2.12E-07	8.30E-06	1.22E-08	5.87E-07	7.96E-09	1.59E-08

* = Chemical did not create a peak response in the chromatogram(s) for that sample.
NA = RPD or RSD could not be calculated because raw data were below detection limit or did not create peak response in chromatograms.
Bold = RPD values
< = Chromatogram produced a peak response for the chemical, but it was below the response for the lowest quantifiable concentration.

After the data were collected from the chromatograms, the HAA9 concentrations in each sample were calculated by taking into account a 1:10 or 1:2 dilution for certain samples. Thus, there were 4 analytical results for each sample type. Of these 4 values, 3 of the closest fitting values were selected, averaged, and a relative standard deviation (RSD) was calculated. If 3 values could not be selected because the values were too dissimilar or below the practical quantification limit (PQL), then two values were averaged and a relative percent difference (RPD) was calculated. For certain HAAs, there were peak responses at the designated retention time, but the detector response translated it to a concentration below the PQL. As a result, an average concentration could not be determined for this HAA species and its value of <PQL is presented

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in Table 3.10. In cases where no chromatographic response was observed, the value on the table is represented as <PQL*. Each average HAA concentration was then converted from "μg/L" to "M" (moles/L) using the molecular weight for the respective HAA. For the HAAs that occurred below detection limit or did not generate a response during GC analysis, the lowest detectable concentration for that HAA was used to convert the units from "μg/L" to "M" to generate concentrations in units consistent with the Plewa et al. (2002) and DeAngelo toxicity studies.

Using the average concentration for each HAA in units of "μg/L", the TOCl, TOBr, and TOX concentration for each sample type was calculated, results for which can be found in Table 3.11. TOCl was calculated by first converting the average concentration for each HAA from "μg/L" to "μg as Cl/L" using a molar ratio of chlorine to the molecular weight of that particular HAA, and then by summing these converted values together. TOBr was calculated similarly: the average concentration for each HAA was converted from "μg/L" to "μg as Br/L" using a molar ratio of bromine to the molecular weight of that particular HAA, and then these converted values were summed together. In order to calculate TOX, the TOBr value must be converted into units of "μg as Cl/L," which is done by multiplying TOBr ("μg as Br/L") by the molar ratio of chlorine to bromine, 35.45:79.90. Once the units have been converted, TOCl and TOBr (as μg Cl/L and as M) are added together to give an organohalogen concentration.

Table 3.11 Organic halogen content of HAA9 from experimental results of Table 3.10.

	Organochlorine (μg/L as Cl)	Organobromine (μg/L as Cl)	Organohalogen Content (μg/L as Cl)	Organohalogen Content (M)
C	2875	44	2919	8.23×10^{-5}
SC B	2247	1034	3281	9.26×10^{-5}
SC I	1783	43	1826	5.15×10^{-5}
Pre-CB	2788	1338	4126	1.16×10^{-4}
Pre-CI	1908	29	1937	5.46×10^{-5}

Looking at the HAA9 results in Table 3.10, Cl_2AA and Cl_3AA were the species that occurred at the highest concentrations, two or three orders of magnitude greater than the other HAAs. $BrCl_2AA$ and $BrClAA$, the next most prevalent compounds, appeared at concentrations between 10^{-7} M and 10^{-6} M, depending on whether or not the sample had been spiked with bromide. ClAA occurred at around the same concentration for each sample type. Even in samples that had not been spiked with bromide (C, SC I, and Pre-CI), there were detectable concentrations of $BrCl_2AA$ and $BrClAA$, indicating the presence of low levels of bromide in the source water. Br_2ClAA was not produced as much as $BrCl_2AA$ and $BrClAA$; however, its concentration was also influenced by whether or not the sample had been spiked with bromide. BrAA and Br_3AA occurred at around the same concentrations, varying between 10^{-8} M and 10^{-7} M, again indicating that elevated bromide content in the samples and reaction with chlorine influenced their formation.

In Table 3.11 the results for organochlorine, organobromine, and organohalogens further show that, as expected, organobromine concentrations were significantly elevated in samples that were spiked with bromide in both solutions in which chlorine was added to bromide before addition of NOM and in which the chlorine and bromide reaction occurred simultaneously. The pre-formed chlorine with bromide sample (Pre-CB) produced more organobromine and

organohalogen than sample SC B. This trend was also seen when comparing only the organohalogen content for SC I and Pre-CI. A graphical representation of this data can be seen in Figure 3.10, where the distribution of organochlorine and organobromine concentrations within the organohalogen content can be seen for each sample. Because the HAA9 analysis did not include iodinated species, organoiodine concentrations could not be calculated.
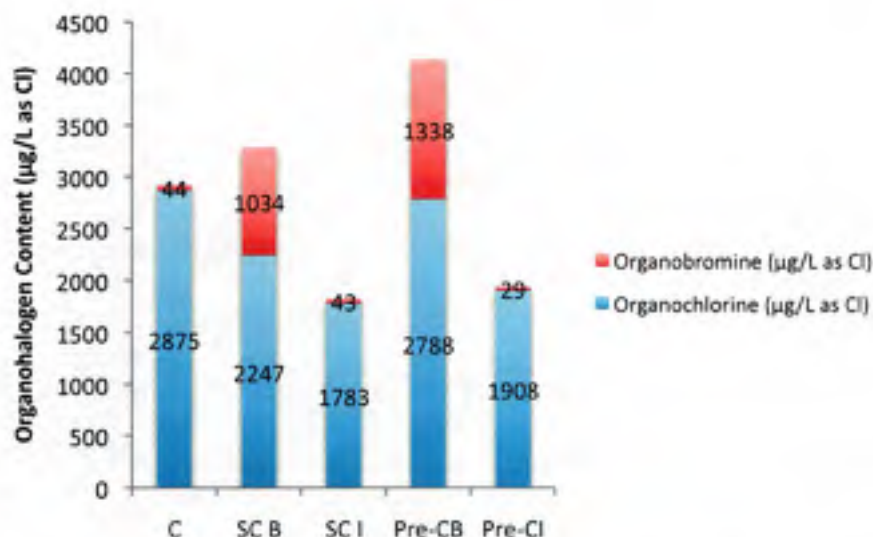

Figure 3.10 Distribution of organohalogen in HAA9 produced during chlorination of RO-concentrated water that was spiked with either bromide or iodide.

3. B.2. GIA Experiment Set 2

The objective of this experiment set was to determine the combined growth inhibitive effects of BrAA and IAA in the same matrix and to evaluate whether the combination of the two chemicals cause a synergistic or antagonistic effect on the growth inhibition of the cells. The matrix for the samples in this experiment was again a 40% solution of sample C in M3:10 Culture Media (a TOC concentration of 23 mg C/L). To ensure that this matrix did not inhibit cell growth at 40% of the initial concentration, sample C (undiluted) was run in duplicate as a

control using the GIA. The other samples that were run in duplicate were 40C BrAA, 40C IAA, and 40C BrAA & IAA.

These samples and their duplicates were each run on a 96-well microplate containing 12 columns each with 8 wells. The first column did not contain any colon cells and was used as a background to which the sample C matrix was added. The second column was used as a control and contained colon cells to which the background matrix was added. Columns 3 – 12 all contained colon cells and each matrix dilution was added to a respective column of wells.

After 3 days holding time in the incubator, the microplates were analyzed for cell density using the Wallac Plate Reader, as described in the Methods section. The Wallac Plate Reader reads the absorbance of each well on the microplate at a wavelength of 600 nm. Average percent cell density per dilution of the sample was calculated in the same way as described in GIA Experiment Set 1. However, since there were duplicates for each sample, the "% of control" (% cell density) values in both plates for the same dilution concentration were averaged. A standard deviation was also calculated using these 16 values. Dose-response curves were created for each sample by graphing concentration with respect to percentage of cell density on a logarithmic scale. These results can be seen in Figures 3.11 – 3.16.

It should be noted that sample 40C IAA was not graphed with its duplicate. For one of the plates that was run, the average "% of control" values for the lowest concentration, 1.00×10^{-6} M, was below 50%, while the value for the other plate was 95%. Thus, the results from the plate with the sub-50% cell density value at the lowest concentration were not included and considered outliers.

The assay cells were exposed to undiluted sample C and at dilutions corresponding to TOC concentrations of 0 (control), 0.3, 0.6, 3, 6, 12, 23, 29, 35, 46, and 58 mg C/L. This sample

was also run in duplicate. Figure 3.11 shows that sample C at most of the dilutions was relatively ineffective at inhibiting cell growth past 50% cell density. At a TOC concentration of 35 mg C/L and above, it is clear that cell growth is beginning to be inhibited. However, at a TOC concentration of 23 mg C/L, equivalent to a 40% dilution of sample C, the average cell density was 129%, indicating that the cells would not be inhibited by growth when exposed to a matrix at that concentration. Thus, the matrices for the subsequent samples were prepared using this TOC concentration of sample C. An explanation for why the average cell density for this dilution was >100% may be because the solution caused the osmolarity within the cells to increase. Also, perhaps the ascorbic acid used for quenching stimulated cell growth. A 40% dilution of sample C was selected as the background matrix for the samples in this experiment set because it is the dilution containing the highest TOC concentration (32 mg C/L) before the cell density begins to steadily decrease with respect to increasing TOC concentration. The first data point seen in this graph represents the average percentage of cell density of the control, which contained only M3:10 Culture Media. Since the graph uses a logarithmic scale, this data point could not be graphed at "0 mg C/L" along the x-axis, so it was plotted at 0.01 mg C/L. The numbers next to each data point represent the average percentage of cell density obtained for that dilution of the sample. Standard deviations for each data point were also calculated (n = 16), represented by the error bars.

Figures 3.12 through 3.16 show the results from the 40C BrAA, 40C IAA, 40C BrAA & IAA, and 40C BrAA & IAA (0.5x) samples. For 40C BrAA and 40C IAA, BrAA and IAA were each spiked at concentrations of 0, 1.00×10^{-6}, 5.00×10^{-6}, 8.00×10^{-6}, 1.00×10^{-5}, 2.00×10^{-5}, 3.00×10^{-5}, 4.00×10^{-5}, 5.00×10^{-5}, 6.00×10^{-5}, and 1.00×10^{-4} M in their respective samples. Sample 40C BrAA & IAA contained a mixture of BrAA and IAA, where each chemical was spiked at

the same concentrations listed for 40C BrAA and 40C IAA. Sample 40C BrAA & IAA (0.5x) also contained a mixture of BrAA and IAA, however each chemical was spiked at half of concentration (0.5x) that was used in the 40C BrAA sample. As stated earlier, all of the samples, except for 40C IAA, were run in duplicate and were graphed using results from both microplates. The results from one of the two 40C IAA microplates were considered to be outliers; thus, only one set of results for this sample was graphed. The first data point on each of these graphs represents the average percentage of cell density of the control, 100%, to which none of samples were added.

From Figures 3.12 and 3.13, IC50 values were interpolated and their values were determined to be 4.5×10^{-6} M and 2.6×10^{-6} M for 40C BrAA and 40C IAA, respectively. IC50 values for Figure 3.14 and Figure 3.15 could also be interpolated for 40C BrAA & IAA (0.5x), which was 1.0×10^{-6} M, but not for 40C BrAA & IAA. However, when these four samples were graphed together (Figure 3.16), their differences in growth inhibition can be seen more visually: it is clear that a mixture of BrAA and IAA had a synergistic effect on inhibiting cell growth when compared to the growth inhibitive effects of the individual BrAA and IAA samples. Instead of interpolating the IC50 values from Figure 3.14 and Figure 3.15 separately, it is possible to do so for the samples graphed together (Figure 3.16) as the only difference between those two samples are that one was prepared at half of the concentration of the other. So the IC50 value of both 40C BrAA & IAA and 40C BrAA & IAA (0.5x) samples is around 6×10^{-7} M.

An equation from Patoczka & Pulliam (1990 cited in Heck et al., 1992) can be used to mathematically calculate whether or not the mixture of BrAA and IAA had a synergistic effect on cell growth:

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$$\frac{1}{IC50_{mix}} = \frac{f_1}{IC50_1} + \frac{f_2}{IC50_2} + \ldots \frac{f_n}{IC50_n}$$

where f_i is the fraction by mass or molar concentration of the ith component in the mixture. If $IC50_1$ and $IC50_2$ represent the IC50 values for 40C BrAA and 40C IAA, respectively, then the expected IC50 value for the mixture, $IC50_{mix}$, is calculated to be 1.65×10^{-6} M, one order of magnitude greater than the actual IC50 value of the mixture. Thus, the BrAA and IAA mixture did have a synergistic effect on cell growth.

It should be noted that the IC50 values that are interpolated are not necessarily accurate. From Figure 3.16, it is evident that the IC50 value for 40C BrAA & IAA (0.5x) has changed from 1.0×10^{-6} M, interpolated using Figure 3.15, to about 8.5×10^{-7} M. While this shift may be minor, considering the fact that for sample 40C BrAA & IAA, the 1.0×10^{-6} M dilution resulted in a cell density percentage of 22%±9, the IC50 for 40C BrAA & IAA (0.5x) is probably incorrect. In order to ensure that the IC50 value is as correct as possible, dilutions at concentrations closer to (greater than and less than) the presumed IC50 value should be made and exposed to the assay cells. This way, the IC50 value can be more accurately interpolated.
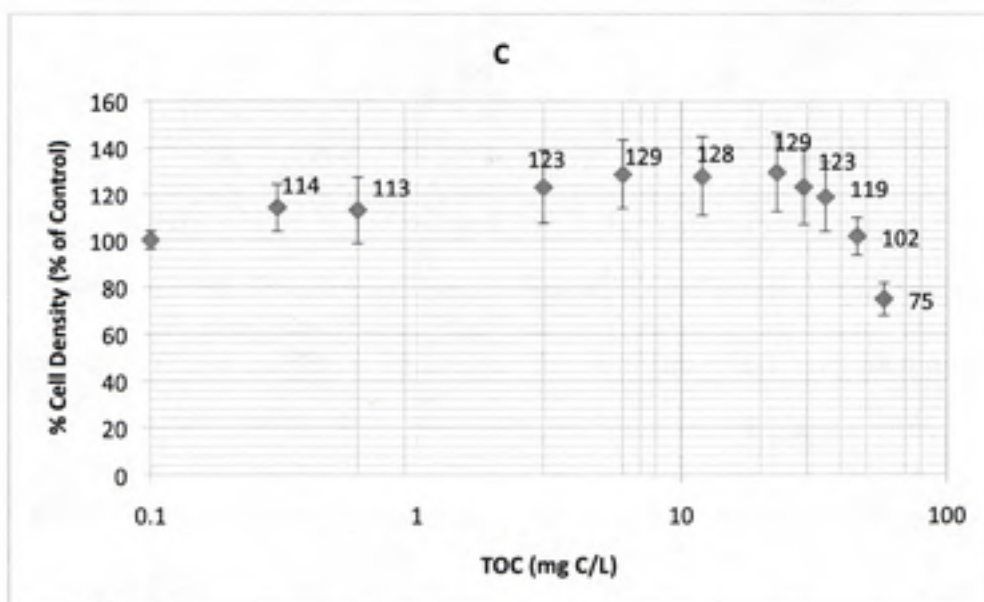
Figure 3.11 Sample C. RO concentrate water dosed with 325 mg/L Cl_2 and quenched for free Cl_2 with 421 mg/L ascorbic acid after 5 days of holding time. The error bars represent the standard deviation for each data point. An IC90 value was determined to be at a TOC concentration of 50 mg C/L.

Table 3.12 Results for sample C.

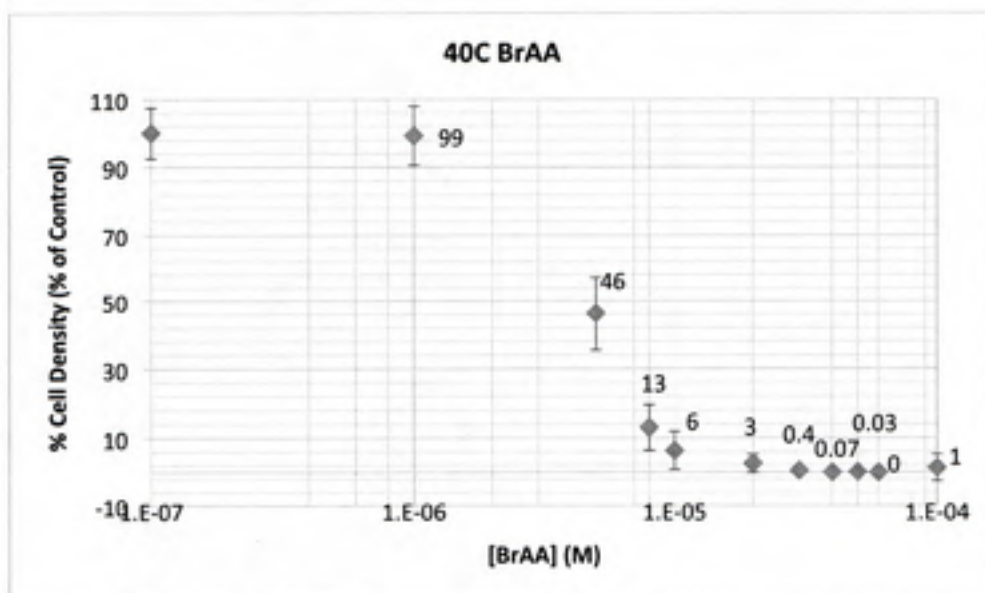| [TOC] (mg C/L)	% Cell Density ± Standard Deviation
0 (control)	100%±4
0.3	114%±10
0.6	113%±14
3	123%±16
6	129%±15
12	128%±17
23	129%±17
29	123%±17
35	119%±5
46	102%±8
58	75%±7

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Figure 3.12 Sample 40C BrAA. BrAA was spiked into a matrix composed of sample C diluted to 40% with M3:10 Culture Media at varying concentrations. The error bars represent the standard deviation for each data point. The IC50 for this sample, which was run in duplicate, is at 4.5×10^{-6} M.

Table 3.13 Results for sample 40C BrAA.

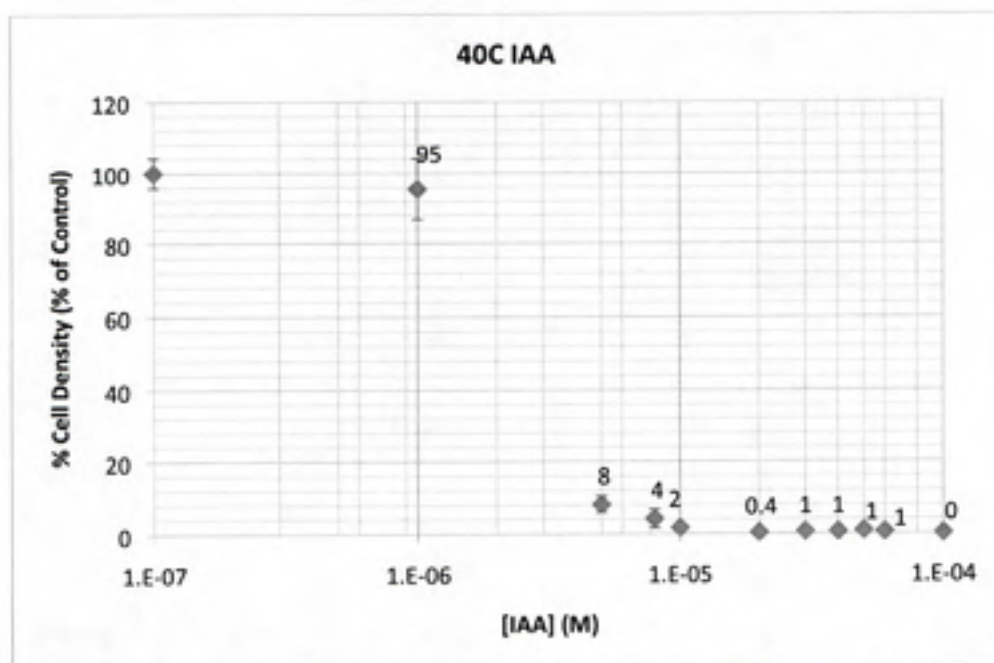| [BAA] (M)	% Cell Density ± Standard Deviation
0 (control)	100%±7
1.00×10^{-6}	99%±9
5.00×10^{-6}	46%±11
8.00×10^{-6}	13%±7
1.00×10^{-5}	6%±6
2.00×10^{-5}	3%±3
3.00×10^{-5}	0.4%±1
4.00×10^{-5}	0.03%±0.3
5.00×10^{-5}	0.07%±0.5
6.00×10^{-5}	0%
1.00×10^{-4}	1%±4

Figure 3.13 Sample 40C IAA. IAA was spiked into a matrix composed of sample C diluted to a TOC of 23 mg C/L (40% of C) with M3:10 Culture Media at varying concentrations. The error bars represent the standard deviation for each data point. The IC50 for this sample, which was not run in duplicate, is around 2.6×10^{-6} M.

Table 3.14 Results for sample 40C IAA.

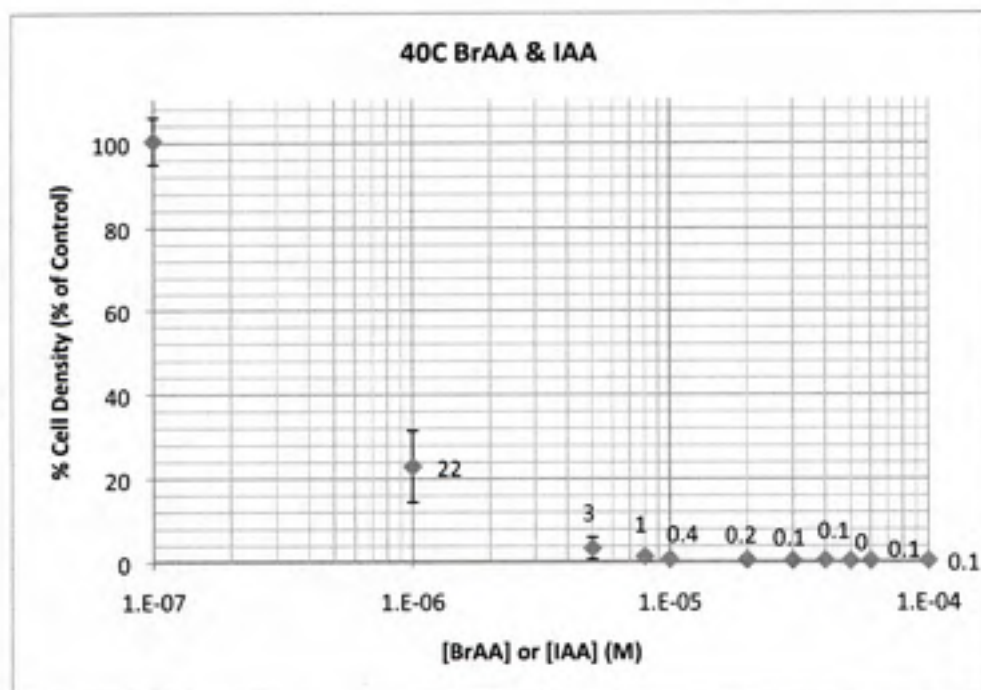| [IAA] (M)	% Cell Density ± Standard Deviation
0 (control)	100%±4
1.00×10^{-6}	95%±9
5.00×10^{-6}	8%±2
8.00×10^{-6}	4%±3
1.00×10^{-5}	2%±1
2.00×10^{-5}	0.4%±1
3.00×10^{-5}	1%±1
4.00×10^{-5}	1%±1
5.00×10^{-5}	1%±1
6.00×10^{-5}	1%±0.4
1.00×10^{-4}	0%

Figure 3.14 Sample 40C BrAA & IAA. BrAA and IAA were spiked together into a matrix composed of sample C diluted to 40% with M3:10 Culture Media at varying concentrations. The error bars represent the standard deviation for each data point. The IC50 for this sample could not be determined.

Table 3.15 Results for sample 40C BrAA & IAA.

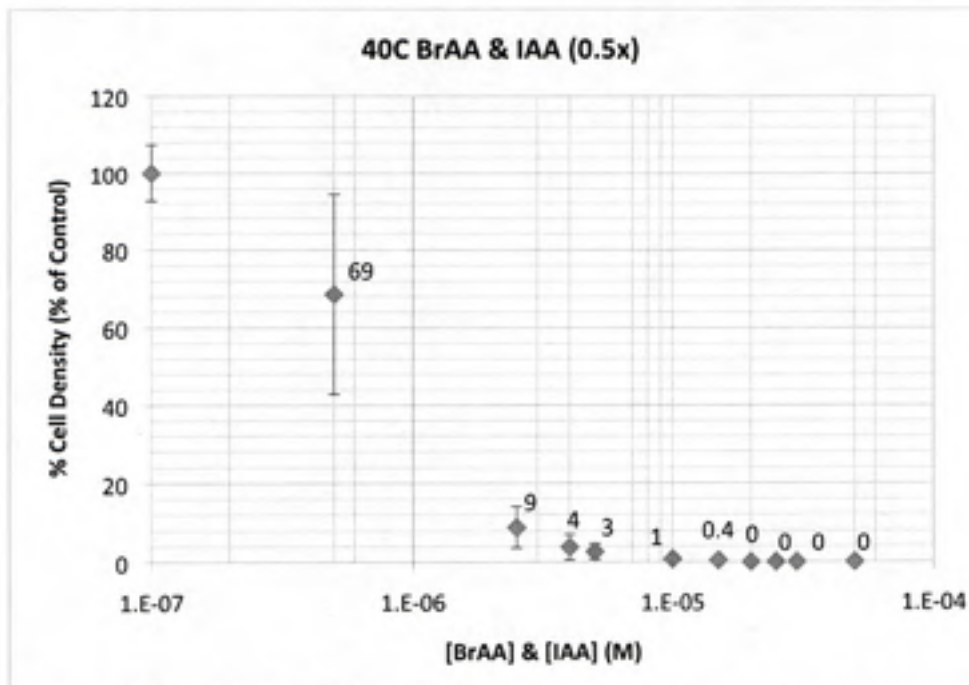| [BAA] or [IAA] (M)	% Cell Density ± Standard Deviation
0 (control)	100%±5
1.00×10^{-6}	22%±9
5.00×10^{-6}	3%±3
8.00×10^{-6}	1%±1
1.00×10^{-5}	0.4%±0.8
2.00×10^{-5}	0.2%±0.4
3.00×10^{-5}	0.1%±0.5
4.00×10^{-5}	0.1%±0.4
5.00×10^{-5}	0%
6.00×10^{-5}	0.1%±0.2
1.00×10^{-4}	0.1%±0.2

Figure 3.15 Sample 40C BrAA & IAA (0.5x). BrAA and IAA were spiked together into a matrix composed of sample C diluted to 40% with M3:10 Culture Media at varying concentrations. The error bars represent the standard deviation for each data point. The IC50 for this sample is 1.00×10^{-6} M.

Table 3.16 Results for sample 40C BrAA & IAA (0.5x)

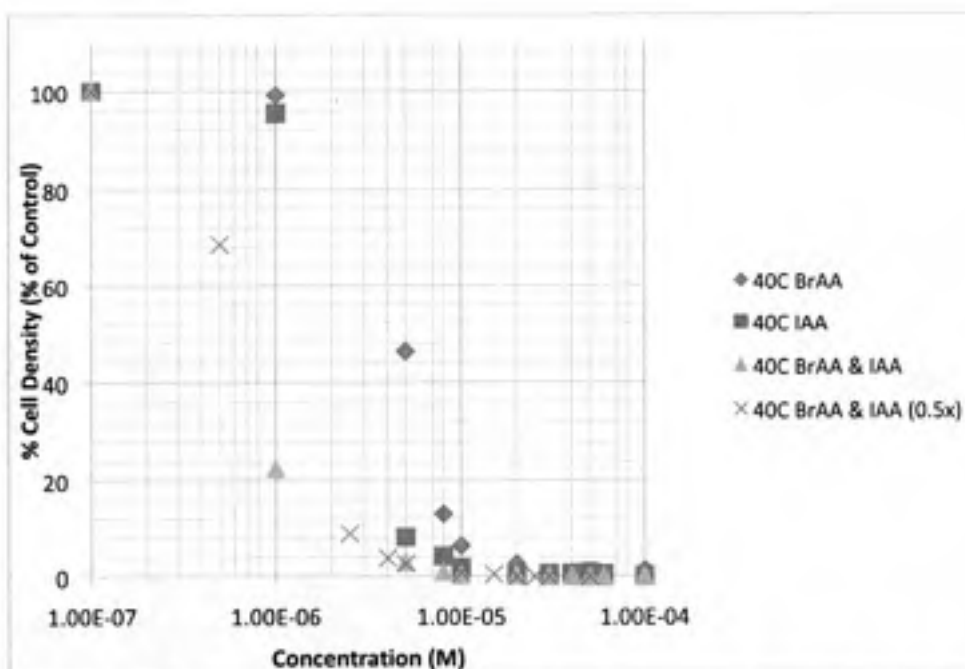| [BAA] or [IAA] (M)	% Cell Density ± Standard Deviation
0 (control)	100%±7
5.00×10^{-7}	69%±26
2.50×10^{-6}	9%±5
4.00×10^{-6}	4%±3
5.00×10^{-6}	3%±2
1.00×10^{-5}	1%±1
1.50×10^{-5}	0.4%±1
2.00×10^{-5}	0%
2.50×10^{-5}	0%
3.00×10^{-5}	0%
5.00×10^{-5}	0%

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Figure 3.16 Samples 40C BrAA, 40C IAA, 40C BrAA & IAA, and 40C BrAA & IAA (0.5x) graphed together. The mixture of the two compounds appears to have had a synergistic effect on cell growth inhibition.

3. C. Why were there no water samples treated with chloramines?

In order to ensure that the colon cells' growth would only be affected by the DBPs in the treated water samples, two criteria had to be met. The first condition is that there must be no residual disinfectant, free Cl_2 from chlorination or total Cl_2 from chloramination, in the water sample, which can be removed using ascorbic acid (also known as vitamin C). Removing residual disinfectant from the water with ascorbic acid is a process called "quenching." Many different quenching agents can be used to remove residual chlorine; however, ascorbic acid was selected because it is a vitamin, it would not inhibit cell growth, and it does not contain nitrogen. Nitrogen-containing compounds have been found to be more toxic than compounds that do not contain nitrogen, evident by comparing the IC50 values of 9 HNMs (Table 3.17) with 6 HAAs (Table 1.4) from using the GIA with CHO cells (Plewa et al., 2004; Plewa et al, 2002). The IC50

values from the 6 HAAs fall within the 10^{-4} M – 10^{-2} M range except for BrAA, while those of HNMs fall within the 10^{-6} M – 10^{-4} M range.

Table 3.17 IC50 values of 9 halonitromethanes (HNMs) from Plewa et al. (2004) using the GIA with CHO cells.

Compound	IC50 (M)	IC50 (µg/L)
Bromonitromethane	7.06×10^{-6}	988
Dibromonitromethane	6.09×10^{-6}	1.33×10^{3}
Tribromonitromethane	8.57×10^{-6}	2.55×10^{3}
Chloronitromethane	5.29×10^{-4}	5.05×10^{4}
Dichloronitromethane	3.73×10^{-4}	4.85×10^{4}
Trichloronitromethane	5.36×10^{-4}	8.81×10^{4}
Bromochloronitromethane	4.05×10^{-5}	7.06×10^{3}
Bromodichloronitromethane	1.32×10^{-5}	2.76×10^{3}
Dibromochloronitromethane	6.88×10^{-6}	1.74×10^{3}

The second condition is that the quenched chlorine/chloramine by-products must not inhibit the growth of the colon cells to prevent a false positive response. Therefore, prior to running Experiment Sets 1 and 2 (as described previously), a series of tests was performed using the GIA to determine whether or not the quenched chlorine and chloramine by-products inhibit the growth of the colon cells.

This experiment involved quenching the chlorine and chloramine disinfectant solutions with a stoichiometric mass of solid ascorbic acid prior to adding it to the RO concentrated water (TOC = ~65 mg C/L), where a residual of 1 mg/L as Cl_2 (MW = 70.906) needs 2.48 mg/L of ascorbic acid (MW =176.12) to be completely quenched. This ratio is based on the equation, also seen in chapter 2:

$$wt \text{ } ascorbic \text{ } acid \text{ } (mg) = \frac{wt \text{ } of \text{ } Cl_2 \text{ } in \text{ } solution \text{ } (mg) \cdot 176.12\frac{g}{mol}}{70.906\frac{g}{mol}}$$

Doing so removes all of the residual disinfectant so its reaction with the NOM in the RO concentrated water is inhibited.

The samples used in this experiment were W1 (control), W1 & BrAA, W2 (control), W2 & BrAA, and BrAA (positive control). These samples were prepared according to the method described in section 2. C in the Methods chapter. Final results are presented in Figure 3.17 and Table 3.18.
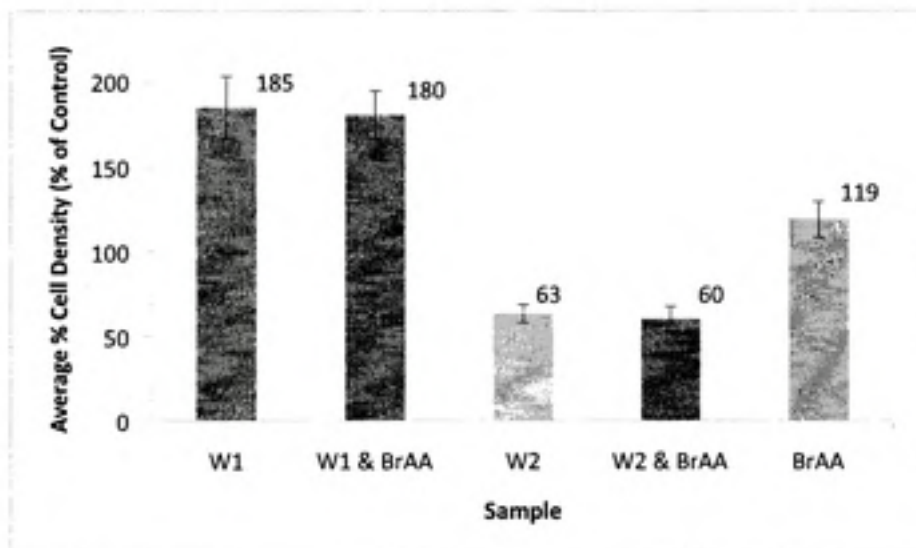

Figure 3.17 Effect of Cl_2/NH_2Cl and ascorbic acid by-products on NCM460 cells using the GIA. Standard deviation for each sample is represented by the error bars.

Table 3.18 Results from the experiment presented in *Figure 3.17*.

Sample	Average % Cell Density ± Standard Deviation	n
W1 (control)	185%±18	8
W1 & BrAA	180%±14	16
W2 (control)	63%±5	8
W2 & BrAA	60%±7	16
BrAA	119%±11	24

From these results, it is clear that the samples that contained the pre-quenched NH_2Cl/ascorbic acid solution, W2 and W2 & BrAA, had a growth inhibitive effect on the colon cells, compared to the samples containing the pre-quenched Cl_2/ascorbic acid solution (W1 and W1 & BrAA). The growth inhibitive effects are not a result of the 1.00×10^{-6} M BrAA spike because the W1 & BrAA and BrAA samples did not inhibit growth of the cells. Additionally, the growth inhibition in samples W2 and W2 & BrAA was not caused by DBPs because the chloramine was pre-quenched with ascorbic acid. Therefore, these results indicate that the pre-quenched NH_2Cl/ascorbic acid solution produced a growth inhibitive by-product, which affected the growth of the cells. It is for this reason why none of the previous experiments were conducted with chloraminated waters.

4. Discussion

The results presented in this report emphasize the importance of evaluating drinking water DBP quality as mixtures rather than as individual chemicals. From the chemical standpoint, it is shown how variable the levels of known and unknown DBPs can be when a water is subjected to different disinfection treatments while from a biological perspective, the toxicity of a mixture of DBPs can be quite different from the predicted toxicity of the sum of the individual compounds. Examining the combined toxicity effects of DBPs is more applicable to real world situations.

4. A. Contra Costa Water District (CCWD) Study DBPs

In a nationwide DBP occurrence study, where water samples were collected from 12 water treatment plants, 4 to 5 times per season from fall 2000 until early spring 2002, researchers found that the median and maximum TOX concentrations were 178 µg/L as Cl⁻ and 284 µg/L as Cl⁻, respectively (Krasner et al., 2006). TOX concentrations of the treated water samples determined from the CCWD findings in the present study generally fell within this range, though some samples (Phase 1B MP400 Cl_2, Phase 2A Cl_2 & LP400 Cl_2, Phase 2B MP400 Cl_2, Phase 3A Pre-NF LP400 Cl_2 & MP400 Cl_2) had TOX concentrations between 300 and 351 µg/L as Cl. The nationwide study also determined median and maximum concentrations for TOCl, 161 µg Cl /L and 206µg Cl /L, and TOBr, as 79µg Br/L (35 as µg Cl/L) and 229 µg Br/L (102 µg as Cl/L). Comparing these values to the results from CCWD (Table 3.1), TOCl concentrations were much lower than the TOBr concentrations, a trend that was correlated to Br and Cl content in THM4 and HAA9. An explanation for this trend is that the CCWD waters were spiked with 1 mg/L of bromide at the beginning of the water treatment process, whereas in the nationwide study the median and maximum bromide content in the raw water were 0.12 mg/L and 0.40

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mg/L, respectively. It is interesting to note that while TOX concentrations in the two studies generally fell within the same range, the distribution trend for TOCl and TOBr concentrations were reversed: there was consistently more TOBr than TOCl in the CCWD samples. This outcome was the same as the Hua & Reckhow (2007) study on waters that had been either ozonated and post-chlorinated or ozonated and post-chloraminated. Hua & Reckhow (2007) also found that there were more bromine-containing THM and HAA species in the ozonated and post-chlorinated samples. The CCWD findings support this outcome where the known organobromine concentrations, in the THM4 and HAA9, were greater than that of the known organochlorine concentrations (Table 3.1). Two reasons why this may have occurred are that perhaps pre-ozonation causes NOM to be more reactive with bromine (Hua & Reckhow, 2007) and that bromine incorporation into NOM is faster than that of chlorine (Symons et al., 1996).

Stated earlier in the Results section and reported in Table 3.1, the most significant and consistent trend throughout all of the CCWD phases, particularly in the phases that used ozone (1A, 1B, 2A, and 2B), was that the percentage of unknown TOBr was greater than that of unknown TOCl. Krasner et al. (2006) did not calculate values for percentage of unknown TOCl or TOBr, so the trend found in the CCWD study could not be compared to the nationwide occurrence study. However, this finding may further support the possibility that bromine incorporation into NOM is facilitated by ozonation (Hua & Reckhow, 2007).

It is known that when ozone reacts with NOM, lower molecular weight compounds are produced and are generally considered to be biodegradeable (Singer & Reckhow, 1999). As discussed in Chapter 1, ozone rapidly reacts with bromide to form hypobromous acid (HOBr), which reacts with NOM to form bromine-containing DBPs. HOBr is a stronger halogenating compound than HOCl and as a result, the kinetics of bromine incorporation into NOM are faster

than that of chlorine (Symons et al., 1996). These facts may further explain why the CCWD samples, which had undergone ozonation, had more bromine-containing DBPs than chlorine-containing DBPs.

Another general trend seen in the CCWD results (Table 3.1) was that the TOX concentrations were consistently higher than the sum of the TOCl and TOBr concentrations (TOCl + TOBr). An explanation for this occurrence is that when TOX is measured using the organic halide analyzer, the instrument measures Cl, Br, and I together in one value. The TOX analyzer cannot differentiate among these three halogens whereas, through IC analysis, the halogens are separated and can be measured individually as TOCl and TOBr. However, TOI (total organic iodine) in the CCWD samples was not measured and hence, TOCl + TOBr $\leq$ TOX. Iodide (I^-) itself was also not measured.

Percentages of unknown TOX, TOCl, and TOBr were calculated for all of the chloraminated samples in the CCWD study; however, for many of the chlorinated samples, these percentages could not be determined. The difficulties with calculating these values may be a result of using three different methods to quantify and calculate the concentrations of TOCl, TOBr, and TOX in these samples. The three methods, THM4 and HAA9 by LLE with GC/ECD, TOX analysis by pyrolysis and coulometric titration, and TOCl and TOBr by IC analysis, all come with their own set of uncertainties and errors that can accumulate through analysis and performing various calculations. Thus, comparing and integrating these methods is possible as demonstrated in the results of Table 3.1; however, complications arise when the sums of the experimental errors of each method are in close proximity to the differences in values calculated.

There has been little research on the production of DBPs through using UV irradiation in drinking water treatment. However, UV irradiation is frequently used to inactivate protozoans

such as *Cryptosporidium parvum* and *Giardia lamblia*, particularly their hardy oocysts (Betancourt & Rose, 2004). Researchers have found that humic acid degradation increases in the presence of UV fluence and H_2O_2 (Wang et al., 2000). Looking at the CCWD data (Table 3.1) a frequent occurrence throughout the phases was that the samples treated with UV irradiation had higher concentrations of TOBr than samples that were not treated with UV irradiation. Additionally, the chlorinated water samples had higher TOBr concentrations than the chloraminated ones. Through these findings, it seems likely that water treatment using a combination of ozone, peroxide, and/or UV irradiation can breakdown NOM into smaller molecules. The CCWD results also support the knowledge that chlorine is more effective than chloramines at converting bromide into HOBr due to the fact that the chlorinated and UV treated samples frequently contained higher concentrations of TOBr than the chlorinated, non-UV treated samples. As a result, the CCWD water quality and treatment conditions were favorable in facilitating the formation of a high proportion of bromine-containing THMs and HAAs.

In a study that examined DBP mixtures in chlorinated (Cl_2) and ozonated/post-chlorinated (O_3/Cl_2) drinking waters, researchers established a number of criteria to evaluate the potential health effects and risks involved with exposures to such treated waters (Simmons et al., 2008). Known as the "4Lab Study" because it involved scientists from four branches within the U.S. Environmental Protection Agency, the source water used in different batches in this study was spiked with mean concentrations of 115 μg/L of bromide and 15 μg/L of iodide to elevate the levels of these ions (Miltner et al., 2008). Once spiked, these waters were either chlorinated or ozonated and post-chlorinated. After treatment, the finished waters were analyzed for a number of DBPs as well as TOX, and a calculation for the incorporation of bromide into these DBPs was undertaken as shown in Table 4.1 where the final bromide concentration in both of the

finished waters is shown in the bottom row.

Table 4.1 Bromine incorporation into DBP in the Miltner et al. (2008) study.

Parameter	Cl$_2$ finished		O$_3$/Cl$_2$ finished	
	μg/L	Percent	μg/L	Percent
Accounted-for Br⁻ in THM4 + HAA9	55.3	41.6	44.6	35.4
Accounted-for Br⁻ in other measured DBPs	12.0	9.0	6.4	5.1
Total accounted-for Br⁻	67.3	50.6	51.0	40.5
Unaccounted-for Br⁻	65.8	49.4	75.0	59.5
[Bromide] (Br⁻)	133.1	100	126	100

For the Cl$_2$ finished water, Miltner et al. (2008) calculated this concentration by subtracting the concentration of bromide lost during chlorination from the initial bromide concentration in the raw water (141 μg/L − 7.9 μg/L = 133.1 μg/L). For the O$_3$/Cl$_2$ finished water, final bromide concentration was calculated by subtracting the bromide equivalent of bromate and the concentration of bromide lost during treatment from 141 μg/L (141 μg/L − 6.1 as μg Br⁻/L − 8.9 μg/L = 126 μg/L). Since bromide was lost during both treatment schemes, should the "lost bromide" not be factored into the "accounted-for" bromide content? This is assuming that the "lost bromide" reacted with the disinfectant to produce bromine-containing DBPs. The CCWD results only included organobromine concentrations from THM4 and HAA9, so to compare percentage of unknown TOBr from the CCWD data to the percentage of unaccounted-for bromide in the 4Lab Study, the percentage of accounted-for bromine in other measured DBPs should not be included in the percentage total accounted-for bromine. Doing so changes the percentages of unaccounted-for bromide to be 58.4% and 64.6% for Cl$_2$ finished and O$_3$/Cl$_2$ finished waters, respectively. There is CCWD data for DBPs other than THM4 and HAA9, but that information was not available at the time this report was written.

The CCWD data in Table 3.1 reports percentages of unknown TOBr for chloraminated

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waters that have been treated with ozone or UV irradiation (Phases 1A & 1B) and with ozone/peroxides or UV irradiation (Phases 2A & 2B). These values fall in the same range as the percentages of unknown TOBr in the Miltner et al. study (2008), or occur in the 70% - 80% range; although, that study did not look at waters that were chloraminated. Because the percentages of unknown TOBr for the CCWD chlorinated samples could not be determined, comparisons between these findings and the Miltner et al. data could not be made.

TOX values from the 4Lab Study were 389 µg Cl/L in Cl_2 finished water and 275 µg Cl/L in the O_3/Cl_2 finished water. These samples were quantified for TOX using a titration of a silver halide, the same method that was used for measuring TOX in the CCWD samples, so the values include presence of chloride, bromide, and iodide. TOX values of the waters in the 4Lab Study indicate that TOX decreases when the waters were ozonated and post-chlorinated, a trend observed in the CCWD study when comparing the TOX results for chlorinated samples in Phases 1A, 1B, 2A, and 2B (which were either ozonated or ozonated and treated with peroxides) with Phase 3A Pre-NF. This trend is also observed in the CCWD data for IC measured TOCl + TOBr. The 4Lab Study reported percentages of accounted-for TOX from THM4 and HAA9 to be 36.1% and 30.4% in the Cl_2 and O_3/Cl_2 finished waters, respectively, values that are not similar to the results from the CCWD study as these values were consistently above 45% for the chlorinated samples. There are sufficiently different aspects of the studies, such as the concentration of bromide in the CCWD study, which can explain the difference.

4. B. Toxicity of Mixtures

It is widely known that, in general, the genotoxicity of DBPs increases from chlorine through bromine to iodine substitution (Richardson et al., 2007). There have been many studies

evaluating the carcinogenicity, mutagenicity, genotoxicity, or cytotoxicty of DBPs; however, they have mostly only focused on the effects of individual DBP compounds (Plewa et al. 2002), and in synthetic solutions, and rarely look at mixtures of DBPs (Simmons et al., 2002), let alone "real" water. Many researchers have shied away from approaching drinking water DBPs as mixtures since over 600 DBPs may exist under specific conditions, but only a small number of them have been positively identified and quantified (Krasner et al., 2006). There are, therefore, many challenges to overcome before determining which types of disinfection contribute the most toxicity to a drinking water and perhaps such toxicity is a result of DBPs that have yet to be identified. Additionally, drinking waters ready for distribution to consumers contain concentrations of DBPs at μg/L or even ng/L concentrations, and the majority of toxicity assays are not sensitive enough at these low concentrations. Thus, the treated drinking water cannot be used "as is," but instead it must be concentrated using various techniques so that the concentrations of DBPs are at a much higher level. Despite these difficulties, treating drinking water as a mixture of DBPs is still extremely important because doing so captures the "real world" contributions to human exposure.

One study involving mixtures of DBPs in real waters using the Ames mutagenicity assay compared the nonvolatile acidic organic extracts (following XAD resin extraction and subsequent elution in ethyl acetate) from two different drinking water sources that were treated with chlorine, chloramines, ozone, and ozone followed by either chlorine or chloramines (DeMarini et al., 1995). The overall results from this study were that the extracts from the chlorinated drinking water (Cl_2) produced the most revertants per liter-equivalent, ozonation plus post-chlorination (O_3/Cl_2) produced the second most, then chloramination (NH_2Cl), ozonation plus post-chloramination (O_3/NH_2Cl) was the second lowest, while ozonation (O_3) alone

produced the fewest revertants. The results from the treated waters were compared to the mutagenicity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(5H)-furanone, also known as MX, a primary mutagen in chlorinated drinking water, since MX accounts for about 60% of the mutagenic activity of some chlorinated drinking water samples and is a very potent bacterial mutagen (Meier et al., 1987).

From a chemist's standpoint, there are several questions regarding the methods used in that study which remain unanswered. As stated earlier, the water used in this study came from two different sources. The waters were treated at their respective treatment facilities and collected after their particular treatment scheme. The residual disinfectant concentrations were reported at the time the water samples were concentrated on XAD resins. For the Cl_2, NH_2Cl, O_3/Cl_2, and O_3/NH_2Cl samples, the residuals were 1.8 mg/L Cl_2, 2.4 mg/L Cl_2, 1.1 mg/L Cl_2, and 2.4 mg/L as Cl_2, respectively. The purpose of a maintaining a residual in the treated drinking water is to control and prevent bacterial and protozoan regrowth. The researchers did not report adding a chemical such as ascorbic acid to quench or remove the chlorine residual in the water prior to sample preparation and subsequent exposure to the *Salmonella* strains. When the waters were passed over the XAD resins, the chlorine residuals could have reacted with the resin beads to form a potentially mutagenic compound that was eluted into the organic layer, or the chlorine residual may have become attached to the resin beads, providing an area where the chlorine could further react with NOM in the remaining water passing through the XAD column thus forming more DBPs. How certain is it that the mutagenicity results were caused by the actual DBPs formed in the water samples rather than by those other processes? Another question with this method is how many of the DBPs were retained through the XAD concentration process? TOX was not measured before or after the water samples were passed through the resins, so it is

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uncertain whether or not the DBPs in the ethyl acetate represents 100% capture of all those in the original samples or whether any fraction may have remained in the aqueous phase. Evaporation of the ethyl acetate to further concentrate the DBPs could have led to loss of the volatile components.

In the 4Lab Study, researchers concentrated the finished drinking waters (Cl_2 and O_3/Cl_2 treated) using reverse osmosis (RO) membranes to prepare the waters for exposure assays (Speth et al., 2008). Prior to RO concentration, the waters were passed through ion-exchange resins to remove hardness and free chlorine. The questions concerning quenching the residual free chlorine in the water samples that were raised from the DeMarini et al. (1995) study are also relevant here. Although the 4Lab Study attempted to remove free chlorine and water hardness (from calcium carbonate) using ion-exchange resins, they reported that the waters that were passed through ion-exchange resins still contained free chlorine.

After RO-concentrating the Cl_2 finished waters 136-fold and the O_3/Cl_2 finished waters 124-fold, the waters were analyzed for percentage recovery of DBPs. Percentage recoveries for TOX were 68% for the Cl_2 finished waters and 62% for the O_3/Cl_2 finished waters. Percentage recoveries for low-molecular weight DBPs, i.e. THMs, were low, likely due to the volatile nature of these compounds. In order to maintain the DBP content of the original, non-RO-concentrated waters (1x) prior to toxicity and evaluation, the DBPs lost after RO concentration were spiked-back into the water concentrates. These "spiked-back" DBPs include $CHCl_3$, $BrCl_2CH$, Br_2ClCH, Cl_2AA, $BrClAA$, Br_3AA, dichloroacetonitrile, and bromochloroacetonitrile. However, this attempt was unsuccessful in replacing all of the lost DBPs because after analysis for THMs and HAAs, the recoveries, for $CHCl_3$ and $BrCl_2CH$ were calculated to fall between 51% and 59% for both types of concentrated waters. DBP recoveries were determined by comparing their

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concentrations in the original waters (1x) with the RO-concentrated waters' 1x equivalent concentrations. The issues with the process of spiking-back the lost DBPs is that the researchers were only spiking-back the DBPs that were identified. As reported in the Krasner et al. study (2006) there is a significant percentage of unknown TOX in treated drinking waters and it is unknown which of these DBPs might have been lost.

Through the data presented in this report and the data published in previous studies, it is clear that measuring TOX, in addition to measuring individual DBP concentrations, is an essential component to water characterization when dealing with water as a matrix containing a mixture of DBPs. Since a TOX measurement is a sum of all of the organic halides in the water, it includes concentrations from all halogenated DBPs that have been identified and from those that have yet to be identified. Between 30% and 60% of halogenated DBPs were accounted for in the TOX of water samples from a 1985 study carried out by the U.S. EPA, which measured chlorinated DBPs at 10 water utilities (Stevens et al., 1990). If these results are extrapolated to treated drinking waters that are typically distributed in the United States, then between 40% and 70% of halogenated compounds are not accounted for in the TOX content. The results from the CCWD study in Table 3.1 support this hypothesis as the percentage of unknown TOX was also generally found fall within and above this range, between 40% and 80%, in samples that were ozonated, treated with/without UV irradiance, and chloraminated. The Krasner et al. (2006) study also found that around 70% of TOX remains unknown, a value determined from the ~30% of TOX that was accounted for on a median basis.

In previous studies examining the mutagenicity, cytotoxicity, or genotoxicity of drinking water DBPs, only individual compounds of DBPs, were used. While it is beneficial to understand the individual exposure effects of these DBPs, the doses that were used in those studies in

synthetic laboratory waters were at much higher than the concentrations that are found in real drinking waters. Moreover, the studies also leave a particular question unanswered: since such a high proportion of TOX in treated drinking water has not yet been identified (Krasner et al., 2006), how much of the exposure effects can be attributed to the identified DBPs and how much should be attributed to the unidentified ones? Because of the uncertainties surrounding this question, it is very important to perform exposure assays with real waters that contain mixtures of DBPs.

4. C. GIA Experiment Set 1

This technical report describes attempts to examine the growth inhibitive effects of an NOM concentrate of surface water, which has been disinfected. In this discussion, the results from these GIA experiments are compared with the toxicological approach used by Plewa et al. (2002) as well as results obtained from assays of individual DBPs by Dr. Anthony DeAngelo, a research toxicologist at the U.S. EPA in Research Triangle Park, NC who modified the Plewa method to use normal human colon cells (NCM 460) instead of Chinese hamster ovary cells (CHO). The DeAngelo method is the one selected for use in this technical report and has been named the growth inhibition assay (GIA).

In their study, Plewa et al. (2002) exposed six HAAs, MX, potassium bromate, and ethylmethanesulfonate (EMS, positive control) to CHO cells at varying concentrations. For the purposes of this technical report, only the HAA data from the study will be considered. Table 4.2 lists the six HAAs with their respective concentration ranges for exposure to the CHO cells. Included in the table are IC50 values that represent the concentrations of each HAA at which 50% of the CHO cell density was reduced.

Table 4.2 Concentration ranges of HAAs used in Plewa et al. (2002) and IC50 values.

HAA	Abbreviation	Concentration range (M)	IC50 (M)	CHO cytotoxicity rank order
Bromoacetic acid	BrAA	$1\times10^{-6} - 5\times10^{-5}$	8.9×10^{-6}	1
Dibromoacetic acid	Br$_2$AA	$1\times10^{-6} - 1.25\times10^{-3}$	5.0×10^{-4}	3
Tribromoacetic acid	Br$_3$AA	$1\times10^{-6} - 0.01$	1.0×10^{-3}	4
Chloroacetic acid	ClAA	$1.0\times10^{-4} - 0.01$	9.44×10^{-4}	2
Dichloroacetic acid	Cl$_2$AA	$5\times10^{-4} - 0.043$	0.0115	6
Trichloroacetic acid	Cl$_3$AA	$1.0\times10^{-3} - 0.043$	0.0175	5

As seen from this data, the order of HAAs from most to least cytotoxic is as follows: BrAA > ClAA > Br$_2$AA > Br$_3$AA > Cl$_3$AA > Cl$_2$AA.

Table 4.3 provides the IC50 values for each of the THM4 and HAA9 compounds obtained in DeAngelo's experiments using the GIA (DeAngelo, personal communication).

Table 4.3 DeAngelo's IC50 values for THM4 and HAA9 exposure to colon cells using the GIA.

	Compound	Abbreviation	Concentration range (M)	IC50 (M)	GIA rank order	Plewa et al. rank order (2002)
THM4	Chloroform	CHCl$_3$	$1.00\times10^{-9} -$ 3.00×10^{-3}	ND		
	Bromodichloromethane	BrCl$_2$CH		ND		
	Dibromochloromethane	Br$_2$ClCH		~3.00×10^{-3}		
	Bromoform	CHBr$_3$		~3.00×10^{-3}		
HAA9	Chloroacetic acid	ClAA	$1\times10^{-6} - 1\times10^{-3}$	2.87×10^{-4}	5	2
	Bromoacetic acid	BrAA	$1\times10^{-6} - 1\times10^{-4}$	1.48×10^{-5}	1	1
	Dichloroacetic acid	Cl$_2$AA	$1\times10^{-6} - 0.5\times10^{-3}$	3.41×10^{-3}	6	6
	Bromochloroacetic acid	BrClAA		5.00×10^{-4}	2	NA
	Trichloroacetic acid	Cl$_3$AA		4.17×10^{-3}	4	5
	Dibromoacetic acid	Br$_2$AA		4.70×10^{-4}	3	3
	Bromodichloroacetic acid	BrCl$_2$AA		2.03×10^{-3}	9	NA
	Dibromochloroacetic acid	Br$_2$ClAA		2.18×10^{-3}	8	NA
	Tribromoacetic acid	Br$_3$AA		2.37×10^{-3}	7	4

"NA" indicates that this HAA was not tested.
ND = Not able to be detected.

A rank order for THM4 could not be determined because two of the species (CHCl$_3$ and BrCl$_2$CH) showed no inhibitory effect even at the highest concentration tested (3.00×10^{-3} M).

The THM4 data in Table 4.3 indicate that this group of DBPs is not particularly growth inhibitive towards the colon cells. The rank order for HAA9 from most to least growth inhibitive is as follows: BrAA > BrClAA > Br_2AA > Cl_3AA > ClAA > Cl_2AA > Br_3AA > Br_2ClAA > $BrCl_2AA$. Comparing the Plewa et al. (2002) and DeAngelo assays (Table 4.3), the rank order for the potency of the HAAs are similar for both methods, although the Plewa et al. (2002) study did not test BrClAA, $BrCl_2AA$, and Br_2ClAA.

As seen in the Results chapter, the GIA results for laboratory generated chlorinated NOM samples shown in Figure 3.1 - Figure 3.5 indicate that these samples were relatively non-growth inhibitive on the colon cells. The HAA9 extraction results for these samples (Table 3.10) show that in spite of the high disinfectant dose and TOC concentration, HAAs ranged from 10^{-9} M to 10^{-5} M, which, according to the IC50 results reported in Table 4.2 were not potent enough to inhibit cell growth by 50% or more. This suggests that both growth inhibition methods are not sensitive enough to detect geno- and cytotoxicity in drinking waters concentrated by a factor of 10.

The 4Lab Study also looked at the toxicity of mixtures of DBPs in real waters; however, their approach was different than the one described in this report. As mentioned earlier, the waters collected in this study were finished drinking waters concentrated 136-fold (Cl_2) or 124-fold (O_3/Cl_2) by RO, chemically analyzed for DBPs, and used in *in vivo* and *in vitro* toxicology assays to determine their exposure effects (Simmons et al., 2008). The *in vivo* assay examined the exposure effects of the RO-concentrated waters (Cl_2 and O_3/Cl_2 finished) on rats (Narotsky et al., 2008). Crosby et al. (2008) examined the biological effects of these concentrates on cultured rat hepatocytes using an *in vitro* gene expression assay, and like the DeMarini et al. (1995) study, Claxton et al. (2008) used the Ames assay as the other *in vitro* assay. In addition to RO

concentration, the waters in that study were also concentrated using XAD resins, eluted with ethyl acetate, concentrated using rotary vacuum evaporation, and dissolved in DMSO to give 8000-fold concentrates (Claxton et al., 2008). An advantage to this approach is that the waters were concentrated roughly 100-fold by RO, 10 times greater than the RO concentration technique used in this report, and 8000-fold using XAD resins. Achieving such high water concentrates and high levels of DBPs through these methods is extremely beneficial when using assays that are not sensitive at lower DBP concentrations: the gene expression assay (Crosby et al., 2008), further discussed later in this chapter, and the GIA. Another advantage to this multi-level approach is that researchers were able to evaluate the toxicity of these RO concentrates on live rats, results that can be used as a model to evaluate human health impacts (Narotsky et al., 2008).

However, some disadvantages from this approach are that the waters were disinfected prior to RO and XAD concentration, residual disinfectant was not completely removed with a quenching agent, and that lost DBPs (from RO concentration) were spiked back to match the proportion of DBPs in the original finished waters. Disinfection before RO/XAD concentration causes loss of volatile DBPs. Failure to completely quench residual disinfectant may result in the residual disinfectant reacting with the XAD resin beads to produce potentially toxic/mutagenic compounds and formation of additional DBPs from attachment of the residual to the resin beads and subsequent reaction with NOM during XAD concentration, both events could create false positive mutagenic results in using the Ames assay. The disadvantage of spiking back the lost DBPs was explained earlier.

The advantages to the approach used in this report are that the waters used in this study were RO-concentrated prior to disinfection and that the residual disinfectant was quenched using

ascorbic acid. Lost DBPs were not spiked back into the waters since they were disinfected post-RO concentration and the only ways the DBPs could be lost were through exposure of the water samples to the air and transfer of the samples during the GIA evaluation process. Disadvantages, however, were that the waters could only be concentrated 10-fold by RO and that the waters were only analyzed for HAA9.

When comparing these approaches, it is evident that the 4Lab Study approach came primarily from the standpoint of toxicology researchers, while the approach described in this report came from the point of view of chemists. Multi-level approaches to evaluating the toxicity of DBP mixtures are still relevant to improving our understanding of exposure effects of finished drinking water though these approaches are complex. For future work, raw waters should definitely be concentrated prior to disinfection. The concentration method could be by RO; however, the concentration factor should certainly be greater than 10. Perhaps XAD resins could also be used to concentrate the raw waters: since disinfection has not yet taken place, there would not be the interferences that were described with waters that had already undergone disinfection. Post-disinfection, the residual disinfectant must be quenched to ensure that the toxicity of the waters is only from DBPs and not from the residual. The same assays (from the 4Lab Study and from this study) could be used depending on the objective and limitations of each study. For example, if one wanted to determine a lifetime toxicity exposure, one could choose to use an *in vivo* assay with rats. If one wanted a quicker and less expensive method to evaluate exposure effects that are more relatable to humans, then the GIA should be used. Additionally, the water samples should be chemically analyzed for THM4, HAA9, halogenated volatiles, TOX, and TOCl/TOBr. This way, the waters can be characterized according to their levels of DBPs and the TOX content (accounted for and unaccounted for) can perhaps be used as

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an indicator for evaluating the water's toxicity.

4. D. GIA Experiment Set 2

Another approach to looking at mixtures involved spiking BrAA and IAA into the same matrix separately (Figure 3.12 & Figure 3.13) and together (Figure 3.15 & Figure 3.16) and evaluating these samples with the GIA to determine whether or not the combination of the two compounds would result in a synergistic or antagonistic growth inhibitive effect on the colon cells. This approach was used because in the GIA Experiment Set 1, IC50 values could not be obtained with those samples even though the waters had a significant dose of disinfectant, indicating that the assay is not sensitive enough at the levels of HAAs that were present in the samples.

There is still insufficient information regarding the effect of DBP mixtures on toxicity assays or on the GIA; however, as stated on page 109 of this report, the expected IC50 value for the BrAA and IAA mixture was 1.65×10^{-6} M, while the actual IC50 value of the mixture was 6×10^{-7} M. Though this difference is only one order of magnitude, it is a clear indication that the mixture had a synergistic effect on cell growth (Figure 3.16), thus indicating that current methods of determining toxicity of DBPs do not represent the true effects of a matrix containing mixtures. Although the samples in GIA Experimental Set 1 that contained a mixture of all nine of these HAAs were not potent enough to produce IC50 values, from a TOC concentration of about 29 mg C/L and onwards, growth inhibition of the cells began to become visible. The individual concentrations of the HAA9 were about two to three orders of magnitude lower than the individual IC50 values that were determined by DeAngelo; however, it is possible that the combination of these HAAs, at a high enough TOC content in the water matrix, started to cause

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the increase in growth inhibition that is seen towards the end of the GIA curves (Figure 3.1 - Figure 3.5).

The necessity for the water samples to have a high concentration of TOC prior to their usage in toxicity assays is represented in the Crosby et al. (2008) study that was mentioned earlier. This study was a component of the 4Lab Study and it examined the gene expression changes in rat liver cells that were exposed to the mixtures of DBPs that formed as a result of concentrating the Cl_2 and O_3/Cl_2 finished waters (Crosby et al., 2008). Researchers determined cytotoxicity by measuring the amount of lactic acid dehydrogenase (LDH) that was released into the medium after exposure to 1:10 and 1:20 dilutions of the Cl_2 concentrate sample, 1:10 dilution of the O_3/Cl_2 sample, and full-strength samples for both types of finished water concentrates. What they noticed was that the full-strength concentrates for Cl_2 and O_3/Cl_2 finished waters were equally cytotoxic when comparing the LDH activity release results of the samples to a control. The dilutions of these concentrates exhibited LDH activities that were 82%, 90%, and 76% of the control value for the 1:10 Cl_2, 1:20 Cl_2 and 1:10 O_3/Cl_2 samples, respectively; thus, indicating that these dilutions were not cytotoxic. The same concerns with regard to the chlorine residual in these water samples arise: how is it certain that the cytotoxicity is not caused by the residual, but only by the mixture of DBPs that are in the concentrates? What would the cytotoxicity of the samples be if the residual chlorine had been quenched? Also, how much do the by-products of quenching contribute to toxicity?

The results from GIA Experiment Set 1 suggest that if the samples contained a higher TOC concentration, more growth inhibition could have been seen. So, although the Crosby et al. (2008) study did not completely remove all of the residual chlorine, it also indicated that a higher concentration of TOC is necessary to see the cytotoxic effects of the samples.

4. E. Toxicity Studies Using Chloraminated Drinking Water

In chapter 3, the results from the effects of quenched chlorine and chloramine by-products on the colon cells in the GIA were documented. Compared with the samples containing a pre-quenched Cl_2/ascorbic acid solution (% cell densities at around 180%), the samples containing the pre-quenched NH_2Cl/ascorbic acid solution were found to have growth inhibitive effects on the cells due to the presence of a toxic by-product from the quenching reaction (% cell densities at around 60%). For this reason, none of the GIA experiments were conducted with waters treated with chloramines. With this new finding, a potential impact on past toxicity studies using chloramine as a drinking water disinfectant is that the results may have false positive biases because, if the residual disinfectant had been quenched with ascorbic acid, then the assays were affected by the toxic by-product from quenching, not from the DBPs. Because real drinking waters have a disinfectant residual to prevent bacterial/protozoan regrowth, this residual must be quenched so that it does not react with other factors in the assays. Thus, toxicity in chloraminated RO water concentrates due to the presence of DBPs alone cannot be studied until there is a quenching agent that can be used that does not produce a toxic by-product when reacted with chloramines.

5. Conclusions

Disinfected drinking water contains a mixture of DBPs, which include THM4, HAA9, and more aggregate measures such as TOX, and TOCl/TOBr/TOI, which indicate the proportions of accounted-for and unaccounted-for organic halogens in the water. The latter can be useful indicators of relative toxicity of water, which together with bioassays such as the GIA discussed in this report can provide valuable information on how to characterize DBP mixtures and evaluate their inherent toxicities. The data presented in this technical report indicate that measuring TOX in addition to the concentrations of other DBPs is an integral component to the evaluation of the overall toxicity of treated drinking waters. The findings from these studies are summarized below.

- In CCWD waters that were spiked with bromide, treated with or without UV irradiance and post-chloraminated, TOBr concentrations remained the same regardless of UV lamp type (low pressure/medium pressure). TOBr concentrations were greater than that of TOCl even though TOX concentrations were generally unchanged (~200 as µg Cl/L). The post-chlorinated counterparts also had TOBr concentrations greater than that of TOCl, with TOX concentrations remaining ~300 µg as Cl/L. All of the post-chloraminated waters had greater percentages of unknown TOBr than unknown TOCl.

- In CCWD waters that were spiked with bromide, treated with ozone, treated with or without UV irradiance, and then post-chlorinated or –chloraminated, TOX concentrations generally occurred around 200 - 300 µg as Cl/L, while TOBr concentrations were consistently greater than those of TOCl. UV irradiance on post-chlorinated and post-chloraminated waters did not significantly lower TOX, TOCl, or TOBr concentrations,

but was generally found to elevate those concentrations. In all of the post-chloraminated waters, the percentage of unknown TOBr was greater than that of unknown TOCl.

- In CCWD waters spiked with bromide, treated with ozone and peroxide, treated with or without UV irradiance, and then post-chlorinated or –chloraminated, TOX concentrations were between ~250 and ~300 µg as Cl/L, while TOBr concentrations were consistently higher than that of TOCl. Waters treated with medium pressure UV lamp at 100 mJ/cm^2 and post-chloraminated had greater TOBr and TOCl concentrations than those that were a) not UV treated and post-chloraminated, b) treated with low pressure UV lamp at 400 mJ/cm^2 and post-chloraminated, and c) treated with medium pressure UV lamp at 400 mJ/cm^2 and post-chloraminated. For all of the post-chloraminated waters, the percentage of unknown TOBr was also higher than that of unknown TOCl.

- Ozonation and ozone with H$_2$O$_2$ (advanced oxidation process, or AOP) treatments provide favorable conditions for forming elevated levels of bromine-containing THMs and HAAs.

- The percentage of unknown TOX in the post-disinfected pilot plant waters was found to fall between 40% and 80%, which is in accord with previous studies.

- It is known that bromine-containing DBPs are more geno- and cytotoxic than their chlorine-containing counterparts. The CCWD treated waters with a high proportion of unknown bromine-containing DBPs would be of potential human health concern. This suggests that UV, ozone, and AOP treatment of bromide-containing waters may not be the optimum combination of processes prior to chlorination or chloramination of such waters.

- RO-concentrated waters were evaluated in two modes: 1) they were spiked with bromide or iodide and then treated with chlorine and 2) a pre-formed bromide/iodide with Cl_2 solution was added to the RO water. In both cases, the treated waters contained individual HAA9 species at concentrations in the range 10^{-8} M – 10^{-5} M. Concentrations of BrAA were in the 3×10^{-8} M - 5×10^{-7} M (<4 µg/L – 72 µg/L) range, which is not high enough to generate a confirmed response in the GIA toxicity measurement. A 10^{-6} M concentration would have been more effective. Nevertheless, there is evidence of cytotoxicity in these waters compared to those where no bromide or iodide was added.

- To effectively determine the toxicity of DBP mixtures in real waters using the GIA, the TOC concentration of the water should be 10 to 100 times greater than the TOC (65 mg C/L) of the RO concentrated water that was used in this study in order to observe a confirmed toxicity response to DBPs generated under disinfection of this water.

- The residual disinfectant in chlorinated water samples must be quenched with ascorbic acid prior to their use in the GIA. Failure to do so can cause false positive responses in the toxicity assay.

- Chloraminated water samples quenched with ascorbic acid cannot be used in the GIA. Doing so creates toxic by-products that cause false positive responses in the assay. No other quenching agent has yet been identified that does not change the speciation and quantities of DBPs present while not affecting the GIA cells.

- The combination of BrAA and IAA in the same matrix creates a synergistic exposure effect in the GIA compared to the effects of the individual species.

These findings indicate that in order to gain a better understanding about characterizing DBP mixtures and evaluating their toxicity in real drinking waters, it is essential to combine

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chemical and sensitive toxicological analytical methods.

6. Recommendations for Future Work

The methods and results from this preliminary study can be taken further for future work with DBP mixtures in treated drinking waters. The following suggestions should be considered.

- The RO concentrated waters should possess a TOC concentration 10 to 100 times greater than 65 mg C/L in order to obtain IC50 values using the GIA.

- Simultaneously analyze the same disinfected water concentrates for THM4, HAA9, halogenated volatiles, TOX, and TOCl/TOBr when using the GIA. This would allow for a better understanding of how TOX can be used as a surrogate indicator for toxicity. Additionally, one could compare the unknown TOBr fraction of one sample to the sample's toxicity from the GIA to see whether or not the unknown TOBr correlates more closely to toxicity than TOX.

- More accurate dose-response curves should be determined, by expanding dilutions of the sample at concentrations closer to (above and below) the projected midpoint value on the curve for that sample.

- Evaluate an approach for removing the disinfectant residual from chloraminated waters without creating toxic by-products, to enable the growth inhibitive effects of chloraminated waters to be compared to those of their chlorinated counterparts.

- A prediction of the dose-response curves concentrations for mixtures of DBPs using computer algorithims would assist in the process of evaluating drinking water toxicity.

Though the toxicity of mixtures of DBPs are difficult to evaluate experimentally, it is mixtures that are present in disinfected drinking waters. While previous studies evaluating the

toxicity of individual drinking water DBPs were helpful, they do not necessarily provide accurate health/exposure endpoints for drinking waters. This research provides some approaches to future risk assessment using a combination of chemical and biological assays that can be used in real drinking waters. The focus of studying health/exposure endpoints should instead be on DBP mixtures because it is a mixture of DBPs in tap water to which consumers are exposed on a daily basis.

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8. Appendix

University of North Carolina

Quality assurance manuals are available at UNC, which include detailed information on methods and procedures, and can be furnished upon request. Abbreviated SOPs for those methods already in place are included here.

8. A. Trihalomethane (THM) Standard Operating Procedure (SOP)

Equipment

- Clear 40-mL, clean, prewashed glass screw cap sample vials with polytetrafluoroethylene (PTFE)-lined silicone septa. Clean vials by washing with Alconox powder detergent solution, rinsing with tap water, and soaking in a 10% ACS-grade HNO_3 bath overnight. The vials should then be rinsed at least three times with tap water and then rinsed three times with laboratory grade water (LGW) and dried in a 180°C oven for at least 24 hours. Repeat the same steps for cleaning the caps and septa except oven temperature should be set at 80°C.
- Gas tight syringes: 25 µL, 50 µL, 100 µL, 500 µL
- 50-250 µL Dade Model J micropipetter fitted with clean glass capillary tips
- Eight 100-mL glass volumetric flasks with glass stoppers
- 10-mL glass beaker
- Two 25 mL glass graduated measuring cylinders.
- 1-L amber bottle mounted with 10-mL pump pipetting dispenser containing PFTE transfer line
- 5-mL amber glass storage vials with crew caps and PFTE-faced silicone septa
- 23-cm disposable glass Pasteur pipettes
- Rubber Pasteur pipette bulb
- 12x32 1.8-mL clear glass gas chromatography autosampler vials with rubber/TFE aluminum seals (Laboratory Supply Distributors, Corp. (catalog #20811-1232)
- Hand crimper for sealing gas chromatography autosampler vials
- vortexer
- Teflon tape
- Stainless steel scupula

Instrumentation

Gas Chromatograph
- Hewlett-Packard GC5890 Series A with autosampler/autoinjector tower
- Capillary Column: HP-1 (Agilent) 30 m length x 0.25 mm inner diameter, 1.0-µm film thickness
- Electron Capture Detector (ECD): Hewlett-Packard Model ECD
- Data System: Hewlett-Packard ChemStation

GC Gases
- Carrier Gas-Ultra High Purity (UHP) helium (He) available through UNC Scientific Storerooms (catalog # SG62350)
- Makeup Gas-Ultra High Purity (UHP) nitrogen (N_2) available through UNC Scientific Storerooms (catalog # SG62750)

GC Supplies
Septa- (Restek, Bellafonte, PA) 11-mm diameter Thermolite Septa (catalog #20365)
- Injector Liner Sleeves- (Supelco, Bellafonte, PA) Split/Splitless Injector Sleeve with deactivated glass wool, 4 mm inner diameter (catalog #20486,05)
- Column Ferrules- (J&W) graphite/vespel 0.5 mm ferrules (catalog #5002025)
- Autosampler Syringes- 10 µL Agilent tapered needle syringe

Reagents

- Laboratory Grade Water (LGW)
- Extraction solvent: Sigma-Aldrich methyl *tert*-butyl ether, 99+%, ACS reagent (catalog #44,380-8)
- Sodium sulfate (Na_2SO_4), (Mallinckrodt, Paris, KY) granular, ACS grade (catalog #8024) from Scientific Storeroom. Bake at 400°C in muffle furnace for 24 hours in a shallow, porcelain dish covered with aluminum foil. Store in glass-stoppered bottle.
- Solvent for dilution of standards: Burdick & Jackson (Muskegan, MI) B&JBrandTM High Purity Methanol for Trihalomethane Analysis (catalog #231235)
- Methanol (for rinsing glassware) HPLC grade
- Ammonium Sulfate ($NH_4)_2SO_4$ (Mallinckrodt, Paris, KY) granular, AR grade, from Scientific Storeroom (catalog #3512)
- Phosphate buffer: Prepare a dry, homogenous mixture of 1% Sodium Phosphate, disbasic (Na_2HPO_4)/99% Potassium Phosphate, monobasic (KH_2PO_4) by weight. Both of these buffer salts should be in granular form and of ACS grade or better.
- THM Calibration Mix, 2000 µg/mL each in methanol. Two sources required (Supelco, Bellafonte, PA) and AccuStandard.
- Internal Standard (IS): Aldrich (Milwaukee, WI) 1,2-dibromopropane neat standard, 99+% (catalog #14,096-1)

Samples
Samples should be collected headspace-free in pre-cleaned 40 mL glass vials with screw caps and PTFE-lined silicone septa containing ~20 mg (8 grains) ammonium sulfate, and 0.7 g phosphate buffer. Samples should be filled head-space free and holding vial at an angle so THM's do not escape through volatilization. Store samples in fridge at 4°C.

Procedure
Internal Standard
Stock solution of Internal Standard (IS) at ~2000 µg/mL in MtBE - prepared by injecting 10µL of the neat standard and injecting into a 5mL-volumetric flask containing 5mL of MtBE

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Primary dilution at 100µg/mL: prepared by injecting 250µL of I.S. stock solution using a micropipette, into a 5mL-volumetric flask containing 5mL of MtBE.

Extracting solution at 50µg/L: prepared by injecting 250µL of I.S. primary dilution using a micropipette, into a 500mL-volumetric flask containing 500mL of MtBE. (Prepare more than needed because there may be bubbles in the dispenser that you need to clear, and will need to pump a few times to start out).

THM Calibration Standards

These are simultaneously prepared from the two suppliers of sock solutions and monitored during each batch of THM analyses.

1. Calibration Standard #1: 100 µg/mL, 100 µL of stock calibration mix to 2 mL high purity methanol.

2. Calibration Standard #2: 20 µg/mL, 20 µL of stock calibration mix to 2 mL high purity methanol.

3. Transfer standards to a 5-mL amber glass vial and store in laboratory standards freezer at -15°C.

4. Check calibration standards a few days before extraction. Standards should be monitored for degradation and contamination by comparing standard chromatographic peak area values obtained on the performance evaluated designated GC to those obtained during initial calibration of standard. The responses obtained on the same instrument are normalized relative to the freshly prepared internal standard to account fpor instrument detector drift and the values for each THM stored on a spreadsheet on the GC computer and backed-up to the external hard drive. New standards should be made from the stock solution if check exceeds 20% drift. If the drift persists, purchase new stock solutions from two suppliers and compare the responses making a note of the stock batch number. The standards assessed are as follows:
   a. 5µl calibration standard #1 into 10 mL extracting solvent (MtBE containing IS) = 50 µg/L each THM
   b. 25µl calibration standard #2 into 2 mL extracting solvent (MtBE containing IS) = 2.5 µg/L each THM

5. Prepare a laboratory reagent blank (the level 1 calibration standard - see step 6) and the laboratory fortified blank (level 3 calibration standard – see step 6) at the beginning of each day and analyze on the GC before extracting samples. If QC criteria fail, troubleshoot and correct the problem reanalyzing these check standards before proceeding to the next step.

6. Prepare calibration standards in 100 mL LGW according to the range of concentrations expected in the samples. An example is shown below.

Level	Calibration Standard	Volume added (µL)	THM conc. (µg/L)
1	NA	0	0
2	20 µg/mL	12.5	2.5
3	20 µg/mL	25	5
4	20 µg/mL	125	25
5	20 µg/mL	200	40
6	100 µg/mL	50	50
7	100 µg/mL	100	100

7. Prepare matrix spike (MS) and matrix spike duplicate (MSD) in 25mL samples → should be ~2-3 times THM levels in samples.

8. Measure 20 mL from all calibration standards using a 50mL measuring cylinder starting from lowest to highest concentration and then follow with the samples all in duplicate and transfer into 40 mL vials. Rinse cylinder 3 times with LGW and once with sample to be measured next between each. Pour at an angle so THMs are not lost through volatilization.

9. Add 4 mL extracting solvent from a solvent dispenser bottle to each 20 mL aliquot. Make sure there are no bubbles in the dispenser addition line.

10. Add ~6 g pre-baked sodium sulfate to each 20 mL sample/calibration standard. (6 g can be measured out in pre-measured marked 10 mL glass beaker) Vortex samples for 1 minute immediately after adding sodium sulfate to avoid clumping. Let samples settle for 5 minutes.

11. Using a disposable 23-cm glass Pasteur transfer ~1.5 mL from the middle of the MtBE layer (top layer) to a GC autosampler vial. Do not transfer any sodium sulfate crystals as they will clog the GC. Cap and crimp vial. Store in the laboratory freezer at -15°C in a tray covered in aluminum foil if not analyzed immediately. Also fill two autosampler vials with MtBE and 2 vials of extracting solvent. Analyze within 8 weeks.

12. Analyze according to specified GC method on the designated GC. Instructions for GC use for this method are provided by the instrument that is available at the time.

Quality Control
Precision is measured as the average and relative percent difference (RPD) of the duplicate analyses of each sample. RPD should be less than 10% otherwise sample has to be flagged as suspect. The coefficient of variation of all the internal standard responses for the complete set of samples must be less than 15%. Individual samples responsible for elevating this value above the threshold should be flagged and considered suspect.

A calibration check standard is prepared in the mid-range of the standard calibration curve and is injected every 10 samples. If the detector response for this sample varies more than 10% from

the previous injection, all samples analyzed between the two injections are flagged for investigation.

Each sample bottle set is accompanied by replicate field and travel blanks

In cases with unknown or mislabeled samples, we will first attempt to determine what sample identification actually is, based on received samples and shipping list information. If a reasonable idea of the sample is determined, it will be analyzed and those data will be qualified in reports and future data analyses.

8. B. Haloacetic Acid (HAA) Standard Operating Procedure

This entire procedure should be performed under a well-ventilated hood. Hazardous reagents will be used during HAA extraction. Double gloving should be used when handling diazomethane or sodium azide.

Materials
1) Several clear 40mL glass vials with open-top screw caps and Teflon-lined septa
2) 10-50µL micropipetter with clean glass capillary tips
3) 50-250µL micropipetter with clean glass capillary tips
4) Several 23cm disposable glass Pasteur pipettes
5) Rubber Pasteur pipette bulb
6) Volumetric flasks & glass toppers: three 25mL, eight 100mL, several 2mL (number depends on number of samples and calibration standards)
7) Two 25mL glass graduated cylinder
8) 10mL glass beaker
9) 1L glass bottle with 10mL pump pipetting dispenser containing PTFE transfer line
10) 500mL amber bottle mounted with 5mL pump pipetting dispenser containing PTFE transfer line
11) 5mL amber glass standard storage vials with open top screw caps and PTFE-lined septa
12) Several 12x32 1.8mL glass GC autosampler vials with rubber/TFE aluminum seals
13) Hand crimper for sealing GC autosampler vials
14) Thermolyne Type 16700 Mixer-MaxiMix I vortexer
15) 1/2-inch Teflon tape
16) Stainless steel scupula
17) Plastic tub for ice bath

Reagents
1) Laboratory Grade Water (LGW)
2) Extraction solvent & standard solvent: Aldrich, 99%+ Methyl tert-Butyl ether (MtBE)
3) Sodium Sulfate (Na_2SO_4): Mallinckrodt, granular. Bake at 400°C in muffle furnace for 24 hours in a shallow porcelain dish covered with aluminum foil. Store in a tightly capped amber jar or in a glass-stoppered bottle.
4) Sulfuric Acid (H_2SO_4): Fisher, concentrated ACS grade or equivalent, stored in 500mL amber bottle mounted with 2mL pump pipetting dispenser.
5) Drying agent: Aldrich, anhydrous magnesium sulfate ($MgSO_4$), 99+% or equivalent
6) Silicic Acid ($SiO_2 \cdot nH_2O$): JT Baker
7) Methanol for rinsing glassware: (HPLC grade or equivalent)
8) Ammonium Sulfate ($(NH_4)_2SO_4$): Mallinckrodt granular, AR grade.
9) Supelco EPA 552.2 Acids Calibration Mix at ~ 1mg/mL each (BrAA, BrClAA, $BrCl_2AA$, ClAA, Br_2ClAA, Br_2AA, Cl_2AA, Br_3AA, Cl_3AA).
10) Internal Standard Stock Solution: Aldrich; 1,2-dibromopropane neat standard, 99+%.
11) Haloester Standard Stock Solution: Supelco; EPA 552.2 Esters Calibration Mix at 200-2000µg/mL in MtBE.

12) Acid Surrogate Stock Standard: Supelco; 2,3-dibromopropionic acid, 99+% at 1mg/mL in MtBE.

Stock Standards
Stock standards are purchased as premixed certified solutions contained in sealed amber glass ampules. Once the glass 1mL sealed ampule of stock solution is opened, the solution is immediately transferred to a 5mL amber vial with a PTFE-lined screw cap. The vial should be immediately capped with the cap and neck of vial wrapped with Teflon tape. The vial should be stored in the laboratory standards freezer at -15°C.

Standards should be monitored frequently for degradation by comparing chromatographic peak area values to those obtained during the initial calibration of the standard. Fresh standards should be prepared if this check exceeds a 20% drift. Stock standards shouldn't be used more than 6 months after opening of sealed ampule.

Internal Standard stock solution prepared at ~2000µg/mL.
      a. Inject with micropipetter 10µL of Aldrich 1,2-dibromopropane neat standard 99+% into a 5mL volumetric flask containing 5mL of Aldrich 99+% MtBE. Fill to the line with MtBE, cap, and invert 3 times.
      b. Transfer immediately to 5mL amber vial capped with open top screw caps lined with PTFE septa. Seal caps with Teflon tape. Label and store vial in lab freezer at -15°C.

Working Dilutions
Internal Standard:
    (i)   Primary dilution at 100µg/mL: prepared by injecting 250µL of I.S. stock solution using a micropipette, into a 5mL-volumetric flask containing 5mL of MtBE.
    (ii)   Extracting solution at 50µg/L: prepared by injecting 250µL of I.S. primary dilution using a micropipette, into a 500mL-volumetric flask containing 500mL of MtBE. (Prepare more than needed because there may be bubbles in the dispenser that you need to clear, and will need to pump a few times to start out).
The volume of the amount of MtBE and IS Extraction Solution will vary based on the number of samples to be extracted. The volume needed can be calculated using the equation below:

N (number of samples to be extracted) × 4mL + 100mL MtBE

Primary Calibration Standards
    HAA Standard Primary Dilution prepare at 20µg/mL.
      c. In the Supelco EPA 552.2 Acids Calibration Mix, each of the nine HAAs is present at a different concentration. Prepare this solution by tracking the HAA with the lowest concentration.
      d. Fill a 2mL volumetric flask to just below the 2mL mark with MtBE.
      e. With a micropipetter, inject 75µL of the EPA 552.2 Acids Calibration Mix into the volumetric flask. Make sure that it is injected below the MtBE surface.
      f. Fill the flask to the 2mL mark with MtBE. Cap the flask and invert three times.

g. Transfer this standard to a 5mL amber vial with an open top screw cap lined with PTFE silicone septa. Seal cap with Teflon tape. Label and store in lab freezer at -15°C.

*Primary dilutions of HAA working standards should be routinely monitored for significant degradation by comparing standard chromatographic peak area values to those obtained during the initial calibration of the standard. Fresh standards should be prepared if this check exceeds a 20% drift.

h. Transfer this standard solution and store it in 1L amber bottle with PTFE pipetting dispenser screw top assembly.
i. Store in lab refrigerator at 4-5°C.

Multicomponent haloester reference standard prepare at 50µg/L - 500µg/L

Fill a 10mL glass volumetric flask with Aldrich 99+% MtBE to the neck of the flask just under the 10mL mark.

Using a micropipetter, inject 250µL of Haloester Standard Stock Solution (200-2000µg/mL) into MtBE.

Add MtBE to 10mL fill line. Cap flask and invert 3 times.

Transfer this standard to a 20mL amber vial with open top screw cap and PTFE-lined silicon septa. Seal cap with Teflon tape.

Label vial and store in lab freezer at -15°C.

Acid Surrogate Additive Standard prepare at 20µg/mL

Add 100µL of 1mg/mL acid surrogate stock standard to a 5mL volumetric flask containing MtBE filled just under the 5mL mark on the neck of the flask.

Fill to the 5mL mark with MtBE. Cap and invert 3 times.

Transfer this standard to 5mL amber vial with open top screw cap and PTFE-lined silicone septa. Seal cap with Teflon tape.

Label vial and store in lab freezer -15°C.

EPA 552.2 Acids Calibration Mix (HAA9) Matrix Spike Standard (MS)

In the Supelco EPA 552.2 Acids Calibration Mix, each of the nine HAAs is present at a different concentration. Make this standard by tracking the HAA with the least concentration. The following steps are written for tracking ClAA at a concentration of 600µg/mL. The final concentration of this dilution is 6µg/mL of ClAA.*

Add 20µL of the Supelco EPA 552.2 Acids Calibration Mix to a 2mL volumetric flask filled with MtBE. Be sure to inject beneath the MtBE layer.

Fill to the 2mL mark with MtBE. Cap flask and invert 3 times.

Transfer this standard to a 5mL amber vial with an open top screw cap lined with PTFE silicone septa.

Seal cap with Teflon tape. Label and store in lab freezer at -15°C.

*The final concentration of this solution should be around 10-20µg/mL.

Instrumentation

1) Gas Chromatograph (GC)

a. Hewlett-Packard GC5890 Series A or Series II, or equivalent with autosampler/autoinjection tower
b. Capillary Column HP-1 (Agilent) 30m length x 0.25mm inner diameter, 1.0μm film thickness, or equivalent.

2) Electron Capture Detector (ECD)
   a. Hewlett-Packard Model ECD
   b. Data System: Hewlett-Packard ChemStation

3) GC Gases
   a. Carrier Gas: Ultra High Purity (UHP) 99.999+% helium (He)
   b. Make-up Gas: Ultra High Purity (UHP) 99.999+% nitrogen (N_2)

4) Miscellaneous GC Equipment
   a. Septa-Restek 11mm diameter Thermolite Septa
   b. Injector Liner Sleeves Supelco Split/Splitless Injector Sleeve with deactivated glass wool, 4mm inner diameter
   c. Column Ferrules J&W graphite/vespel 0.5mm ferrules
   d. Autosampler Syringes 10μL Agilent tapered needle syringe

Samplevials

Samples should be collected in pre-cleaned 40mL glass vials with open top screw caps and PTFE-lined silicone septa. Prepare vials in the lab before collecting samples by pipetting 50μL of the 80mg/L sodium azide solution directly into the vial and adding approximately 20mg (8 grains) of ammonium sulfate. Cap and label all vials appropriately. Samples should be extracted within 14 days from date of collection.

Test Mixes: Preparation & Procedure

1. In order to test the instrument performance the day before sample analysis, prepare a dilution of internal standard at 50μg/L in MtBE in a 25mL volumetric flask. Analyze the MtBE before preparing the dilution. Transfer about 1mL of this dilution to a small GC vial. Cap, label the vial, and analyze by the appropriate chromatographic method.

2. Analyze an aliquot of the multicomponent haloester standard prepared In the range 100-200 μg/l in the extraction solvent to determine analyte retention times.

3. If these reagents are clean and the GC is functioning properly, extract samples within three weeks of the date the samples were collected.

4. GC data should be removed from the GC computers within one month.

Calibration Standards

1) Prepare all standards in **100mL of LGW**.
2) Label 6 separate 100mL volumetric flasks with the concentrations to be prepared. The lowest concentration should not be below 0.1μg/L.
3) Fill each 100mL volumetric flask with **LGW** to just below the fill line on the neck of the flask.

4) With an appropriate micropipetter that uses glass capillary tips, put a measured amount of the primary calibration standard directly into **LGW** below the surface. The amount of primary calibration standard will vary depending on the desired concentration of the secondary calibration standards.
5) Fill the volumetric flask to the fill line with **LGW**, cap the flask, and invert three times.
6) Two blanks should be prepared by filling two 40mL clear glass vials with **20mL LGW**.
   a. Measure **20mL LGW** with a clean glass 25mL graduated cylinder.
   b. Label, then cap vials using open top screw caps with PTFE-lined septa.
7) Rinse the 25mL graduated cylinder three times with **LGW**.
8) Using the cleaned 25mL graduated cylinder, transfer 20mL of the secondary calibration standards in the 100mL volumetric flasks to 40mL glass vials. Again, these glass vials are capped with open top screw caps and PTFE-lined septa.
   a. Make duplicates of these 20mL secondary calibration standards.
   b. Make sure the vials are labeled accordingly.

Matrix Spike Addition
1) Matrix spike (MS) and matrix spike duplicate (MSD) samples should be prepared. These samples should be chosen randomly from the duplicates of collected samples. One set of MS and MSD samples should be prepared for each analytical batch.
2) Add **25µL of the HAA9 Matrix Spike Standard** to a 25mL of the matrix spike sample.
3) Make a duplicate 25mL aliquot of sample.
4) Measure 20mL using a graduated cylinder for each spike solution into a clean 40mL glass vial. Label each standard as a MS or a MSD.

Sample Preparation
1) Remove samples (stored in 40mL glass vials) from refrigerator and let them warm to room temperature while preparing calibration standards.
2) Use a clean, glass 25mL graduated cylinder to measure out 20mL of each sample.
3) Dispose remaining amount of sample into a waste beaker.
4) Pour the measured 20mL sample back into its 40mL glass vial.
   a. IMPORTANT: Pour samples on the side of the glass (graduated cylinder or vials) to reduce the samples' interaction with air.
5) Between measurements, rinse graduated cylinder 3 times with **LGW**. Pre-rinse graduated cylinder one time with the next sample to be transferred.

NOTE: Wash all used glassware 3 times with LGW and once with methanol.

Acid Surrogate Addition
1) Add **20µL of the acid surrogate additive standard at 20µL/mL** to all 20mL calibration standards, samples, and matrix spike samples using a micropipetter.
2) Stir in the surrogate with the pipetter tip. Do NOT cap and invert samples. Change pipette tip between samples.

Acidification
1) Using a glass pipette, add **1.5mL of concentrated sulfuric acid (H_2SO_4)** to all 20mL of calibration standards, samples, and matrix spike samples.

2) Let vials cool in an ice bath for 20-30min.
3) Swirl these vials gently to mix water and acid.

Internal Standard Addition
1) Using a pump pipette dispenser, add **4mL of extracting solvent** to each 20mL sample and calibration standard.
2) When using the pump pipette dispenser, make sure there are no bubbles in the addition line.
3) Two layers will be visible: an organic top layer of MtBE and an aqueous bottom layer.

Sodium Sulfate Addition and Extraction
1) Add about **10g of baked sodium sulfate** to each sample and calibration standard. This mass is measured out in a pre-measured glass beaker especially for this step.
2) Immediately after adding sodium sulfate, vortex all samples and calibration standards for 1 minute to prevent solidification of sodium sulfate.

Solvent Transfer to 2mL Volumetric Flasks
1) For each sample and calibration standard: With a clean, glass, 23cm Pasteur pipette, transfer **2mL of the top layer** (MtBE + IS layer) to a clear, glass 2mL volumetric flask capped with screw caps and PTFE-lined septa.
2) Use a clean pipette for each transfer. Be sure not to transfer any water and sodium sulfate crystals.

Derivatization
To all MtBE extracts in 2mL volumetric flasks:
1) Add 1/2 of a small, rounded scoop of **anhydrous powdered magnesium sulfate**. Re-cap the flask. DO NOT MIX!
2) Add **225µL cold diazomethane** with a micropipette. Re-cap the flask and DO NOT MIX!
3) Store these flasks in the refrigerator for 15 minutes.
4) Check for a yellow color in all samples. Note the samples that are not yellow in color.
5) Allow samples to warm to room temperature (about 15 minutes).
6) Add a small rounded scoop of **silicic acid n-hydrate powder**. The extract should become colorless because silicic acid quenches residual diazomethane.
7) Remove enough of the extracts from the 2mL volumetric flasks to fill GC vials about 70% full. Make sure no solids are in the vials, and then cap the vials.
8) Label each vial with the sample location and date.
9) Place these samples in a tray and wrap them in aluminum foil.
10) Label foil with name, date, and test. Store in the freezer before GC analysis.

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8. C. Total Organic Halogen (TOX) Standard Operating Procedure

Analysis of Total Organic Halogen (TOX) in Finished Drinking Water

Use laboratory coat, gloves, close-toed shoes, and goggles during the entire procedure.

Instrumentation
1) Adsorption Module (Tekmar-Dohrmann)
   Model: AD-2000 Adsorption Module
   Model n°: 890-161
   Serial n°: 99292009
   For 100mL sample size: Range: 4-1000 µg AOX/L; Precision: ±2 µg/L or ±2%

2) Organic Halide Analyzer
   Model: DX-2000 Organic Halide Analyzer
   Model n°: 890-162
   Serial n°: 99292009
   For 100mL sample size: Range: 4-1000 µg TOX/L; Precision: ±2 µg/L or ±2%
   For 10mL sample size: Range: 40-10,000 µg TOX/L; Precision: ±20 µg/L or ±2%

3) Software
   AOX/TOX by column – Copyright 1993-1996 Rosemount Dohrmann Div. – Version 2.10

The process of the system operates in such a way that it meets international methods including:
- EPA Methods 9020A, 9076, 450.1, and 1650
- ASTM-D-4744
- Standard Methods 5320B
- DIN 38409-H14
- ISO Method 9562
- SCAN-W Method 9:89
- NEN Method 6402

Reagents
- Lab grade water (LGW, deionized water)
- 70% by wt. Acetic Acid (Glacial, Fisher Scientific) in LGW
- 80% H_2SO_4 (ACS Plus Grade, Fisher Scientific) in LGW
- Concentrated H_2SO_4 (ACS Plus Grade, Fisher Scientific)
- 40 mg/mL Na_2SO_3 (anhydrous, ACS Grade, Fisher Scientific) in LGW
- 5,000 mg NO_3^-/L of KNO_3 (ACS Grade, Fisher Scientific) in LGW
- 200 ng Cl/µL of NaCl (ACS Grade, Fisher Scientific) in LGW
- 500 ng Cl/µL 2,4,6-Trichlorophenol (98%, Aldrich) in high purity methanol
- Sodium bicarbonate (Industrial grade, Fisher Scientific)
- Methanol (halogen free, highest purity, LCMS Grade, Fisher Scientific)
- Dry GAC (100-200 mesh GAC, <1.0µg/40mg, Rosemount Dohrmann)
- Glass-packed Carbon Columns 2mm ID (CPI International)
- Helium, 99.9+% purity, 220ft^3
- Oxygen, 99% purity, 220ft^3

Sample Collection & Dechlorination

Samples for TOX analysis should be collected in 125mL or 250mL amber bottles with open-top PTFE-lined septa. To quench about 3mg/L of free chlorine, 40μL of a 40mg/mL solution of sodium sulfite (Na_2SO_3) should be added to the sample bottles prior to collecting the samples.

Before collecting samples, the sample tap should be opened and allowed to run to waste for 2-3 minutes. The flow should then be reduced, the bottle placed at a slant and the water allowed to run down the side. When the bottle is almost full, cap the bottle with the teflon side of the liner facing inwards. Invert the bottle to mix and then open the cap and completely fill so that no air bubbles remain. Invert to confirm absence of air.

After collection, samples should be kept in a refrigerator at 4°C until analysis, which should take place within 14 days of collection.

Sample Pre-Treatment

Before the analysis, allow sample bottles to achieve room temperature. Then, add 25 drops of concentrated sulfuric acid (A.C.S. Plus) to the 125 mL samples, or 50 drops to 250 mL bottles, with a glass Pasteur pipette.

Sample Preparation – Adsorption

The sample volume, adsorption rate of sample to the carbon columns, channel fill rate, and use of sample prime can be adjusted in the control panel using the arrow keys after selecting the channel in use and pressing the keys "SAMPLE" and "MENU."

Program used for sample channels (1-4):
Sample volume: 100 mL
Adsorption rate: 2 mL/min.
Fill rate: Slow (33 mL/min)
Sample prime: NO
Priming volume: 0 mL

Program used in the nitrate channel for nitrate wash:
Sample volume: 2 mL
Adsorption rate: 1 mL/min

Before sample adsorption, make sure that the previous user rinsed the sample channels with LGW. This information should be written in the TOX logbook. Label the carbon columns appropriately.

To load the samples in the channels, choose one of the channels (1-4) keys, press the "START/STOP" key, connect the sample to the channel using one of the fill tubes (Figure 1a) and press "OK." After the desired volume of sample is in the channel, the screen will display the message "Connect columns (then press OK)."

Disconnect the fill tube, pierce the endcaps of two glass carbon columns, connect the columns in series using a connector (Figure 1b), and press the "OK" key. The aqueous sample is then passed through the two carbon columns, a top column and a bottom column for breakthrough, connected in series at a flow rate that permits complete adsorption of the organic halogens. The sample will drip out at the end of the bottom carbon column. Collect this waste in a beaker. Water samples may be discarded in the drain.
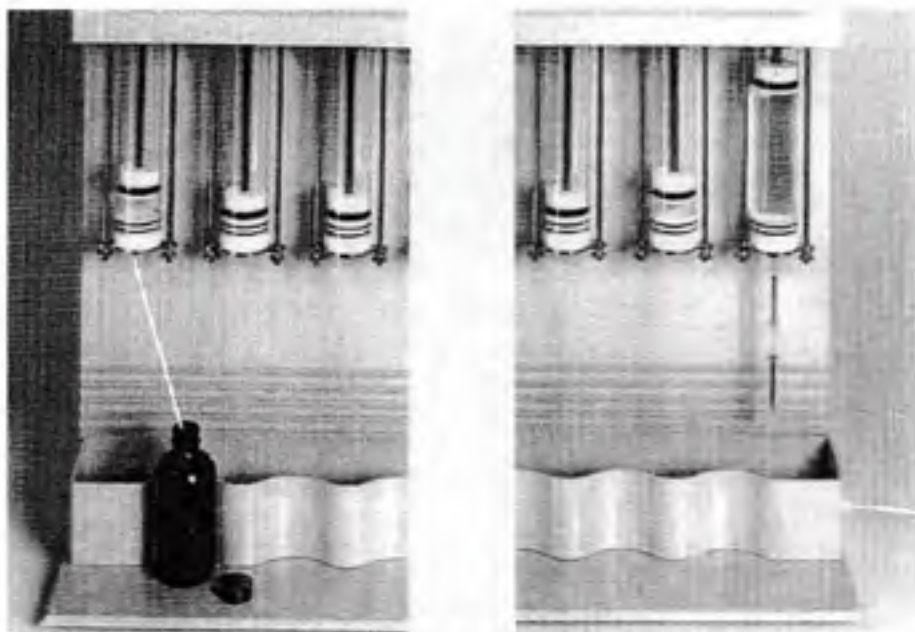
Figure 1: Adsorption module; a) Sample loading connection; b) Column connection.

When the run is complete, disconnect the sample columns from the adsorption module. Minimize their contact with the air by wrapping aluminum foil around both ends of each column. Keep the sample columns upright in a beaker covered with aluminum foil.

To remove inorganic chloride ions, the samples must be rinsed with a nitrate wash. Connect the top column to the nitrate wash channel and wash the column with 2 mL of the nitrate wash solution (5,000 mg NO_3^-/L KNO_3) at a rate of 1mL/min. Do the same for the bottom column. If the sample columns are not ready to be analyzed on the DX-2000 Organic Halide Analyzer, cover the ends of the columns with aluminum foil again and store in a beaker covered with foil. For sample columns that are ready to be analyzed, transfer them to the DX-2000 Organic Halide Analyzer.

Throughout the entire sample adsorption process, two nitrate blanks must be made, each blank in a single column, typically at the beginning and at the end of the adsorption process.

DX-2000 Organic Halide Analyzer Instrument Preparation

Before using the DX-2000 Organic Halide Analyzer module for sample analysis, make sure that the gas supplies, oxygen for combustion and helium as carrier gas, are enough. Do not use the instrument if the pressure of both gases is at 500 psi or lower. Change gas tanks when pressure of the gas tanks reaches 500 psi.

Use laboratory coat and gloves during all the procedure and laboratory goggles whenever handling acids.
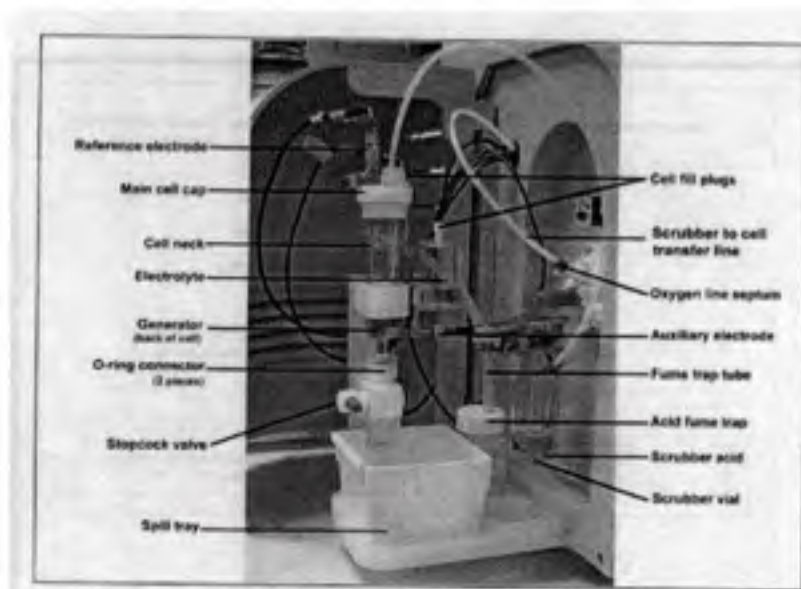
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Figure 2: The titration cell parts.

Scrubber vial

When the instrument is not working, the scrubber should always be disconnected from the combustion tube. To change the acid in the scrubber vial, pour the old acid into an acid waste container. Add fresh 80% H_2SO_4 to the marked line on the scrubber vial. This acid should be changed weekly.

Spill Tray & Acid Fume Trap

Change the sodium bicarbonate in the spill tray after making sure that the acid is neutralized. If acid is still present in the tray, use more sodium bicarbonate to neutralize it, and then empty the tray into aluminum foil and empty the contents in the garbage. Before throwing it in the garbage, be sure that no acetic acid smell can be detected. Keep it in the hood until this condition is met.

After rinsing the tray with tap water and drying it, fill it to about 1/3 with sodium bicarbonate to neutralize cell electrolyte. Empty the acid fume trap contents in the sink, rinse it with LGW and insert sodium bicarbonate to about 1/4 full and LGW to about 1/2 full as shown in Figure 3.

Figure 3: Acid fume trap.

Coulometric Cell

Change acid in the cell by removing the cell fill plugs, opening the stopcock valve to drain the acid from the cell to the spill tray, closing the valve, and filling the cell to the cell neck with fresh 70% acetic acid.

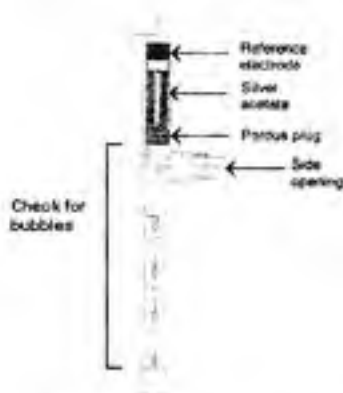Check if the reference electrode (Figure 4) has bubbles.

Figure 4: Reference electrode.

Do not remove the metal reference electrode from the reference electrode assembly. Problems in obtaining a stable baseline are likely to occur if the metal reference electrode is moved as it connects to the silver acetate reagent.

If there are bubbles present in the reference electrode, remove it from the cell and *take it to a hood with a moving window that will protect you from a possible spill*. Remove the side-opening plug and insert a syringe filled with 70% acetic acid in the side opening. With the part of the reference electrode that contains silver acetate facing down, slowly inject the acid from the syringe to the electrode. Repeat this procedure three times making sure that all the bubbles are removed by moving the electrode.

Be careful when doing this step, as the syringe can suddenly detach from the side opening and release acetic acid.

DX-2000 Organic Halide Analyzer Settings

To begin sample analysis in the computer program, choose the icon "AOX/TOX by Column" and click "OK." Make sure that the gas tank valves are open and that the acetic acid in the coulometric cell and H_2SO_4 in the scrubber vial are bubbling vigorously.

- Gas & Temperature Settings

    In the "System Setup" menu bar click "Open" and turn the system to a "Ready" position that will turn on the gas flow and increase the furnace temperature to 850°C from its standby temperature of 550°C in increments of 50°C. When the temperature reaches the desired ready mode temperature, an orange light in the temperature control panel indicator will appear in the "Ready" light.

    Connect the scrubber vial to the combustion tube and close the injection port lid. In the front panel check that the gas flow for oxygen is around 50mL/min and helium is around 25 mL/min. Adjust the flow up and down by turning the knobs, if necessary.

- Baseline Monitor

    Select in the "System Checks" menu the "baseline monitor" option and wait for about 15 minutes until the baseline is stable. The voltage reading should be higher than 250 V. If the voltage reading is lower than 250 V, flush the cell with fresh 70% acetic acid, or inject 5 μL of the 200 ng Cl/μL NaCl solution until the desired voltage value is obtained.

    In the "System Setup" menu bar click "Open" and turn the cell to the "ON" position. In the "System Checks" menu, choose again the "Baseline Monitor" option and wait until the

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baseline is stable. If you wish to see the current instead of the voltage reading, select in the "Options" menu the "Graph Mode" option of your choice. Both current and voltage will always be displayed in the bottom of the computer screen.

- Cell Check

 To verify that the cell is working properly, inject a 5 μL of a 200 ng Cl/μL NaCl solution and check the recovery obtained.

 In the "System Checks" menu choose the "Cell Check" option and fill the "Run Info" menu with the information of the solution injected. Before pressing the "Start" key have the syringe ready for the injection. Press "Start," wait for a message saying, "Inject to cell then press OK," remove the white cell fill plug (Figure 2) from main cell cap, inject the desired volume into the titration cell and click "OK."

 If the resulting percentage recovery is between 90-110%, then the cell is working properly. Perform this check three times for consistent results.

- Clean Boat

 Before analyzing the samples, the boat has to be cleaned. Select the "Clean Boat" option in the "System Checks" menu.

- Combustion Check

 To verify the furnace performance, inject 2 μL of a 500 ng/μL 2, 4, 6-trichlorophenol (TCP) solution into the boat on top of a scoop of dry GAC and check the recovery obtained.

 Carefully place a scoop of dry GAC in the boat and close the lid tightly. In the "System Checks" menu choose the "Combustion Check" option, fill the "Run Info" menu with the information of the solution injected. Before pressing the "Start" key have the syringe ready for the injection. Press "Start", wait for a message saying, "Inject to boat then press OK", carefully inject the volume through the lid septum and press "OK."

 Make sure that the furnace is completely pyrolyzing the carbon: fresh carbon is black, while pyrolized carbon is a light orange color. If the carbon is not completely pyrolizing, check for gas leaks. The lid above the boat should be sealed tightly. 90-110% recovery of the TCP solution indicates good recovery. Perform this check three times for consistent results. Remove the pyrolized carbon from the boat using a vacuum tube attached to a trap. Perform this check during analysis of samples to verify the furnace's performance.

- Sample Analysis

 Remove the plastic endcaps from the sample column. Open the injection port lid and use the T-shaped ejector tool to inject the sample-adsorbed carbon into the glass boat. *Be careful to not touch the boat with the tool, as the boat is extremely fragile.* Close the lid and make sure that the seal is tight by checking the bubbling in the titration cell and the scrubber vial.

 Under the "Run" menu, select "Manual Run." Select the type of sample (blank, sample, standard). Next select common run parameters: print results, enter comments, sample ID name, and enter adsorption volume. Select the column parameters for the type of sample (sample, blank, standard) to be analyzed: top/bottom column, blank value, dilution factor, standard concentration if the sample is a standard, nitrate if the sample is a nitrate blank. Verify that the information is correct. Click "OK" to save and "Start Run" when ready to run the analysis. A graph of voltage (or amperes) vs. time in seconds will appear on the computer screen during the analysis. When the sample has finished undergoing combustion, the computer will output a raw TOX value in "μg Cl." Vacuum the boat once analysis is over. Repeat the procedure for other samples.

- TOX Measurements

The following expressions can be used to determine the TOX concentration in mgCl/L and the breakthrough percentage, which should be lower than 10%. An average of the nitrate blank values should be used as "blank" in the expressions below.

$$Final\ result(\mu g\,Cl\,/\,L) = \frac{(top + bottom) - (2 \times blank)}{Adsorption\ Volume}$$

$$Breakthrough(\%) = \frac{(bottom - blank) \times 100}{(top + bottom - (2 \times blank))}$$

System Shutdown and Power Off

To maximize system reliability and minimize downtime, do not turn off the system power every day. Instead, set the system to the "Standby" operating mode. Power should only be turned off when:

- The system will not be used for an extended period of time
- Directed to do so during maintenance and parts replacement, etc.

Shutdown

First check that there are no runs in progress. Next, clean the boat as necessary. Set the system into Standby mode. Switch off the valves on the gases.

Power Off

The system should only be turned off if it is not being used for an extended period of time, in emergencies, or for maintenance. First, clean the boat as necessary. Set the system to Standby. Turn off operating power by performing the Power On steps in the reverse order.

Quality Assurance/Quality Control

Cell & Combustion Check Recoveries

To ensure the validity of the data collected, it is extremely important to perform cell checks and combustion checks before analysis and sporadically during the sample analysis process. If the recoveries obtained range between 90-110% the system is being effective in the determination of the total organic halide content of the samples.

Haloacetic Acids & Trihalomethanes Recoveries (from Vanessa Pereira, MSEE 2001)

Standard haloacetic acids (HAA) and trihalomethane (THM) solutions were analyzed using the absorption module and the DX-2000 Organic Halide Analyzer to measure their TOX content. The percent recoveries obtained with this instrument can be found in Table I. The cell check recovery obtained was 107% and the combustion check recovery was 96% when these tests were performed.

Table I: Percent recoveries obtained in the analyses of standard concentrations prepared of the HAA and THM individual species ([std]).

	Sample ID	[std] (mg/L)	[std] (mg as Cl/L)	Final result (mg Cl/L)	Recovery (%)
HAA	chloroacetic acid	100	38	35	93
	dichloroacetic acid	100	55	71	129
	trichloroacetic acid	100	65	75	115
	dibromoacetic acid	100	33	41	125
	bromochloroacetic acid	100	41	47	115
	bromoacetic acid	100	26	35	138
	bromodichloroacetic acid	100	51	35	69
	tribromoacetic acid	100	36	26	73
	chlorodibromoacetic acid	100	42	46	110
THM	chloroform	100	89	60	68
	bromodichloromethane	100	65	43	66
	dibromochloromethane	100	51	34	66
	bromoform	100	42	29	68

The conversion of µg/L to µg as Cl/L is based on the molecular weight of the compound and in the Cl and Br content as shown in the following expression:

$$[std](\mu g\, as\, Cl\,/\,L) = [std](\mu g\,/\,L) \times \frac{Mr(Cl)}{Mr(compound)} + [std](\mu g\,/\,L) \times \frac{Mr(Br)}{Mr(compound)} \times \frac{Mr(Cl)}{Mr(Br)}$$

To identify if the low recoveries obtained in the THM standard solutions were due to weak adsorption into the carbon columns, a direct injection of bromoform was made in the boat. The percent recovery obtained was 69.9% indicating that the low percent recoveries obtained are not explainable by a weak adsorption onto the carbon columns.

8. D. Total Organic Chloride (TOCl) and Total Organic Bromide (TOBr) Detection on the Dionex Ion Chromatograph

Use laboratory coat, gloves, close-toed shoes, and goggles during the entire procedure.

Instrumentation

1) Dionex Ion Chromatograph
   Conductivity Detector, Serial #911003E930302 XTC: uses a Dionex AS14 analytical column, a Dionex AG4 guard column, 250µL sample volume, Dionex AMMS III-4mm ion suppressor
   Gradient Pump: set to use 100% of eluant at 1.0mL/minute
   Eluent Gas Module: uses a mobile phase (eluent) at 3.5mM sodium carbonate & 1.0mM sodium bicarbonate
   Regenerant Pump: uses 25mN H_2SO_4 at ~2mL/minute

2) Adsorption Module (Tekmar-Dohrmann)
   Model: AD-2000 Adsorption Module
   Model n°: 890-161
   Serial n°: 99292009
   For 100mL sample size: Range: 4-1000µg AOX/L; Precision: ±2µg/L or ±2%

3) Organic Halide Analyzer
   Model: DX-2000 Organic Halide Analyzer
   Model n°: 890-162
   Serial n°: 99292009
   For 100mL sample size: Range: 4-1000µg TOX/L; Precision: ±2µg/L or ±2%
   For 10mL sample size: Range: 40-10,000µg TOX/L; Precision: ±20µg/L or ±2%

Heating tape must be attached around the area where the glass combustion tube is exposed to the air. The tape should heat above 100°C so that the vapors from pyrolizing the sample do not condense on the glass.

4) Software
   Ion Chromatograph: Dionex PeakNet Copyright 1992-2001, Version 5.1
   AOX/TOX by Column: Copyright 1993-1996 Rosemount Dohrmann Division, Version   2.10

Ion Chromatograph (IC) Reagents

- Lab grade water (LGW, deionized water)
- 3.5mM Sodium carbonate (anhydrous, granular, ACS Grade, Mallinckrodt)
- 1.0mM Sodium bicarbonate (ACS Grade, Mallinckrodt)
- Concentrated H_2SO_4 (ACS Plus Grade, Fisher Scientific)
- NaCl (ACS Grade, Fisher Scientific)
- NaBr (ACS Grade, Fisher Scientific)
- Methanol (halogen free, highest purity, LCMS Grade, Fisher Scientific)
- Helium, 99.9+% purity, 220ft^3
- 47mm & 0.45µm membrane filter (hydrophilic polyether sulfone, Gelman Sciences/Pall Corp.)

TOX Reagents

- Lab grade water (LGW, deionized water)

- 70% by wt. Acetic Acid (Glacial, Fisher Scientific) in LGW
- 80% H_2SO_4 (ACS Plus Grade, Fisher Scientific) in LGW
- Concentrated H_2SO_4 (ACS Plus Grade, Fisher Scientific)
- 40mg/mL Na_2SO_3 (anhydrous, ACS Grade, Fisher Scientific) in LGW
- 5,000 mg NO_3^-/L of KNO_3 (ACS Grade, Fisher Scientific) in LGW
- 200ng Cl/μL of NaCl (ACS Grade, Fisher Scientific) in LGW
- 500ng Cl/μL 2,4,6-Trichlorophenol (98%, Aldrich) in high purity methanol
- Sodium bicarbonate (Industrial grade, Fisher Scientific)
- Pentachloroacetone (85% PW, Aldrich)
- (±)1,2-dibromopropane (97%, Aldrich)
- Methanol (halogen free, highest purity, LCMS Grade, Fisher Scientific)
- Dry GAC (100-200 mesh GAC, <1.0μg/40mg, Rosemount Dohrmann)
- Glass-packed Carbon Columns 2mm ID (CPI International)
- Helium, 99.9+% purity, 220ft^3
- Oxygen, 99% purity, 220ft^3

Sample Collection & Dechlorination

Samples for TOCl/TOBr analysis should be collected in 125mL or 250mL amber bottles with open-top PTFE-lined septa. To quench about 3mg/L of free chlorine, 40μL of a 40mg/mL solution of sodium sulfite (Na_2SO_3) should be added to the sample bottles prior to collecting the samples.

Before collecting samples, the sample tap should be opened and allowed to run to waste for 2-3 minutes. The flow should then be reduced, the bottle placed at a slant and the water allowed to run down the side. When the bottle is almost full, cap the bottle with the teflon side of the liner facing inwards. Invert the bottle to mix and then open the cap and completely fill so that no air bubbles remain. Invert to confirm absence of air.

After collection, samples should be kept in a refrigerator at 4°C until analysis, which should take place within 14 days of collection.

Sample Pre-Treatment

Before the analysis, allow sample bottles to achieve room temperature. Then, add 25 drops of concentrated sulfuric acid (A.C.S. Plus) to the 125mL samples, or 50 drops to 250mL bottles, with a glass Pasteur pipette.

Sample Preparation – Adsorption

The sample volume, adsorption rate of sample to the carbon columns, channel fill rate, and use of sample prime can be adjusted in the control panel using the arrow keys after selecting the channel in use and pressing the keys "SAMPLE" and "MENU."

Program used for sample channels (1-4):
Sample volume: 50mL
Adsorption rate: 2mL/min.
Fill rate: Slow (33mL/min)
Sample prime: NO
Priming volume: 0mL

Program used in the nitrate channel for nitrate wash:
Sample volume: 2mL
Adsorption rate: 1mL/min

Before sample adsorption, make sure that the previous user rinsed the sample channels with

LGW. This information should be written in the TOX logbook. Label the carbon columns appropriately.

To load the samples in the channels, choose one of the channels (1-4) keys, press the "START/STOP" key, connect the sample to the channel using one of the fill tubes (Figure 1a) and press "OK." After the desired volume of sample is in the channel, the screen will display the message "Connect columns (then press OK)."

Disconnect the fill tube, pierce the endcaps of two glass carbon columns, connect the columns in series using a connector (Figure 1b), and press the "OK" key. The aqueous sample is then passed through the two carbon columns, a top column and a bottom column for breakthrough, connected in series at a flow rate that permits complete adsorption of the organic halogens. The sample will drip out at the end of the bottom carbon column. Collect this waste in a beaker. Water samples may be discarded in the drain.
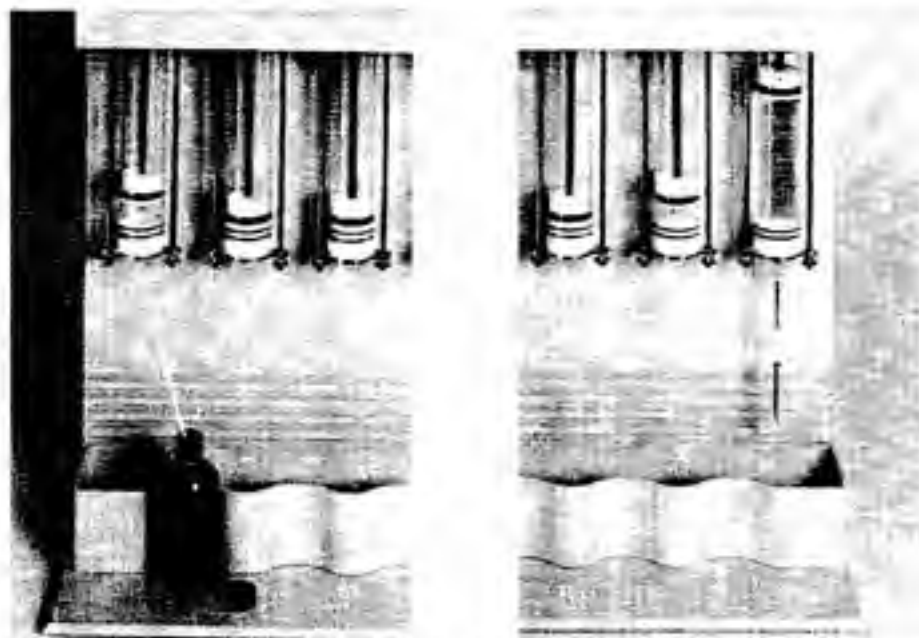

Figure 1: Adsorption module; a) Sample loading connection; b) Column connection.

When the run is complete, disconnect the sample columns from the adsorption module. Minimize their contact with the air by wrapping aluminum foil around both ends of each column. Keep the sample columns upright in a beaker covered with aluminum foil.

To remove inorganic chloride ions, the samples must be rinsed with a nitrate wash. Drain the old nitrate wash solution into a waste container. Fill the nitrate channel with fresh nitrate wash solution (5,000mg NO_3^-/L KNO_3). Connect the top column to the nitrate wash channel. Press the "Nitrate" channel button, followed by the "Start/Stop" button, then the "OK" button. This program will wash the column with 2mL of the nitrate wash solution (5,000mg NO_3^-/L KNO_3) at a rate of 1mL/min. Do the same for the bottom column. If the sample columns are not ready to be analyzed on the DX-2000 Organic Halide Analyzer, cover the ends of the columns with aluminum foil again and store in a beaker covered with foil. For sample columns that are ready to be analyzed, transfer them to the DX-2000 Organic Halide Analyzer for TOCl/TOBr sample collection. Throughout the entire sample adsorption process, two nitrate blanks must be made, each blank in a single column, typically at the beginning and at the end of the adsorption process.

Instrument Preparation of Total Organic Halides (TOX) Analyzer

Before using the DX-2000 Organic Halide Analyzer module for sample analysis, make sure that the gas supplies, oxygen for combustion and helium as carrier gas, are enough. If so, open the gas valves.

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Do not use the instrument if the pressure of both gases is at 500psi or lower. Change gas tanks when pressure of the gas tanks reaches 500psi. Because the detector components of the TOX analyzer are not used in this method, the cell readings do not have to be high, but stable so that the instrument performs correctly.

In the computer, select the "TOX Talk" shortcut icon on the desktop and double click on "TOX by Column." Select "System Setup" in the menu bar, click "Open," turn the system to "Ready" mode, and turn the cell "On." Increase the furnace temperature to 850°C from its standby temperature of 550°C in increments of 50°C. When the temperature reaches the desired ready mode temperature, an orange light in the temperature control panel indicator will appear in the "Ready" light. While increasing the furnace temperature, plug the heating tape plug into a power outlet so that it will be at the right temperature during sample collection.

Next, check the coulometric cell. Even though the cell is not needed for any detection, the level of acetic acid in the cell always needs to be at the neck of the cell so the instrument does not malfunction. If the acetic acid level is below the neck, fill up the cell with acid. Check if there are any bubbles in the electrode. If bubbles are present, remove it from the cell by disconnecting the wiring at the top (pull the wire off of the electrode, do NOT pull the orange plug out of the electrode itself), and pull the electrode out of the cell. Take the electrode and the bottle of 70% acetic acid into the hood. Remove the orange plug located on the SIDE of the electrode. Turn the electrode upside down with the white spongy end facing upwards. Insert the tip of the acetic acid bottle into the side opening and fill the electrode with acid. Turn the electrode on its side while filling so that it can be filled to the top of the opening. Then, insert the orange septa back into the SIDE opening while rotating the electrode so that the white spongy end is again facing upwards. Doing so should push any bubbles in the electrode out of this end and leave the tube free of air. If not, repeat the process again until all of the bubbles are gone. While it may seem pointless to do so for this method, since the electrode is not being used as a detector, it prevents the electrode from being damaged.

If everything is fine with the cell and the electrode, empty the contents of the scrubber vial into an acid waste container and fill to the marked line with fresh 80% H_2SO_4. Connect the scrubber vial to the combustion tube and insert the plastic connector into the side of the vial that connects the scrubber to the cell. In the front panel of the TOX instrument, make sure that the gas flows are at the correct positions. In the front panel check that the gas flow for oxygen is around 50mL/min and helium is around 25mL/min. Adjust the flow up and down by turning the knobs, if necessary.

Once the baseline is stable, when the voltage and current are not changing, under "System Checks," select "Clean Boat." When the graph appears, click on "Start," which sends the boat into the oven and makes the system burn off any residue left on the boat from previous runs so that it will not be transferred to the samples.

After the boat has been cleaned, the system is ready to start making the TOCl/TOBr samples. Fill a clean, dry scrubber vial (not the same one used for the 80% H_2SO_4) with 7mL of LGW. DO NOT attach it to the glass combustion tube yet. Remove plastic endcaps from the sample column and with the T-shaped tool, inject the sample into the boat. Close the lid and make sure that it is sealed tightly. Select "Clean Boat." When the graph appears on the screen, disconnect the scrubber vial containing 80% H_2SO_4, disconnect the tubing that connects the scrubber to the cell, connect the scrubber vial containing 7mL LGW to the glass combustion tube, and immediately click "OK." The boat will start to move into the furnace and sample collection will begin.

Watch the bottom left corner of the computer screen to determine at which step the sample process is. When the prompt "Boat to cool" appears, which occurs at around 220 seconds, disconnect the sample scrubber vial from the combustion tube, reconnect the acid scrubber vial, and reattach the tubing that connects the scrubber vial to the cell. It is very important to reattach the tubing that connects the scrubber to the cell. Failure to do so causes the baseline to drop, which causes the instrument to fall out of equilibrium. It takes a long time for the system to equilibrate, so reattaching the tubing is necessary.

Take the sample scrubber vial containing the collected TOCl/TOBr to the hood. Measure out 3mL of LGW in a graduated cylinder. Pour the collected sample into a 20mL glass vial, labeled

appropriately, with an open-top cap and PTFE-lined septa. Rinse the vial and removable glass tubing with the 3mL of LGW using a glass Pasteur pipette. Pour this rinse into the 20mL glass vial with the rest of the sample. Store this sample at 4°C. Rinse the sample vial and removable glass tubing three times with LGW and invert over a KimWipe until next use. Rinse the graduated cylinder with LGW. Use a new Pasteur pipette each time.

Before collecting another sample, the boat should be vacuumed. After vacuuming, the TOX analyzer is ready for the next sample collection. Follow these steps with every sample column until all of the samples are collected in 20mL glass vials. When finished, store the samples at 4°C until analysis on the IC.Unplug the heating tape from the power outlet, disconnect the acid scrubber, wipe off the red septa at the end of the tube that connects the acid scrubber vial to the cell with a KimWipe and LGW, and go to "System Startup" and switch the analyzer to "Standby" mode (550°C). Close the gas valves and clean up the space around the instruments.

Matrix & Scrubber Spikes

Matrix spike samples are aqueous samples into which an organic halogen has been added into the matrix, or the actual water sample. For this spike, a stock solutions of pentachloroacetone and ($\pm$)1,2-dibromopropane were prepared at concentrations of 30mg/mL in MtBE and 40mg/mL in MtBE, respectively. The pentachloroacetone stock solution was diluted to a working solution at 0.25g Cl'/L in MeOH, while the dibromopropane stock solution was diluted to a working solution at 0.30g Br'/L in MeOH.

Select a water sample at random for matrix spiking. To determine the matrix spike concentrations, look at the TOX data for that particular water sample (TOX data should have been collected already for this sample). Assume that half of this TOX value is chloride and the other half is bromide. Since the TOX value is given in units of "µg as Cl'/L," convert the bromide half into ""µg as Cl'/L." Double these theoretical chloride and bromide values, because the matrix spike concentrations should be two times the expected, which will be the concentration of pentachloroacetone and ($\pm$)1,2-dibromopropane that should be spiked in the sample. Use the working solutions of pentachloroacetone and ($\pm$)1,2-dibromopropane.

Spike these concentrations of pentachloroacetone and ($\pm$)1,2-dibromopropane into the water sample filled up to the neck in a 50mL volumetric flask. After spiking, fill to the 50mL mark and invert three times. Now the sample is ready to be adsorbed onto carbon columns and run through the TOX to collect the TOCl/TOBr sample. Once this sample is collected, it is transferred to a 20mL glass vial with an open-top cap and PTFE-lined septa and stored at 4°C until analysis on the IC.

Scrubber spike samples require 5mL of the 10mL TOCl/TOBr samples collected using the TOX analyzer. The samples are spiked after sample collection with 10µL of an inorganic chloride and bromide calibration standard at 100mg/L, to give a 200ppm spike. Then the spiked sample is analyzed on the IC.

The scrubber sample to be spiked is selected at random. 5mL of this scrubber sample is placed in a 5mL volumetric flask. 10µL of the 100mg Cl' and Br'/L calibration standard is injected into the sample and the flask is inverted three times. The spiked sample is transferred to a glass vial with an open-top cap and PTFE-lined septa and stored at 4°C until analysis on the IC.

Calibration Standards

Stock solutions of inorganic chloride and bromide are prepared at 1000mg/L for each ion species. These solutions are good for six months and must be stored in 250mL amber glass bottles with open-top caps and PTFE-lined septa at 4°C. Using these stock solutions, a calibration standard at 100mg/L is prepared, which lasts for one month. The calibration standard is used to make the calibration points.

The following calibration points should be made:

Concentration (μg Cl⁻ and Br⁻/L)	Total Volume in LGW (mL)
0	100
50	100
100	100
200	100
300	100
500	100
1000	100
1500	100
5000	100

All of the calibration points should be made in 100mL volumetric flasks. They should be filled to three-fourths of the way to the line with LGW, the appropriate amount of 100mg/L calibration standard added, LGW added to the fill line, and inverted three times. These calibration solutions should be placed in appropriately labeled 40mL glass vials at 4°C until IC analysis. Calibration points should be run every day before samples are run. After every 10 samples, the 200μg/L calibration point is injected to as a performance check. Calibration solutions should be made fresh weekly.

Dionex Ion Chromatograph Preparation

The mobile phase for this procedure is 3.5mM Na_2CO_3/1mM $NaHCO_3$ and should be filtered using a 47mm, 0.45μm hydrophilic polyether sulfone filter. The regenerant for this procedure is 25mN H_2SO_4. Connect the sample loop, guard column, and column. Do not yet attach the suppressor to the mobile phase line or the regenerant line; however, the suppressor can be attached to the column.

Check to see if the helium gas tank pressure is at 500psi or lower. If so, the tank needs to be changed. If the tank pressure is above 500psi, open the valves to start the gas flow.

Switch on the Conductivity Detector (the square, blue button on the top left corner). Make sure the cell is OFF and that it is in LOCAL mode. Switch on the Gradient Pump (switch is located at the back of the pump). Make sure that it is in LOCAL mode and that the mobile phase is on STOP. Switch on the Eluent Degas Module (main silver switch beside the pressure gauge). Adjust the pressure to 5psi. Switch on the ACI so that the computer and the IC can communicate between each other.

Flip the mobile phase switch 4 up to the ON position and loosen the cap on the 3.5mM Na_2CO_3/1mM $NaHCO_3$ labeled bottle. Flip switch 2 to ON position for LGW and loosen the cap. Locate sparge line 4 and 2 and remove the plugs. Flip the top switches to the SPARGE position. Place the lines in a small vial of methanol one at a time to check that the gas is flowing. Wipe the tip off thoroughly with a KimWipe and connect the line to the sparge port on the mobile phase bottle and on the LGW bottle. Allow mobile phase and LGW to sparge for 10 minutes for old mobile phase and LGW and 20 minutes for fresh ones.

Remove the sparge lines, tighten the caps on the bottles, re-plug the sparge lines, flip switches 2 and 4 down to turn them off, and flip switches 2 and 4 from SPARGE to PRESSURIZE. Test pressure lines for gas flow in methanol and wipe the tips thoroughly. Connect the pressure lines to pressure ports on their respective bottles. Make sure that the pressure is still around 5psi. Check the helium tank for

leaks by turning off the tank, tapping the gauge to see if pressure changes, and turning the tank back on. If any change in the set level gauge is seen, then there is a leak.

On the gradient pump, make sure that the pump is set at the proper flow rate and percent. The LGW bottle at location 2 should be at 100% at a flow rate of 1mL/min. To change the percent, press "%, 2, 1, 0, 0." Make sure the other reservoirs are at 0%. Attach a 3mL syringe to the clear port on the gradient pump. Turn the silver bar towards the syringe so that it is parallel to the syringe and it is pointing towards you. Push START on the gradient pump so the LGW will run. Draw back on the plunger of the syringe to start filling it with LGW. Fill the syringe with 3mL of LGW and push STOP to stop the flow. Flip the silver bar parallel to the pump to close the valve. Disconnect the syringe and empty the contents into a waste beaker. Repeat this procedure two more times, but after filling the syringe a third time, do not disconnect the syringe after pushing STOP unless there are air bubbles, in which case the syringe will need to be disconnected and the bubbles removed before proceeding. When bubbles have been removed, turn the black knob, located above and to the right of the syringe, to the left to loosen it. Slowly push the contents of the syringe back into the pump while watching the clear tubing below the black knob. Make sure all of the bubbles in the tubing are evacuated by tapping on the tubing. Turn the black knob into the closed position. Return the silver bar to the original, closed position. Disconnect the syringe and empty any leftover LGW into the waste beaker. Repeat this procedure for the mobile phase bottle at location 4.

Press START on the mobile phase flow and watch the pressure. If pressure rises dramatically and continues to do so beyond the pressure at which it was stabilized during the last run, stop the flow. There is a blockage in the line and will have to be removed before proceeding. Also, check the mobile phase line in the sink to see if it is dripping. If no mobile phase is dripping out, there is a blockage in the line. Once the mobile phase is dripping out of the eluent line, connect it to the "ELUENT IN" port on the suppressor. The "ELUENT OUT" port should be connected to the detector.

While pressure is equilibrating, tighten the cap on the regenerant bottle and tighten the connection between the regenerant bottle port and the line going to the pump. Turn on the gauge to ~1-2mL/min, but not higher than 3mL/min or else the suppressor membrane will be damaged. Once the regenerant starts flowing out of the line, connect the line to the "REGEN IN" port on the suppressor. The "REGEN OUT" port should be connected to the regenerant waste line. Watch the line for any bubbles. If so, tap the suppressor until they flow out. Check the flow of the regenerant waste line by using a graduated cylinder and timing for 1 minute to measure the flow rate. Let the pressure and pump equilibrate for about 30 minutes. Keep the cell OFF.

After the pressure has stabilized, record this value in the logbook. Turn the cell ON on the detector and allow it to equilibrate. Record this value in the logbook, as well. The value should always be around the value the last time the IC was used. If it varies considerably, try the following tests to detect the problem:

- Stop the flow and turn the cell off.
- Disconnect the column, plug the ends, and screw the two lines together.
- Start the pump and turn the cell on.
- If pressure decreases, the column is causing the problem.
- If the pressure is still high, remove the suppressor connection in the same fashion as above and see what the cell reading returns.
- If the pressure is still high, reconnect everything as normal, start the flow, and turn the cell on. Check for leaks in the system.

Switch the cell and the pump to REMOTE settings. Open PeakNet program on the computer. Make sure that the data will be saved in the appropriate folder (see below for details). Get a new syringe and rinse it with LGW. Wipe the end of the syringe clean, and then rinse the syringe with a little bit of sample. Discard into a waste container. Draw about 1mL of sample into the syringe and attach syringe to the sample valve. The system is now ready for IC analysis. Type in the sample name, inject the sample into the valve, and click RUN. This sample will run for 15 minutes, after which another sample must be

injected. After each sample run, rinse the syringe with LGW once and one time with a small volume of the next sample to be analyzed.

Computer Preapration of Dionex IC
Data Storage
　　In the Data folder in the PeakNet folder, create a folder with the user's name. Within this folder, create a new folder named using today's date. Select "Methods" in PeakNet's main menu and select the method "toclman." Change the directory to the file that was just created. Save changes and close the methods.

Run
　　Click on RunACl on the PeakNet main menu. Close the "IC 2" window. Make sure the pump and the detector are set to REMOTE, which were set to LOCAL originally. Click LOAD at the top of the screen and select "Method." Select the "toclman" method and click "OK." A prompt will appear that says "Loading GPM events..." When this message disappears, click "Run," "Start," enter the sample name, and then click "Start Run."

Shutting Down the IC
　　Switch all the modules to the LOCAL mode, turn the cell OFF, and STOP the pump. Switch the pump to 100% for LGW, location 2. Let LGW flow through the column for 10 minutes. Doing so protects the column for the next use. STOP the pump, switch settings back to 100% for the mobile phase in location 4 for the next use. Turn off the regenerant gauge, loosen connection at port, and loosen the cap. Disconnect the regenerant and eluent lines and plug up the ports on the suppressor.

　　Turn the gas module to sparge position, loosen the caps on the mobile phase and LGW bottles, turn off the entire module, turn off the switch for the second and fourth bottle locations, switch to pressurize, and tighten the caps.

　　Turn off the pump, the detector, and the ACI. Turn off the helium tank valve. Record the number of injections made in the logbook. Store samples in the refrigerator.

Data Analysis

　　To view the chromatograms, click on "Optimize" in the main menu and then open the desired file. Click on the "Manual Integration" icon to view the peak areas. Chloride's retention time occurs at around 5.20 minutes, while bromide's retention time occurs at around 7.30 minutes. Calibration curves, one each for chloride and bromide, should be created in Excel. Create a best-fit line, display the equation and R-squared value on the graph. An R-squared value of 0.997-0.999 is desired. The calibration curve should be used only for the samples that were run on the same day as the calibration points.

　　Check the chromatograms for the samples. Make sure that the integration is uniform. If not, manually adjust the integrations so that the peaks are all integrated at baseline. Use the calibration curves to determine the chloride and bromide concentrations in the samples. Average the nitrate blanks and subtract them from the final chloride and bromide concentrations.