COMPARATIVE CYTOTOXICITY OF DRINKING WATER DISINFECTION
BY-PRODUCT MIXTURES PRODUCED DURING CHLORINATION AND
CHLORAMINATION


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# ABSTRACT

REBECCA MILSK: Comparative Cytotoxicity of Drinking Water Disinfection
By-Product Mixtures Produced During Chlorination and Chloramination
(Under the direction of Dr. Howard Weinberg)

Chlorinated and chloraminated waters containing characterized natural organic

matter (NOM) were compared on the basis of cytotoxicity, disinfection by-product (DBP),

and total organic halogen (TOX) levels.  Cytotoxicity was evaluated using a growth

inhibition assay (GIA) in NCM460 human colon cells.  Without adding iodide or nitrate

or using medium pressure ultraviolet (UV) pretreatment, the chlorinated water was more

cytotoxic than the chloraminated water.  At elevated iodide levels and using pretreatment

with medium pressure UV, the chloraminated water was the most cytotoxic of all

disinfected samples evaluated.  This is likely due to the formation of iodinated DBPs,

possibly enhanced by degradation of the NOM with UV but present at levels below

detection of the analytical methods used.  This current research shows that the GIA is

able to detect these differential cytotoxic responses, and can provide more insight into

changes in water quality than DBP and TOX measurements alone.

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**CHAPTER 1**

INTRODUCTION

**DBP Regulation**

**A. History of Drinking Water Regulation and Disinfection By-Product Discovery**

Ever since Dr. John Snow identified a contaminated well as the source of a cholera outbreak in London in 1855, drinking water quality has been established as a critical public health concern. The incident highlighted the need to examine characteristics of drinking water beyond its apparent taste, smell, and color. After Louis Pasteur's discovery in the late 1880s that microbes were capable of transmitting disease, scientists and engineers began to develop methods to identify and remove these pathogens from drinking water. In the U.S. in the early 1900s, slow sand filtration was used to remove turbidity, the cloudiness observed in water due to the presence of particles or suspended solids. Turbidity is related to microbial risk because microbes can attach to these particles. Although slow sand filtration did reduce waterborne disease outbreaks, the introduction of disinfectants led to much greater efficacy in this regard. The first recorded use of a disinfectant in the U.S. was in 1908 in Jersey City, New Jersey, where chlorine was employed [1].

In 1914, the U.S. Public Health Service set the first federal drinking water regulation, which restricted bacteriological levels in water delivered to interstate carriers such as ships and trains. Various other drinking water regulations were set over the next 50 years, culminating in the Safe Drinking Water Act (SDWA) of 1974, which provided

a framework for regulating chemical contaminants in public drinking water supplies. Specifically, the SDWA called for the U.S. Environmental Protection Agency (U.S. EPA) to draw up national interim drinking water regulations based on 28 standards that had been set in 1962 by the Public Health Service, and to revise these standards as necessary via review by the National Academy of Sciences [1].

As the SDWA was being passed in 1974, Rook in the Netherlands and Bellar, Lichtenberg, and Kroner at the U.S. EPA separately discovered that trihalomethanes (THMs) were produced during chlorination of surface waters used in the production of drinking water [2, 3]. These represented the first identified drinking water disinfection by-products (DBPs), or compounds formed when a disinfectant reacts with naturally occurring organic matter, iodide, and bromide present in the water. The occurrence of chloroform and other THMs in treated drinking water was confirmed in a 1975 survey of 80 drinking water treatment plants, with at least four THMs detected in all plants utilizing free chlorine as a disinfectant [4]. This discovery sparked an investigation of the health implications associated with chloroform exposure through drinking water. Carcinogenicity was observed in rats and mice at high chloroform dosage levels [5], leading to the 1979 enactment of trihalomethane regulation, which set the maximum contaminant level (MCL) for total trihalomethanes (TTHM), which is the sum of chloroform, dibromochloromethane, bromodichloromethane, and bromoform concentrations, at 0.10 mg/L [6].

By the mid-1980s, only 23 drinking water contaminants including the four THMs had been regulated and only one standard had been revised. Congress was dissatisfied with the slow progress being made on the regulatory front, and in 1986 passed an

amendment to the SDWA requiring that EPA set maximum contaminant level goals (MCLGs) and MCLs for 83 contaminants, and to regulate contaminants beyond these 83 within a certain time frame. MCLGs are non-enforceable guidelines that represent the level below which no health effect is expected. MCLs, on the other hand, are enforceable guidelines that take into account both technological and cost limitations, while staying as close to the MCLGs as possible [1].

Concern about the feasibility of the plan for regulating new contaminants and the possibility that the 83 contaminants listed in the 1986 amendment may not represent high-priority contaminants led to a 1996 amendment that required EPA to set contaminant regulation priorities. These priorities were to be based on health effects data, occurrence information, and the estimated reduction in health risk provided by regulation [1]. Following this amendment, the EPA now earmarks drinking water contaminants for further investigation and possible regulation by first generating a contaminant candidate list (CCL). These contaminants are known or predicted to occur in drinking water and are known or predicted to cause adverse health effects. The EPA makes regulatory decisions on at least five CCL contaminants every five years [7]. Data used to evaluate potential health effects are obtained from epidemiological, clinical or case studies, *in vivo* or *in vitro* toxicological laboratory studies, and biological activity or effects models [8].

The 1996 amendment also led to the development of the Stage 1 Disinfectants/ Disinfection Byproducts Rule (DBPR), which was issued by EPA in 1998. This rule set an updated MCL for TTHM (0.080 mg/L), and created MCLs for bromate (0.010 mg/L), chlorite (1.0 mg/L), and HAA5 (0.060 mg/L), which is the sum of the five haloacetic acids, monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, monobromoacetic

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acid, and dibromoacetic acid. Bromate and chlorite are primarily by-products of ozone and chlorine dioxide treatment, respectively, while haloacetic acids are formed at highest levels during chlorination. Maximum residual disinfectant levels (MRDLs) were set at 4.0 mg/L as $Cl_2$ for both chlorine and chloramine [9]. Drinking water treatment plants' compliance with Stage 1 MCLs and MRDLs was measured using running annual averages of all monitored locations along the distribution system, meaning that if one location had a high contaminant or disinfectant level, this number could be balanced out by a lower level at another location. The Stage 2 DBPR, issued by EPA in 2006, maintained the MCLs and MRDLs outlined in the Stage 1 DBPR, but changed the calculation for compliance to a more stringent, locational running annual average. This rule, which is currently in effect, requires that the running annual average at each monitored location comply with the MCLs and MRDLs [10].

**B. Limitations of the Current Regulations**

In order to meet the MCLs for TTHM and HAA5 outlined in the Stage 2 DBPR, drinking water utilities are increasingly choosing disinfectants other than chlorine, such as chloramine. However, different disinfectants are associated with formation of different classes of DBPs, which may be more toxic than the regulated DBPs. While over 600 DBPs have been identified [11, 12], many DBPs remain unknown. For example, in a bench-scale study, approximately 60% of total organic halides (TOX) were unaccounted for in chlorinated water, while approximately 80% of TOX was unknown in chloraminated water [13]. Moreover, the occurrence levels and health effects of most known DBPs have not yet been evaluated [14]. While disinfection is necessary for the

elimination of pathogens, the EPA must continuously assess DBP formation associated with the various disinfectant technologies in order to reduce public health risk.

**Use of chloramination versus chlorination**

**A. Disinfectant technology**

Chlorine remains the most commonly used disinfectant in the U.S. and is added to water either as a gas or as a hypochlorite salt. Upon addition of chlorine gas to water, the following reaction takes place:

$$Cl_2 + H_2O \leftrightarrow HOCl + Cl^- + H^+$$

Hypochlorous acid (HOCl) is a weak acid ($pK_a \approx 7.5$) and dissociates into hypochlorite ion ($OCl^-$) and hydrogen ion. Hypochlorous acid and hypochlorite ion both inactivate microbes in water by denaturing enzymes or proteins, or by disrupting the cellular membrane, but hypochlorous acid is the stronger disinfectant. In the case of a hypochlorite salt, such as calcium hypochlorite ($Ca(OCl)_2$) or sodium hypochlorite ($NaOCl$), the same active species, HOCl, is formed upon addition to water [15].

Chloramine is produced by adding ammonia to hypochlorous acid, during which the following reactions can take place:

$$NH_3 + HOCl \leftrightarrow NH_2Cl + H_2O \qquad (1)$$
$$NH_2Cl + HOCl \leftrightarrow NHCl_2 + H_2O \qquad (2)$$
$$NHCl_2 + HOCl \leftrightarrow NCl_3 + H_2O \qquad (3)$$
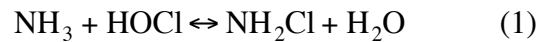
Drinking water utilities aim to produce monochloramine ($NH_2Cl$), which is the active disinfectant. The amounts of monochloramine ($NH_2Cl$), dichloramine ($NHCl_2$), and trichloramine ($NCl_3$) formed are influenced by pH and the ratio in which ammonia and chlorine are combined. For example, a $Cl_2$:N (w/w) ratio of 7 leads to formation of trichloramine, which is undesirable [15].

**B. Advantages/Disadvantages**

Chloramine provides a more stable residual than chlorine and leads to lower formation of regulated DBPs [15]. However, chlorine is much more effective at killing pathogens, with chloramine requiring 25 to 100 times longer inactivation periods to be as effective as chlorine at equivalent concentrations [16]. Because of this, water utilities that use chloramine typically first apply a primary disinfectant, such as chlorine, ozone, chlorine dioxide, or UV light, in order to kill pathogens, and then use chloramine as a secondary disinfectant to leave a residual in the distribution system [17].

While chloramine produces lower levels of the regulated THMs and HAAs, it is associated with increased formation of iodinated and nitrogenous DBPs. In a nationwide DBP occurrence study, iodo-THMs, which are not currently regulated, were found at highest concentrations (0.2-15 μg/L for individual compounds) in a plant that used chloramine without prechlorination. Iodo-acids were discovered in this same plant [18, 19]. Still, chloramine is associated with overall lower halogenated DBP formation, as indicated by TOX levels [13].

**Overview of toxicological assays**

**A. *Salmonella* Mutagenicity Assay**

The *Salmonella* test employs histidine requiring *S. typhimurium* strains to determine mutagenicity. Different strains possess different types of mutations, such as frameshift or base substitution mutations, that lead to this inability to produce histidine. When *Salmonella* cultures that have been treated with a suspected mutagen are plated on minimal media, only revertant colonies, which can produce histidine independently, will grow. The number of revertants corresponds to the ability of the chemical agent to

directly induce mutations. *S. typhimurium* assays are useful in that microsomal mono-oxygenases can be added to the bacterial suspensions to see if DBPs are metabolically activated, indirect-acting mutagens. Rat liver enzyme, S9, can be used for metabolic activation [20]. If a mutagen is also cytotoxic, then mutagenicity can be underestimated because dead cells will not grow on a plate as revertants. By measuring cytotoxicity in addition to mutagenicity, the sensitivity of the mutagenicity assay can be increased [21].

**B. Mammalian Cell Cytotoxicity and Genotoxicity Assays**

Because tests on bacteria may not be applicable to mammalian cells, Plewa et al. developed quantitative, comparative mammalian cell cytotoxicity and genotoxicity assays in Chinese hamster ovary (CHO) cells [22, 23]. In the cytotoxicity assay, different concentrations of a specific DBP in growth medium are applied to CHO cells in 96-well microplates, with the inclusion of a blank control column containing only medium and a negative control column containing only cells and medium. These plates are covered with AlumaSeal to prevent cross-contamination and placed in a 37°C incubator at 5% $CO_2$ for 72 hours, after which the wells are aspirated, the cells are fixed with methanol, and the cell membranes are stained with crystal violet. The plates are washed, 200 μL aliquots of deionized water are added to each well, and the plates are read on a microplate reader at 595 nm [23]. Reductions in absorbance as compared to the negative control correspond to reduced cell density due to the impact of the DBP on cell survival and growth kinetics [22].

The genotoxic effects of DBPs in CHO cells can be measured using the single-cell gel electrophoresis (SCGE) assay. In this assay, CHO cells are grown overnight in a 96-well microplate at 37°C, then washed with Hank's balanced salt solution (HBSS) and

treated with different concentrations of DBP in growth medium. Plates covered with

AlumaSeal are placed in a 37°C incubator at 5% $CO_2$ for 4 hours, after which the cells are

washed, and trypsin and EDTA solutions are added to detach the cells from the bottom of

the wells. An aliquot is taken for acute cytotoxicity measurement using trypan blue, and

the remaining volume is added to 1% low melting point agarose. Aliquots of this

suspension are placed on duplicate slides, which have previously been coated with 1%

normal melting point agarose and allowed to dry. A final layer of 0.5% low melting

point agarose is added, and the slides are placed in lysing solution overnight at 4°C to

remove the cellular and nuclear membranes. The slides are denatured in an

electrophoresis tank containing alkaline buffer and electrophoresed, and then neutralized

with Tris buffer, rinsed with deionized water, and dehydrated with methanol. Dried

slides are stored in a covered slide box until rehydration using deionized water and

staining with ethidium bromide. Slides are analyzed using a fluorescence microscope

with an image analysis system that can measure SCGE parameters, such as tail moment.

The tail moment value represents the density of DNA that has migrated away from the

nucleus multiplied by the distance traveled, and is a direct indicator of DNA damage [23].

The importance of using mammalian cells in toxicity studies was highlighted

when the same DBP compounds were analyzed using both CHO and *Salmonella* cells.

Although the cytotoxic and genotoxic rank order in CHO cells and *S. typhimurium* are

similar for the haloacetic acids, there are some discrepancies in cytotoxicity and

genotoxic/mutagenic potency values between CHO and bacterial cells, such as for the

DBP, 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX). Further analysis

confirmed that *S. typhimurium* cannot be used to quantitatively predict DBPs' effects in

mammalian cells. In fact, the *S. typhimurium* assay would not have found that bromoacetic acid is more cytotoxic and genotoxic than MX. This was only discovered by using CHO cell assays [23]. However, a recent study using a human-derived hepatoma line (HepG2) to measure genotoxicity of several DBPs with the SCGE assay reported some findings that were not coincident with the CHO results. For example, chloroacetic acid was not genotoxic in HepG2 cells, but was genotoxic in CHO cells. Also, di- and trichloroacetic acid were positive in HepG2 cells, but negative in CHO cells. These results further illustrate the differing sensitivities of cell lines [24].

Differences in sensitivity may in part be due to metabolic deficiencies. Much of the metabolic activity present *in vivo* is lost *in vitro*, so it is often desirable to use exogenous (e.g. S9 mix) or endogenous (e.g. P450 genes transfected to a cell line) systems to activate xenobiotic metabolism [25]. In a recent study, an exogenous S9 mix was developed that effectively activates nitrosamine DBP compounds such as N-nitrosodimethylamine (NDMA) in the CHO genotoxicity assay [26]. The importance of metabolic activation has also been highlighted for brominated THMs, which are activated in a *Salmonella* strain (RSJ100) that has been transfected with the rat theta-class glutathione S-transferase T1-1. A dose-dependent increase in revertant colonies was not observed in a control strain (TPT100) that lacks this transferase [27, 28].

**DBP toxicity studies**

**A. Single compound**

CHO assays have been used to assess the cytotoxicity and genotoxicity of over 60 individual DBPs [14]. Previous applications have shown that halonitromethanes are more cytotoxic and genotoxic than their regulated haloacetic acid counterparts. When

occurrence levels were taken into account as well, halonitromethanes were found to pose a bigger toxic threat in drinking water than the regulated haloacetic acids [29]. The CHO assays have also indicated that haloacetonitriles and haloacetamides are more cytotoxic and genotoxic than the regulated haloacetic acids [30, 31]. Structure-activity relationship analysis accompanying these studies also provides insight into the mechanisms of DBP toxicity, such as which functional groups are correlated with greater toxicity.

**B. Complex mixture**

While toxicological analysis of single compounds provides insight into individual mechanisms of DBP toxicity and helps shape prioritization of DBPs for regulation, unknown DBPs cannot be included in single compound studies. Complex DBP mixture studies are, therefore, critical to understanding toxicity related to real world DBP exposures. The *Salmonella* mutagenicity assay has been used to evaluate toxicity of drinking water extracts and concentrates for the past 30 years [14]. Drinking water extracts are typically prepared by concentrating water on an XAD resin and extracting with an organic solvent. DeMarini et al. found that chlorinated drinking water extracts had 1.5 to 1.8 times higher mutagenic potency than chloraminated extracts. However, use of XAD for concentration caused volatile DBPs to be lost, so these were not included in the extracts [32]. Another study investigated both mutagenicity and TOX levels in chlorinated water extracts prepared using XAD and found a high correlation (r=0.95) between the two parameters [33], indicating that perhaps a higher TOX level might explain the elevated mutagenic potency in the chlorinated extract.

In EPA's "Four-Lab Study," reverse osmosis (RO) was used to generate aqueous drinking water concentrates suitable for *in vivo* toxicological studies in rodents [34].

Bromide and iodide-spiked source water that had undergone coagulation, flocculation and clarification, followed by either chlorination or ozonation/post-chlorination, was concentrated by factors of 136x and 124x, respectively [35, 36]. One issue that arose was that volatile DBPs were lost during the concentration process, and so attempts were made to evaluate the DBP losses and spike these levels back into the waters before they were fed to the animals [36]. However, DBPs with concentrations below 2 $\mu$g/L were excluded from the spiking, as were nonhalogenated DBPs, which may also be toxicologically significant. Nevertheless, DBP analysis of the concentrates indicated that representative bromine and iodine-containing DBPs were formed, and that expected differences in DBP formation between the chlorinated and ozonated/post-chlorinated water were observed [37]. It was further confirmed that DBP levels were relatively stable over the 10-day rodent exposure. Drinking water extracts were also prepared using an XAD resin in order to ascertain differences in performance compared to RO concentration. DBP analysis of both XAD extracts and RO concentrates indicated that the RO concentration was similar or better than XAD extraction in concentrating most of the DBPs detected [37].

The XAD extracts and RO concentrates generated in the "Four-Lab Study" were analyzed with the *Salmonella* mutagenicity assay, and it was found that the RO concentrates exhibited less than 50% of the mutagenic response of the XAD extracts. The volatile (from the RO only) and non-volatile organics were more mutagenic in the chlorinated samples than the ozonated/post-chlorinated samples, with addition of S9 attenuating the mutagenic response [38]. The RO concentrate was also used in an *in vivo* developmental toxicity screen in Sprague-Dawley rats. This screen, which fed the

concentrates to rats on gestation days 6-16, did not detect any effects on prenatal or postnatal survival, or on pup weight, in either the chlorinated or ozonated/post-chlorinated treatment groups. It is possible that longer treatment times are required before any developmental effects are observed [39].

## C. Research gaps

Two-year cancer rodent bioassays are important for predicting human health effects of DBPs and shaping regulatory decisions, but these experiments are costly and it is not feasible to evaluate each of the hundreds of DBPs in this manner. In order to focus research efforts, DBPs have been prioritized for future carcinogenicity testing and other toxicity studies using occurrence levels and structure-activity relationships analysis [40]. However, the identity of many DBPs remains unknown and, therefore, these are not included in the prioritization process. As utilities switch to alternative disinfectants, the occurrence of DBPs in treated waters also changes, further complicating prioritization efforts. At the same time, single-compound DBP studies do not take into account the interactions that take place among a mixture of DBPs and other components in real water matrices, which could alter the toxic response. In order to address these concerns, there is significant interest in evaluating DBPs as complex mixtures, either as a real-world complex mixture—a sufficiently similar mixture that has been produced using a reproducible disinfection scenario, or a defined mixture [41].

**Statement of research question and objectives**

This project sought to address the following research questions:

1) Are DBPs produced by free chlorine more cytotoxic than those produced by monochloramine?

2) If so, are differences in toxicity correlated with the presence of specific DBPs, including the regulated DBPs, or the amount of total organic halogen (TOX)?

The specific research objectives were as follows:

(I) To compare the NCM460 human colon cell cytotoxicity of DBPs formed during chlorination to those formed during chloramination using simulated drinking water prepared under each of the following conditions (targeting 3 mg/L as $Cl_2$ residual free chlorine or monochloramine):

- 24 hour contact time with chlorine or monochloramine

- Spiked with iodide prior to 24 hour contact time with chlorine or monochloramine

- Spiked with nitrate prior to 24 hour contact time with chlorine

- 72 hour and 96 hour contact times with chlorine or monochloramine to evaluate changes that might occur in the distribution system

(II) To measure TOX and representative known DBPs in the chlorinated and chloraminated samples and relate to observed cytotoxicity.

Hypothesis: Given the reduced TOX levels, which are indicative of overall lower DBP formation, typically observed in chloraminated waters as compared to chlorinated waters [13], it is predicted that the chloraminated water will be less cytotoxic than the chlorinated water in NCM460 human colon cells.

In order to test this hypothesis, the experimental approach was to:

(a) Prepare a simulated drinking water source

- Using commercially available NOM RO isolates, which are freeze-dried solids derived from source waters, and can be rehydrated to obtain desired dissolved organic carbon (DOC) concentrations

(b) Evaluate isolates from two different natural source waters, Nordic Lake and Suwannee River, in order to assess how cytotoxic response and DBP formation may vary based on source

(c) Evaluate the cytotoxicity of each of the following matrix components separately, in order to determine concentrations that the NCM460 cells could tolerate:

- Each type of NOM by itself

- Phosphate buffer used to control pH at 7.1 during disinfection

- Residual free chlorine or monochloramine in laboratory-grade water (in the presence of non-cytotoxic concentration of phosphate buffer)

- Iodide spike (in NOM matrix)

- Nitrate spike (in NOM matrix)

(d) Use selected DOC concentrations for Suwannee River and Nordic Lake NOM (goal was to use as high a DOC concentration as possible in order to later generate a high level of DBPs) and desired spiking conditions to run chlorine or chloramine demand tests over designated contact times (24, 72 or 96 hours) in order to determine the disinfectant dose required to leave a target 3 mg/L as $Cl_2$ residual (relevant to full-scale disinfected waters and determined to not be cytotoxic to NCM460 cells)

(e) Prepare disinfected samples using calculated doses from (d) and measure DBPs, TOX, and cytotoxicity

(f) Analyze chemical and toxicological data and determine how chloraminated samples differed from chlorinated ones and if TOX and specific DBP levels are correlated with higher cytotoxicity

# CHAPTER 2

## MATERIALS AND METHODS

### Chemicals and Reagents

Nordic Lake and Suwannee River Natural Organic Matter (NOM) Reverse Osmosis isolates were purchased from the International Humic Substances Society (St. Paul, MN).  Laboratory grade water (LGW) was prepared using a Dracor (Durham, NC) system that passes influent 7 MΩ house deionized water through a 1 μm pore size filter followed by an activated carbon resin, removing residual disinfectants and reducing the total organic carbon (TOC) level to below 0.2 mg C/L.  The system also includes a mixed bed ion exchange resin, which further removes ions to 18 MΩ.  Phosphate buffer solutions were prepared using sodium phosphate monobasic monohydrate and sodium phosphate dibasic heptahydrate, both certified ACS grade from Fisher Scientific (Fair Lawn, NJ).  Nitrate and iodide spikes were prepared using sodium nitrate (99.3%, certified ACS grade) from Fisher Scientific and potassium iodide (99.0%, certified ACS grade) from EMD Chemicals (Gibbstown, NJ), respectively.  L-ascorbic acid (SigmaUltra grade) was obtained from Sigma (St. Louis, MO), sodium azide was obtained from Acros (NJ), and both ammonium sulfate (granular) and sodium sulfite (anhydrous, granular) were certified ACS grade and obtained from Mallinckrodt (Paris, KY).  Sodium hypochlorite solution (5.65-6%, laboratory grade) was purchased from Fisher Scientific, and stored in the dark at 4°C.  Ammonium chloride (granular, 99.8%, certified ACS grade) was obtained from Mallinckrodt.  Sodium thiosulfate and sodium

hydroxide (50% w/w) were certified ACS grade, and concentrated sulfuric acid and hydrochloric acid were certified ACS plus grade, all from Fisher Scientific. DPD free chlorine, DPD total chlorine, and monochlor-F reagent powder pillows were purchased from the Hach Company (Loveland, CO). Diiodoacetamide (99%) was obtained from CanSyn Chem. Corp. (Toronto, ON), and a stock solution was prepared in methanol suitable for purge and trap analysis from Sigma-Aldrich (St. Louis, MO). Methyl *tert*-butyl ether (>99.99%, OmniSolv MtBE) was obtained from EMD Chemicals.

The normal derived colon mucosa (NCM460) human epithelial cell line used for the growth inhibition assay (GIA) was obtained from INCELL Corporation (San Antonio, TX), as were M3:10A cell culture medium complete and SMX supplement mix. Phosphate buffered saline (PBS pH 7.4, calcium chloride and magnesium chloride-free), TrypLE Express (with phenol red), Fungizone Amphotericin B (250 μg/mL), fetal bovine serum (FBS), and minimum essential medium (MEM) powder (with Earle's salts and L-glutamine but no sodium bicarbonate) were obtained from GIBCO Invitrogen (Grand Island, NY). Sodium bicarbonate (cell culture tested) and 0.4% trypan blue were purchased from Sigma, and dimethyl sulfoxide and methanol, both HPLC grade, were purchased from Fisher Scientific. Isoton II diluent was obtained from Beckman Coulter (Fullerton, CA). Crystal violet was obtained from Fluka (St. Louis, MO).

**Cell line used in this project**

This project used normal derived colon mucosa (NCM460) cells from normal human colon epithelium. The doubling time of these cells is approximately 32 hours, and normal growth characteristics are expressed [42]. The ratio of theta-class glutathione S-transferase T1-1 (GSTT1-1) activity to cytochrome P450 activity for the NCM460 cells is similar to that of rat large intestine and higher than that of rat liver [43, 44]. Because

GSTT1-1 is associated with activation of THMs and cytochrome P450 is associated with detoxication [44], this strain is thought to favor activation of THMs. The cytotoxic response of these cells to about 50 regulated and unregulated DBPs has been measured using a growth inhibition assay adapted from the cytotoxicity assay used by Plewa [23]. When responses were compared to those obtained using the CHO cell line, statistically significant concordances were observed [45].

**NOM Source Preparation by IHSS**

Nordic Lake and Suwannee River natural organic matter (NOM) reverse osmosis isolates used for this study were obtained from the International Humic Substances Society (IHSS). These isolates could be used to make up waters with high carbon concentrations, which are necessary to generate a high level of DBPs and observe cell responses. Nordic Lake is a drinking water reservoir in Vallsjoen, Skarnes, Norway. IHSS collected water samples from there between October 29 and November 3, 1997. The DOC concentration of the water was 10.7 mg/L as C and the pH was 5.6. Suwannee River rises in Okefenokee Swamp in southern Georgia, USA, and flows southwest to the Gulf of Mexico. DOC concentrations range from 25 to 75 mg/L as C and pH is less than 4.0. The NOM is isolated from water using reverse osmosis, then is desalted and freeze-dried [46].

**Utility of TOX measurement**

Total Organic Halogen (TOX) represents all of the halogenated organics present in the water. When used in concert with DBP occurrence levels, the percent of the TOX that is made up of known, quantified DBPs can be calculated, along with the remaining unknown, or unidentified, portion of TOX. While TOX does not include inorganic or non-halogenated DBPs, it is a useful metric for determining how well the known DBPs

approximate the composition of the DBP mixture as a whole.  For example, the Nationwide DBP Occurrence Study that surveyed 12 full-scale drinking water treatment plants that utilized varying disinfectants, including chlorine, chloramine, ozone and chlorine dioxide, found that the median value of known TOX was only 30% [19].  The high percentage of unknown TOX indicates that many DBPs still need to be identified, and that the toxicity of real world DBP mixtures that contain this unknown fraction needs to be investigated.

**Glassware Preparation**

Glassware was cleaned by soaking overnight in Alconox powder detergent solution, rinsing with tap water, soaking in a 10% ACS-grade nitric acid bath, and rinsing with LGW.  Non-volumetric glassware was dried in a 180°C oven for 24 hours, while volumetric glassware was rinsed with methanol and dried at room temperature.  Plastic caps and polytetrafluoroethylene (PTFE)-lined silicone septa were cleaned by soaking in Alconox solution, rinsing with LGW followed by methanol, and drying at room temperature.

**Sample Preparation**

Nordic Lake and Suwannee River NOM stock solutions were each prepared by dissolving the NOM reverse osmosis isolates in LGW and filtered under vacuum through a 0.45 μm pore size nylon membrane filter with a 47 mm diameter (Whatman International, Maidstone, England) using a glass Millipore filtration system (Bedford, MA).  Immediately after the two stock solutions were prepared, a 1:50 dilution of each was made in LGW, and the dissolved organic carbon (DOC) levels were measured using a Shimadzu TOC-V$_{CPH}$ Total Organic Carbon Analyzer with a TNM-1 Total Nitrogen Measuring Unit (Shimadzu Corp., Atlanta, GA) following Standard Method 5310 [47].

Samples were analyzed in duplicate, and two out of three 100 μL injections were required to have a relative percent difference of <2%. A standard operating procedure for TOC analysis is provided in Appendix A.

For the first growth inhibition assay experiment evaluating the two types of NOM, stock solutions were prepared with DOC concentrations that exceeded the desired final concentration of 120 mg/L as C and were then diluted with LGW as necessary during sample preparation. Subsequently, the percent carbon by weight for each NOM was used to determine the masses of NOM required to prepare stock solutions with DOC levels of approximately 120 mg/L as C. The percent carbon by weight for Suwannee River NOM and Nordic Lake NOM was approximately 39.3% and 27.5%, respectively. For the first experiment comparing the two types of NOM, no buffer was added to control the pH, but for later experiments in which 20 mM phosphate buffer was used to control the pH at 7.1, the phosphate buffer salts were added directly to the NOM stock solution and the pH was adjusted using 10M NaOH prior to filtration and preparation of a 1:50 dilution for TOC analysis. NOM stock solutions were stored in amber glass bottles at 4°C, and were typically used within one month of preparation. When using NOM stock solutions stored for over one month, the solutions were filtered and the TOC levels in a 1:50 dilution were measured again to confirm stability. Stock solutions of sodium nitrate and potassium iodide were also prepared in LGW and stored at 4°C.

Prior to using the sodium hypochlorite (NaOCl) stock solution for sample dosing or monochloramine preparation, its concentration was measured according to Standard Method 4500-Cl B [40]. A chlorine working solution targeted at ~1000 mg/L as $Cl_2$) was prepared by adding 2 mL of the NaOCl stock to a 100 mL volumetric flask and bringing

it to volume with LGW.  To a 125 mL Erlenmeyer flask containing a stir bar, 40 mL of

working solution was added, followed by ~10 drops glacial acetic acid to adjust the pH to

between 3 and 4, and 1 g potassium iodide.  The resulting brown solution was titrated

with 0.1 N sodium thiosulfate ($Na_2S_2O_3$, prepared in LGW) until a pale yellow color

appeared, at which point 1 mL of starch was added dropwise to the solution.  The

resulting dark purple solution was titrated with further 0.1 N $Na_2S_2O_3$ until it turned clear.

This titration was repeated with another 40 mL of working solution, and the average

volume of $Na_2S_2O_3$ required was used to calculate the chlorine stock solution

concentration, as follows:

$$mg/L\,as\,Cl_2 = \frac{(X\,mL\,Na_2S_2O_3\,used\,to\,titrate)(0.1N\,Na_2S_2O_3)(35.45g\,Cl_2/mol)(1000mg/g)(100mL\,LGW/2mL\,Cl_2)}{(40\,mL\,sample\,titrated)}$$

This measurement was carried out approximately once a month.

A monochloramine stock solution was prepared fresh daily by first making up 100

mL of a 24 mM ammonium chloride solution in LGW and adjusting the pH to 8 with 1M

NaOH and transferring to a beaker.  Using the NaOCl stock solution concentration

determined previously, for example 55390 mg/L as $Cl_2$, the volume of NaOCl stock

required to obtain an $N:Cl_2$ molar ratio of 1.2:1 with a final monochloramine

concentration of 1400 mg/L as $Cl_2$ was calculated, as follows:

$$V_{Cl_2}(mL) = \frac{1400mg/L\,as\,Cl_2 \times 100mL}{55390mg/L\,as\,Cl_2\,measured} = 2.5mL$$

This calculated volume of NaOCl stock solution was added dropwise to the 24 mM

ammonium chloride solution while rapidly stirring.  The pH adjustment to 8 and slow

addition of NaOCl are necessary to avoid dichloramine formation.  The 1.2:1 $N:Cl_2$ molar

ratio was used in order to avoid breakpoint chlorination reactions, which occur as the

N:Cl$_2$ ratio decreases, and involve reduction of residual disinfectant and production of nitrogen gas, nitrate, and nitrogen trichloride. A 1:20 dilution of the monochloramine stock solution was made in LGW, and the monochloramine and dichloramine concentrations were determined from absorbance measurements taken at 245 nm and 295 nm wavelengths using a Hitachi U-3300 UV-VIS spectrophotometer (Hitachi, Tokyo, Japan**)**. Given a path length ( $l$ ) of 1 cm and molar absorptivity ($\varepsilon$) values for monochloramine and dichloramine at each of the two wavelengths, the concentrations of monochloramine (c$_1$) and dichloramine (c$_2$) were calculated using two simultaneous equations (one for each wavelength) in the following form, from Beer's Law:

$$A(\lambda) = c_1 l\varepsilon_1(\lambda) + c_2 l\varepsilon_2(\lambda)$$

The level of free chlorine present in a 1:1000 dilution of the monochloramine stock solution in LGW was measured using a Hach kit, as described under the Disinfectant Residual Measurement heading. For example, in a 1295 mg/L as Cl$_2$ monochloramine stock solution, the free chlorine concentration was relatively low at 90 mg/L as Cl$_2$. The monochloramine stock solution was stored at 4°C until use on the same day.

Disinfectant demand tests for each NOM were carried out over 24 hours for each sample condition. Three different doses of disinfectant were used within each test, and these were plotted versus the corresponding residual. The line equations for the plots were then used to calculate the disinfectant doses required to obtain the desired 3 mg/L as Cl$_2$ residual. For the 72 and 96 hour chloramine treated samples, a formal demand test was not carried out, and the chloramine dose used to obtain residuals of approximately 5 and 3 mg/L as Cl$_2$, respectively, was determined using trial and error. Disinfected samples were placed in headspace-free and demand-free glass vials with open top plastic

caps and PTFE-lined silicone septa, and were kept at 25°C in the dark for the duration of the treatment time. Demand-free glassware was prepared by filling with 20 mg/L as $Cl_2$ sodium hypochlorite solution made from the stock in LGW, letting sit for 24 hours, rinsing with LGW, and drying.

Disinfected NOM samples were prepared in a 70 mL volume by measuring out the NOM stock solution with a graduated cylinder into 125 mL amber glass bottles, adding chlorine or chloramine doses as determined from the demand tests, covering with open top plastic caps and PTFE-lined silicone septa, inverting slowly and transferring headspace-free to 60 mL demand-free glass vials. These vials were then stored as described for the demand tests. Note that although the chloramine doses required to leave a 3 mg/L as $Cl_2$ residual were lower than the chlorine doses, the monochloramine stock solution had a much lower concentration (~1400 mg/L as $Cl_2$) than the chlorine stock solution (~55390 mg/L as $Cl_2$) and therefore the volume of monochloramine stock added for the chloraminated samples slightly diluted the NOM concentration. In order to maintain the 70 mL final sample volume for the chloraminated samples, the volume of NOM stock solution was adjusted to account for the added volume from the monochloramine stock. For example, 1.6 mL monochloramine stock solution was added to 68.4 mL Nordic Lake NOM during preparation of the chloraminated Nordic Lake sample. After the treatment time, 12 mL of sample was used for the growth inhibition assay, 45 mL was used for disinfection by-product (DBP), total organic halogen (TOX), and total organic chloride/bromide/iodide (TOCl/Br/I) analysis, and the remaining volume was used to measure the residual disinfectant.

The 45 mL of sample used for chemical analysis was first added to 180 mL LGW (a 1:5 dilution) so that the DBP concentrations would be within the calibration range. These volumes were measured into a 250 mL amber glass bottle using 50 and 250 mL graduated cylinders, respectively, with the disinfected sample transferred by pouring slowly onto the side of the cylinder or bottle in order to minimize volatile losses. The glass bottle was capped and slowly inverted to mix the diluted sample, which was immediately transferred to vials prepared for the individual analytical methods later described.

**Disinfectant Residual Measurement**

Free and total chlorine residuals were measured in 10 mL quartz glass cells using the Hach Chlorine Pocket Colorimeter (Hach Co., Loveland, CO). Method 8021 was used to measure free chlorine (0 to 2.00 mg/L $Cl_2$) with DPD free chlorine reagent powder pillows, and Method 8167 was used for total chlorine (0 to 2.00 mg/L $Cl_2$) with DPD total chlorine reagent powder pillows, both methods equivalent to Standard Method 4500-Cl G [40]. Monochloramine residuals (0-4.50 mg/L $Cl_2$) were measured in 10 mL plastic cells using the Hach DR/890 Datalogging Colorimeter (Hach Co., Loveland, CO) and Method 10171, an indophenol method, with monochlor-F reagent powder pillows. When residuals exceeded the range of the colorimeters or sample volume was limited, dilutions were made in LGW using 10 mL volumetric flasks. The consistency between the U-3300 spectrophotometer and the Hach DR/890 in measuring monochloramine concentrations was assessed by making a 1:1000 dilution of the monochloramine stock solution in LGW and measuring the absorbance with the Hach kit, which automatically selects the wavelength upon choosing a particular method. In one such evaluation, the

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monochloramine concentration measured with the Hach kit was 1410 mg/L as $Cl_2$, which was relatively close to the 1295 mg/L as $Cl_2$ concentration measured on the U-3300.

**Preliminary Experiments**

Before analyzing disinfected NOM samples with the growth inhibition assay (GIA), the effects of the sample matrix on NCM460 human colon cell growth were evaluated. The samples that were tested included phosphate buffer (up to 50 mM) in LGW at pH 7.1, the NOM itself (up to 120 mg/L as C), as well as iodide (up to 5 mg/L as I) and nitrate (up to 498 mg/L as N) spikes in the presence of Nordic Lake NOM (up to 112 mg/L as C) and up to 20 mM phosphate buffer, once it was determined that 20 mM phosphate buffer at pH 7.1 did not adversely affect cell growth. Unquenched chlorine (up to 36.9 mg/L as $Cl_2$) and monochloramine (up to 43 mg/L as $Cl_2$) residuals in LGW with 20 mM phosphate buffer were also analyzed with the GIA. From this experiment, it was decided that chlorine or chloramine residuals of up to 5 mg/L as $Cl_2$ do not adversely affect cell growth, and residuals were targeted to not exceed this level during subsequent experiments.

**Growth Inhibition Assay**

Unless otherwise noted, solutions were sterile and procedures were carried out in a biological safety hood. On the first day of the growth inhibition assay (GIA), 25 cm$^2$ culture flasks of NCM460 cells were removed from a 37°C incubator, the media was aspirated, and the flasks were rinsed with 5 mL phosphate buffered saline (PBS). The PBS was then aspirated, 5 mL TrypLE Express was added, and the flasks were placed in a 37°C incubator for 10 to 15 minutes, until cells were detached from the bottom of the flasks. The trypsin reaction was stopped by adding 1 mL of M3:10A medium with 0.5% fungizone to each flask and pipetting rapidly to mix. The cells from each flask were

added to a 50 mL centrifuge tube, which was briefly vortexed.  The tube was centrifuged

(Fisher Scientific Marathon 3000R centrifuge, Thermo IEC, Needham Heights, MA) at

800 rpm and 4°C for 5 minutes.  The supernatant was aspirated and the cell pellet was

resuspended in 4 to 10 mL of M3:10A medium with 0.5% fungizone.  A 1:100 dilution of

the cell mix was made in Isoton solution, and the cells were counted using a Z1 Dual

Coulter Particle Counter (Beckman Coulter, Fullerton, CA).   Cell viability was

determined by adding an aliquot of cell mix to an aliquot of 0.4% trypan blue and

counting at least 100 cells using a hemocytometer (Hausser Scientific, Horsham, PA) and

a Nikon Diaphot Microscope (Nikon Instruments, Melville, NY).  Based on the

concentration of viable cells, the volume of cell mix needed to obtain a concentration of

$5 \times 10^4$ cells/mL in a 20 mL volume was calculated and added to 20 mL of M3:10A

medium with 0.5% fungizone.  The resulting cell suspension was vortexed, poured into a

reagent reservoir, and 200 μL added to each well ($1 \times 10^4$ cells/well) in columns 3 through

11 in a sterile, 96 well cell culture flat bottom plate with a low evaporation lid (Corning

Inc., Corning, NY).  To the column 2 wells, 200 μL M3:10A medium with 0.5%

fungizone was added to constitute the blank.  Covered plates were placed in a 37°C

incubator overnight.

On the second day of the GIA, samples were prepared in cell culture medium and

placed on the cells in a range of concentrations.  For standard compounds, such as the

positive control diiodoacetamide, working solutions were prepared in M3:10A medium

with 0.5% fungizone, and then dilutions of these were made, also in M3:10A with

fungizone.  However, for the NOM samples, preparing a working solution with M3:10A

medium was undesirable because it would dilute the carbon concentration considerably

and diminish the toxicological response.  The NOM samples were therefore combined with dry medium and a relatively small volume of supplements.  The highest sample concentration corresponded to an 80% dilution of the original sample.  In these cases, a "Minimal Essential Medium (MEM) blank" solution was first prepared for use in dilutions of the sample.  For a 120 mL MEM blank solution (enough for six 96-well plates, or three duplicate samples), 1.14 g MEM and 264 mg $NaHCO_3$ were added to 96 mL LGW.  The pH was adjusted to 7.1~7.2 using 2M HCl, and 12 mL Fetal Bovine Serum (FBS) and 12 mL SMX supplements mix were added.  The solution was vacuum filtered using a Nalgene 0.2 μm pore size, 50 mm diameter NYL filtration unit (Nalge Nunc International, Rochester, NY).  An "MEM concentrate" solution was then prepared for combination with disinfected samples.  To prepare 14 mL MEM concentrate (enough for three samples), 665 mg MEM and 154 mg $NaHCO_3$ were added to 7 mL sterile FBS and 7 mL sterile SMX, the pH was adjusted to 7.1~7.2, and the solution was filtered using a Millipore Steriflip 0.22 μm pore size vacuum filtration unit.  For a 15 mL final sample volume, 12 mL of sample (e.g. chlorinated Nordic Lake NOM) was combined with 3 mL of MEM concentrate.  Dilutions of the sample, now containing MEM, were then prepared using the MEM blank solution.  When running non-disinfected samples as in the preliminary experiments involving phosphate buffer, NOM itself, and NOM with iodide or bromide spikes, loss of volatile DBPs was not a concern, so instead of using an MEM concentrate, the sample was combined directly with the MEM and $NaHCO_3$, pH adjusted, and filtered upon addition of FBS and SMX.

During cell treatment, the old media was removed on sterile gauze, and 200 μL aliquots were placed in each well.  Columns 2 and 3 contained only MEM blank solution,

and columns 4 to 11 contained increasing concentrations of sample, with one sample concentration per column. The wells were covered with Alumaseal II (Research Products International) and plates were placed in 37°C incubator for 72 hours. After this period, plates were stained with crystal violet at the lab bench, as now described. The plates were taken out of the incubator, the media was removed on a gauze mat, and wells were rinsed with 200 μL PBS. The cells were then fixed to the bottom of the wells by adding 100 μL methanol to each well and letting it sit for 20 minutes. The methanol was removed, and 100 μL of 1% crystal violet in methanol was added for 20 minutes. The plates were rinsed in running tap water until no dye was observed when tapping the plate on a clean gauze mat. After the plates were allowed to dry, 50 μL dimethylsulfoxide was added to each well, the plates were wrapped in foil and placed on a shaker at 110 rpm for 30 minutes. The absorbance values were measured using a Wallac Victor 1420 Multilabel Counter microplate reader at 600 nm (Perkin Elmer Wallac Inc., Gaithersburg, MD). The dates on which the different samples were analyzed using the GIA are shown in Table 1.

**GIA Data Handling**

Each sample was run on duplicate plates, which were prepared on the same day, except for the iodide-spiked UV-treated and chloraminated sample, which was run in duplicate on two separate days (four plates total), with each column (8 wells) on the plate containing a different concentration of sample (1st column contained only medium, 2nd column contained only cells and medium). Each point on the GIA plot therefore represents n=16 replicates. For each plate, the average absorbance value of the background (only medium) was subtracted from the absorbance values of all the other wells. Then, the average absorbance value of the negative control (only cells and

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medium, no sample) was set as 100% cell growth, and the absorbance values of the remaining wells, which contained sample, were divided by this average absorbance value of the control to get cell density values as "percent control." The background-subtracted absorbance values of the control wells were also divided by this average absorbance value of the control to get cell density values as "percent control," in order to ascertain the variation amongst the control wells. By correcting the data for each plate using absorbance values from its own negative control wells, any variations in growth that might occur between plates are taken into account, allowing data from duplicate plates to be combined. The 16 cell density values for each concentration, including the negative control, were placed in SigmaPlot 11.0 (Systat Software, San Jose, CA), where a one-way analysis of variance (ANOVA) test was used to determine if the sample induced a significant cytotoxic response. If a significant $F$ value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was carried out to determine the lowest concentration at which a cytotoxic response was observed. The Holm-Sidak test involves comparing the 16 cell density as percent control values for the control wells to the 16 cell density as percent control values for each of the concentrations, and testing the null hypothesis that there is no difference between these values for the control wells and the wells containing a particular sample concentration. The mean cell density values were then plotted versus concentration, which was on a log scale, to obtain a dose-response curve. The curve was fit using three parameter sigmoidal regression, and the $R^2$ value represents how much the data varies from the sigmoidal model. Error bars represent the standard deviation. Two-way ANOVA was used to compare mean cell density values among the disinfected samples at each concentration level, with statistical

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significance (p<0.0001) denoted with an asterisk above that concentration level on the figure.

**Halogenated Volatile DBP Extractions**

      The 1:5 dilution of disinfected NOM described in the Sample Preparation section was added headspace-free to a 60 mL vial and quenched with 1.95 mg L-ascorbic acid. This amount was added as an aliquot of 19.5 mg/mL ascorbic acid solution, which was prepared in LGW in a 5 mL volumetric flask. The pH was adjusted to 3~3.5 using 1N $H_2SO_4$, which was prepared from the concentrated acid in LGW. These samples were stored at 4°C until analysis, which unless otherwise noted in the results, took place within 24 hours of quenching. Halogenated volatiles and haloacetamides were analyzed in 30 mL sample aliquots using liquid-liquid extraction with MtBE, followed by analysis on a Hewlett Packard 5890 Series II gas chromatograph with an autosampler/autotower injector, HP-1 (Agilent Technologies, Palo Alta, CA) capillary column (30m X 0.25mm id X 1μm film thickness), and a Hewlett-Packard Model Electron Capture Detector (ECD), according to the standard operating procedure provided in Appendix A. The DBPs extracted included THM4, six iodo-THMs, four haloacetonitriles (trichloro-, dichloro-, bromochloro-, dibromo-acetonitrile), haloketones (1,1-dichloropropanone, 1,1,1-trichloropropanone), chloral hydrate, halonitromethanes (trichloro-, tribromo-, bromodichloro- and dibromochloro-nitromethane), and haloacetamides (eleven chloro-, bromo- and iodo-substituted).

**Cyanogen Chloride Extraction**

      The 1:5 dilution of disinfected NOM described in the Sample Preparation section was added headspace-free to a 60 mL vial and quenched with 1.95 mg L-ascorbic acid, as previously described for the halogenated volatile DBP extraction. The pH was adjusted

to 3~3.5 using 1N $H_2SO_4$. These samples were stored at 4°C until analysis for cyanogen chloride using liquid-liquid extraction with MtBE followed by GC-ECD analysis according to the standard operating procedure provided in Appendix A. The aqueous sample for cyanogen chloride analysis was prepared separately from the sample for halogenated volatiles analysis because the extraction procedure for cyanogen chloride has specific requirements such as keeping the samples on ice and at a lower pH to stabilize the compound. Halogenated volatiles analysis must be completed within 24 hours of quenching, while cyanogen chloride analysis can be carried out at a later date.

**Haloacetic Acids Extraction**

The 1:5 dilution of disinfected NOM described in the Sample Preparation section was added headspace-free to a 40 mL vial and quenched with 8 grains of ammonium sulfate. For sample preservation, 2 mg sodium azide was also added as an aliquot of 20 mg/mL sodium azide solution that was prepared in LGW in a 25 mLvolumetric flask. These samples were stored at 4°C until analysis. Nine haloacetic acids were analyzed in 20 mL sample aliquots using a liquid-liquid extraction with MtBE followed by derivitization with diazomethane and analysis by GC-ECD according to the standard operating procedure provided in Appendix A. The haloacetic acids measured included chloro-, bromo-, dichloro-, trichloro-, and dibromoacetic acid, which make up HAA5, as well as bromochloro-, bromodichloro-, dibromochloro-, and tribromoacetic acid, which together with the HAA5 compounds make up HAA9.

**Total Organic Halogen (TOX) and Speciation Analysis**

The 1:5 dilution of disinfected NOM described in the Sample Preparation section was added headspace-free to two 25 mL vials and quenched with 0.534 mg of sodium sulfite, which was added as an aliquot of 5.34 mg/mL sodium sulfite solution that was

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prepared in LGW in a 5 mL volumetric flask.  These samples were stored at 4°C until

analysis for total organic halogen (TOX) and its speciation, total organic

chloride/bromide/iodide, according to the standard operating procedures provided in

Appendix A.

# CHAPTER 3

## RESULTS

**Preliminary Growth Inhibition Assay Experiments**

The two types of NOM used in this project, Nordic Lake and Suwannee River, were prepared in LGW in the absence of phosphate buffer and tested for cytotoxicity up to a concentration of 96 mg/L as C (Figure 1). Note that this highest concentration was generated upon addition of FBS and SMX supplements mix to a 120 mg/L as C sample, resulting in an 80% dilution, as described in the Methods chapter. While the y-axes in the dose-response curves are properly labeled "cell density as percent control," for the sake of brevity, the cell density values discussed here will only be referred to as "cell density." Discussions on statistical significance refer to the results of the one-way ANOVA test and/or the Holm-Sidak multiple comparison versus the control group analysis, also described previously. At 96 mg/L as C, the cell density was 82±10% for the Nordic Lake NOM, and 91±17% for the Suwannee River NOM. The results of the Holm-Sidak test indicated that the growth inhibition observed at 96 mg/L as C for Nordic Lake NOM was significantly different from that observed in the control group (100±13% cell density), but that the growth inhibition observed at that same level for Suwannee River NOM was not significantly different from the control (100±9% cell density). While the highest DOC concentration of Nordic Lake NOM did generate a significant cytotoxic response, the statistical analysis was not completed until after disinfection experiments were already carried out, so the approximately 96 mg/L as C level was still

used for the disinfection experiments.  It is also important to note that while it may seem

that the Nordic Lake NOM is more cytotoxic than the Suwannee River NOM at the

highest concentration used, the 96 mg/L as C concentration represents an approximately

nine-fold concentration factor over the Nordic Lake source water (DOC level=10.7 mg/L

as C) and only about a two-fold concentration factor over the Suwannee River source

water (for Suwannee River, the IHSS reports a DOC range of 25-75 mg/L as C, so 50

mg/L was used for this calculation).  This difference in concentration factor may also

play a role in the differences in cytotoxicity later observed between the two NOM types

upon disinfection.

The cytotoxicity data for the phosphate buffer experiment are shown in Figure 2.

Although at lower concentrations (5 mM and below) of buffer, it appeared that there may

be stimulation of cell growth (110±10% cell density at 5 mM phosphate buffer), the cell

density values at these levels were not statistically different from the control (100±8%

cell density).  At higher concentrations (20 mM and above), however, there were

significant reductions in cell density (86±13% cell density in the wells containing 20 mM

phosphate buffer).  At the 15 mM phosphate buffer concentration, 94±13% cell density

was observed, and this was not statistically different from the control.  It was, therefore,

decided to use 20 mM phosphate buffer to control the pH at approximately 7.1 during

disinfection experiments because the 20 mM phosphate buffer is ultimately diluted to 15

mM upon addition of SMX and FBS when preparing the samples for the GIA.

The cytotoxicity of the disinfectants, chlorine and chloramine, was then tested

(Figure 3), with the presence of 20 mM phosphate buffer at pH 7.1 in each of the original

samples, prior to addition of SMX and FBS as described above.  For chlorine, at 3.69

mg/L as $Cl_2$, there was $109\pm12\%$ cell density, which was not statistically different from the control ($100\pm7\%$ cell density), but at 7.38 mg/L as $Cl_2$, there was $118\pm11\%$ cell density, which was a significant increase as compared to the control, as indicated by Holm-Sidak analysis. For chloramine, however, none of the concentrations tested, even the highest, 34.4 mg/L as $Cl_2$ ($96\pm18\%$ cell density), produced significant reductions or increases in cell density versus the control ($100\pm15\%$ cell density), also as indicated by the Holm-Sidak test. In the proceeding 24-hour disinfection experiments, the disinfectant residual was targeted at 3 mg/L as $Cl_2$ in order to minimize possible stimulation of cell growth due to the presence of unquenched disinfectant. This target residual also meets the U.S. EPA's maximum residual disinfectant level for chlorine and chloramine, which is set at 4.0 mg/L as $Cl_2$ [10].

The cytotoxicity of nitrate and iodide spikes in the presence of Nordic Lake NOM (up to 112 mg/L as C) and phosphate buffer (up to 20 mM) was then evaluated, in order to determine the level of spiking that the cells could tolerate. Suwannee River NOM was not used for experiments involving spiking due to an apparent increase in cell density with exposure to increasing concentrations of chloraminated Suwannee River NOM, which was not readily explained. The cytotoxic response for nitrate-spiked Nordic Lake NOM versus nitrate concentration is shown in Figure 4. Note that the NOM and phosphate buffer concentrations were diluted along with the nitrate concentration. The cell density was $101\pm13\%$ at 100 mg/L as N, and $87\pm12\%$ at 149 mg/L as N, the latter of which represented a significant reduction as compared to the control ($100\pm15\%$). At the highest nitrate concentration, 398 mg/L as N, there was only $39\pm6\%$ cell density. Although up to 125 mg/L as N would have been acceptable in the original sample, due to

the 80% dilution that takes place during GIA sample preparation, it was decided to use 100 mg/L as N for the nitrate-spiked disinfection experiments.  Figure 5 shows the cytotoxic response for the Nordic Lake NOM spiked with nitrate up to 398 mg/L as N as shown previously in Figure 4, but plotted versus DOC concentration, along with the response for Nordic Lake NOM alone, shown previously in Figure 1.  Together these response curves indicate that the cytotoxicity observed in the combined sample at the third and higher dilutions is due to the presence of nitrate, not due to the presence of NOM.  The use of a 112 mg/L as C concentration of DOC in this study represents an over 10-fold concentration factor from the original Nordic Lake source water (10.7 mg/L as C).  Nitrate concentrations are typically no greater than 1 mg/L as N in surface waters [48], which means that the 100 mg/L as N spiking level used in this study represents at least a 100-fold concentration factor.  While the nitrate concentration is scaled up to a greater degree than the DOC concentration, the elevated spiking level was chosen in order to better understand how these spikes influence DBP formation and cytotoxicity.

For the iodide-spiked Nordic Lake NOM, the cell density observed at the highest iodide concentration, 4 mg/L as I, was 85±12% (Figure 6), which was a significant reduction as compared to the control (100±18% cell density).  However, when the cytotoxic response was plotted versus DOC concentration and compared to the response to Nordic Lake NOM alone, this reduction in cell density appeared to be due to the presence of NOM, not the presence of iodide (Figure 7).  It was therefore decided to use 5 mg/L as I, which is diluted to 4 mg/L as I during GIA sample preparation, for the iodide-spiked disinfection experiments.  While iodide concentrations are not reported for the Nordic Lake source water, in an occurrence study carried out at drinking water

treatment plants in 22 U.S. cities and one Canadian city, the source waters for these plants had iodide levels ranging from 0.4 to 104.2 µg/L (when detected) with a median of 10.3 µg/L [49].  Using this median iodide level as a representative source water concentration, the 5 mg/L iodide level used for spiking in this study represents a 485-fold concentration over what is typically found.  The iodide levels used in this project are therefore scaled up higher in relation to actual waters than the DOC levels, as described for nitrate.

**Cytotoxicity, DBP, and TOX levels in Disinfected Samples**

The NOM type, spiking conditions, disinfectant doses, residuals, and demands for each of the samples are listed in Table 2, along with TOX, TOCl, TOI, %known TOX, %known TOCl and %known TOI values.  Although the objective of this study was to assess differences between chlorinated and chloraminated samples, a concurrent study in our laboratory was investigating the effects of UV treatment on DBP formation, and in the interest of evaluating the sensitivity of the growth inhibition assay to these different treated NOM samples, UV treatment was incorporated as well, using 500 mJ/cm$^2$ medium pressure UV [50].  The concentrations of the 10 THMs and other halogenated volatile DBPs are shown in Tables 3 and 4, respectively.  Levels of haloacetamides, haloacetic acids, and cyanogen chloride, are listed in Tables 5, 6, and 7, respectively. Note that these concentrations represent the levels present in the original disinfected sample containing ~120 mg/L as C.  The lowest significant cytotoxic concentration, $IC_{10}$, $IC_{20}$, and $IC_{50}$ values for each sample are shown in Table 8.  The regression model coefficients and $R^2$ values are listed in Table 9.  The two-way ANOVA test results used to determine statistical difference ($p<0.0001$) by comparing the mean cell density values among samples (grouped by figure) at each concentration level are listed in Table 10.

These p-values represent the probability that the observed difference among the mean cell density values of the samples in the figure at a particular concentration level would occur, assuming that the null hypothesis is true. Known TOX values were calculated by summing the concentrations of measured DBPs (trihalomethanes, haloacetonitriles, haloketones, halonitromethanes, chloral hydrate, haloacetamides, haloacetic acids, and cyanogen chloride) as mg/L chloride. Known TOCl/TOBr/TOI levels were calculated by summing the concentrations of chlorinated, brominated, and iodinated DBPs, respectively.

Of the halogenated volatile DBPs, which include trihalomethanes, haloacetonitriles, haloketones, halonitromethanes, and chloral hydrate, the following compounds were below the detection limit in all samples: dibromochloromethane, bromochloroiodomethane, and dibromoiodomethane. Most of the haloacetamides measured were below the detection limit in all samples, including bromochloroacetamide, dibromoacetamide, chloroiodoacetamide, bromodichloroacetamide, bromoiodoacetamide, dibromochloroacetamide, tribromoacetamide, and diiodoacetamide. Of the haloacetic acids, bromoacetic acid, dibromoacetic acid, dibromochloroacetic acid, and tribromoacetic acid were below the detection limit in all samples. TOBr was also below the detection limit (<0.24 mg/L) in all samples, and TOI was only detected in iodide-spiked samples. Low levels of brominated DBPs were expected because none of the samples were spiked with bromide. For the iodide-spiked samples, higher levels of iodinated DBPs were expected to be formed during chloramination as compared to chlorination, as has been shown in previous studies [19, 49, 51, 52]. Because iodinated DBPs have also been found to be highly toxic [49, 53, 54], it was predicted that the

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iodide-spiked chloraminated samples might be more cytotoxic than the corresponding

chlorinated samples.

For ease of viewing trends, the cytotoxicity data for the different samples were

plotted in various combinations, as now described. The cytotoxicity curves for

chlorinated and chloraminated Nordic Lake NOM, with the curve for untreated NOM

(same as in Figure 1) included for comparison, are shown in Figure 8. The cytotoxicity

curves for Suwannee River NOM treated with these same disinfectants are shown in

Figure 9. First, it was observed that chlorinated Nordic Lake NOM was more cytotoxic

than chlorinated Suwannee River NOM. The lowest dose that induced a significant

cytotoxic response using the Holm-Sidak test was 23.0 mg/L as C (corresponding to

83±10% cell density) for the chlorinated Nordic Lake NOM, but 92.0 mg/L as C

(corresponding to 69±17% cell density) for the chlorinated Suwannee River NOM.

However, the 23.0 mg/L as C level represents an approximately two-fold concentration

factor over the Nordic Lake source water, and the 92.0 mg/L as C level also represents

about a two-fold concentration factor over the Suwannee River source water, indicating

that the concentration factor rather than the source water type is playing a role in these

observed cytotoxic responses. TOX values were similar between these two samples, with

25.1 mg/L as Cl in the chlorinated Nordic Lake NOM and 24.2 mg/L as Cl in the

chlorinated Suwannee River NOM. TOCl levels were slightly higher in the chlorinated

Nordic Lake sample (22.7 mg/L as Cl versus 18.6 mg/L as Cl). The %known TOX was

also similar between the two samples, at approximately 60%, while the %known TOCl

was slightly higher in the chlorinated Suwannee River sample (77% versus 68%).

Similar levels of halogenated volatile DBPs were observed in both chlorinated samples,

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except for bromodichloromethane and trichloronitromethane, which were present at approximately four and five times higher concentrations, respectively, in the Nordic Lake sample compared to the Suwannee River sample. The haloacetic acid levels were also similar between the two samples, except for bromochloroacetic acid and bromodichloroacetic acid, which were present at approximately three times higher concentrations in the Nordic Lake sample. The two haloacetamides detected, dichloroacetamide and trichloroacetamide, were both at slightly higher levels in the Nordic Lake sample, at 39 μg/L and 101 μg/L, respectively. Cyanogen chloride concentrations were similar between these two samples, at 4.2 μg/L in the Nordic Lake sample and 3.3 μg/L in the Suwannee River sample.

In Figure 8, it was also observed that the chlorinated Nordic Lake NOM was much more cytotoxic than the chloraminated Nordic Lake NOM, with lowest cytotoxic concentrations at 23.0 mg/L as C (corresponding to 83±10% cell density) and 89.9 mg/L as C (corresponding to 86±10% cell density), respectively. The TOX level was higher in the chlorinated sample (24.3 mg/L as Cl) versus the chloraminated sample (3.35 mg/L as Cl), which was expected because chlorine is a stronger halogenating agent than chloramine with a higher demand and, hence, higher dose is required to leave the same residual. TOCl levels followed this same trend. Both %known TOX and %known TOCl were also much higher in the chlorinated sample than in the chloraminated one, indicating that few of the chloraminated DBPs were being accounted for in this study and in fact reflect the difficulty in current chemical methods. Most of the halogenated volatile DBPs, such as the trihalomethanes, were present at higher concentrations in the chlorinated versus the chloraminated sample, except for 1,1-dichloropropanone (3.8 μg/L

in chlorinated sample and 75.1 µg/L in chloraminated sample). However, in the presence of a complex mixture of DBPs, this higher level of 1,1-dichloropropanone did not appear to have much cytotoxic impact in the chloraminated sample. For the haloacetamides, dichloroacetamide was found at a slightly higher level in the chloraminated sample (54 µg/L versus 39 µg/L), while trichloroacetamide was present at an approximately 67-fold higher level (101 µg/L) in the chlorinated sample compared to the chloraminated sample. Like the trihalomethanes, the haloacetic acids occurred at much higher levels in the chlorinated sample. This observation was expected because the lower formation of these regulated DBPs upon chloramination is the main reason utilities are increasingly choosing chloramine for their secondary disinfectant. Cyanogen chloride levels were higher in the chloraminated sample (10.1 µg/L) compared to the chlorinated sample (4.2 µg/L), but as for 1,1-dichloropropanone, these higher levels of DBPs did not translate into overall high levels of cytotoxicity for the chloraminated sample. However, they are suggestive of pathways for chloramine DBPs at the lower doses and weaker oxidizing properties of chloramine compared to chlorine.

One unexpected result was the apparent stimulation of cell growth upon exposure to increasing concentrations of chloraminated Suwannee River NOM (Figure 9). The lowest concentration at which the mean cell density was statistically different from the control ($100 \pm 13\%$) was 44.6 mg/L as C, corresponding to $122 \pm 22\%$ cell density. Differences in TOX, TOCl, and DBP levels between this sample and chloraminated Nordic Lake NOM, which did not stimulate cell growth, were not apparent. The Suwannee River NOM did have a higher chloramine residual, at 5.0 mg/L as $Cl_2$, compared to 2.7 mg/L as $Cl_2$ for the Nordic Lake NOM. While the preliminary

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experiment that tested the cytotoxicity of chloramine residuals up to 34.4 mg/L as $Cl_2$ in LGW indicated that none of these concentrations induced significant cytotoxic responses (Figure 3), the standard deviations for the cell density as percent control values were large, ranging from 11% to 20%, and the presence of a higher residual may have still had an impact on the results of the chloraminated Suwannee River experiment.  Because the goal of this project was to evaluate differences in cytotoxicity between chlorinated and chloraminated samples, and it was not immediately clear what factors had led to the stimulation in cell growth for the chloraminated Suwannee River sample, only Nordic Lake NOM was used for later disinfection experiments, which involved different treatment and spiking conditions.  This chloraminated Suwannee River sample was run again about a month later, on October 20, 2011, using the same NOM stock solution, and a similar result was obtained (see Appendix B).  However, when the chloraminated sample was prepared using a newly made NOM stock solution and evaluated again on February 8, 2012, an increase in cell density was not observed (see Appendix B). Perhaps a contaminant was present in the original stock solution that reacted with chloramine and stimulated growth.

The cytotoxicity curves for all of the chlorinated Nordic Lake samples evaluated in this study are shown in Figure 10.   These include samples that were: chlorinated only; iodide-spiked and chlorinated; UV-treated prior to chlorination; nitrate-spiked and chlorinated; and nitrate-spiked and UV-treated prior to chlorination.  Taking into consideration the large error bars for the cell density as percent control values, the dose-response curves appeared similar among all of the chlorinated samples.  However, the two-way ANOVA results show that the mean cell density values were statistically

different (p<0.0001) among these samples at the two highest DOC concentrations.  The

lowest cytotoxic concentration for these samples ranged from 11.2 mg/L as C for the

nitrate-spiked (no UV) sample, to 44.8 mg/L as C for the iodide-spiked sample.  These

lowest cytotoxic concentration values, however, do not provide the full picture of the

cytotoxic potency of samples, as some of the curves have steeper reductions in cell

density at the higher sample concentrations.  For example, although the nitrate-spiked (no

UV) sample induced a significant cytotoxic response at a lower concentration than the

other chlorinated samples, the smallest $IC_{50}$ value, which is the concentration at which

50% reduction in cell growth is observed, was that of the nitrate-spiked UV-treated

sample, with an $IC_{50}$ of 61.8 mg/L as C.  The nitrate-spiked (no UV) sample had an $IC_{50}$

value of 75.0 mg/L as C, while the iodide-spiked sample had the highest $IC_{50}$ of all the

chlorinated samples, at 80.6 mg/L as C.  TOX values ranged from 24.2 mg/L as Cl for the

chlorinated only sample, to 30.5 mg/L as Cl for the unspiked UV-treated sample.  The

TOCl values ranged from 19.1 mg/L as Cl for the unspiked UV-treated sample, to 24.4

mg/L as Cl for the iodide-spiked sample.  These variations in TOX and TOCl values did

not seem to greatly affect cytotoxic response.  The %known TOX was approximately 60-

70% in the chlorinated samples.  Halogenated volatile DBP levels were similar among

these chlorinated samples, except that chloral hydrate levels in the UV-treated samples

(1857 µg/L in UV-treated; 1981 µg/L in nitrate-spiked, UV-treated) were almost double

those observed in the non-UV-treated samples.  Haloacetic acid levels were also similar,

with a notable difference observed only in the bromochloroacetic acid concentration,

which was higher in the chlorinated only sample (85 µg/L versus ~10 µg/L levels in the

other four chlorinated samples).

The cytotoxicity curves for all of the chloraminated samples are shown in Figure 11. As compared to the chlorinated samples, more variation in cytotoxic responses were observed among the different conditions, which included: chloraminated only; iodide-spiked and chloraminated; UV-treated prior to chloramination; and iodide-spiked and UV-treated prior to chloramination. The latter sample was produced and evaluated twice, first on October 13, 2011, and then on January 16, 2012. Because the cytotoxicity data looked different between the two experiments, these data sets were plotted as separate curves, rather than averaged, in order to show this variation. The two-way ANOVA test results showed that the mean cell density values were statistically different among the samples beginning at the ≈22 mg/L as C concentration level. This result contrasted with the chlorinated samples, which were not found to exhibit statistical difference in mean cell density until much higher concentration levels, ≈67 and 90 mg/L as C. Based on the $IC_{20}$ values, the cytotoxic rank order (from most toxic to least toxic) among the chloraminated samples was: iodide-spiked and UV-treated prior to chloramination (1/16/2012) > iodide-spiked and UV-treated prior to chloramination (10/13/2011) > UV-treated prior to chloramination > iodide-spiked and chloraminated > chloramination only. TOX levels did seem to relate somewhat to these differences in cytotoxicity, in that the chloramine only sample had the lowest TOX at 3.35 μg/L Cl, the iodide-spiked sample was higher at 4.39 μg/L Cl, and the three UV-treated samples were highest, ranging from 4.63 μg/L to 5.07 μg/L Cl. No apparent correlations were observed between TOCl or TOI values and cytotoxic responses. Not including the iodinated THMs, the halogenated volatile DBPs were generally higher among the UV-treated samples, with THM4 levels ranging from 125 to 153 μg/L. Iodinated THMs, including chlorodiiodomethane and

triiodomethane, were only detected upon inclusion of an iodide spike, making THM10 levels highest in these samples. Chloral hydrate was only detected in the three UV-treated samples, bromodiiodomethane (1.1 µg/L) was only detected in the iodide-spiked and UV-treated sample from October 13, 2011, and dichloroiodomethane (22 µg/L) was only detected in the iodide-spiked and UV-treated sample from January 16, 2012. HAA5 and HAA9 levels in the unspiked UV-treated sample and one of the iodide-spiked UV-treated samples (from 10/13/2011) were almost two times the levels observed in the two samples that were not treated with UV. Interestingly, the iodide-spiked UV-treated sample from January 16, 2012, had HAA5 and HAA9 levels that were approximately 200 µg/L lower than the other two UV-treated samples. Chloroacetic acid was only detected in the unspiked samples, at 46 µg/L in the chloraminated only sample and 131 µg/L in the UV-treated, chloraminated sample. Bromochloroacetic acid was highest in the chloraminated only sample, at 27 µg/L. Cyanogen chloride levels were higher in the UV-treated samples than the non-UV-treated, with the highest concentration (50.8 µg/L) observed in the iodide-spiked UV-treated sample from January 16, 2012.

While the cytotoxicity data for the iodide-spiked samples have already been presented in Figures 8 and 9, these curves were also plotted on the same graph (Figure 12) in order to better view differences between chlorination and chloramination. As was previously observed in the unspiked samples (Figure 8), for the iodide-spiked samples, the chlorinated one ($IC_{20}$=46.4 mg/L as C) was more cytotoxic than the chloraminated one ($IC_{20}$=58.3 mg/L as C). The samples that were UV-treated and then chloraminated were the most cytotoxic of the iodide-spiked samples, with an $IC_{20}$ value of 37.8 mg/L as C for the experiment run on October 13, 2011, and an $IC_{20}$ value of 17.8 mg/L as C for

the one run on January 16, 2012. In terms of why these $IC_{20}$ values differed on the two dates, differences in chemical composition, such as DBP levels, between the two samples are a possibility. However, while elevated cyanogen chloride and dichloroiodomethane concentrations were detected in the sample from January 16, 2012, compared to the one from October 13, 2011, it seems unlikely that these two DBPs fully account for the greater cytotoxic response observed with the January 16th sample. Another possible explanation is that there were issues with the cell growth observed during the entire set of experiments run on January 16th, which would affect the cell density levels. Indeed, upon looking at the cytotoxicity curves for diiodoacetamide, the positive control run concurrently with each experiment, an unusually high level of cytotoxicity was observed on January 16, 2012 (Figure 13). Using an unpaired two-sample t-test, it was found that the mean cell density values on the two dates were statistically different (p<0.0001) beginning at the $9x10^{-6}$ M diiodoacetamide concentration level. This topic will be discussed further in the Discussion section. While TOX and TOCl values, as well as %known TOX and %known TOCl, were highest in the chlorinated samples, TOI levels and %known TOI were highest in the chloraminated samples. It was notable that even though the TOX value for the chlorinated sample (27.8 mg/L as Cl) was much higher than that of the UV-treated and chloraminated sample (5.07 mg/L as Cl), the latter sample was more toxic. This result indicates that while TOX is a useful measure of DBP content, it is a not a perfect indicator of overall toxicity. THM4 was highest in the chlorinated sample, as were trichloroacetonitrile, chloral hydrate, trichloronitromethane, and 1,1,1-trichloropropanone. As described previously, chlorodiiodomethane and triiodomethane were only detected in the chloraminated samples. Similar to THM4, the

HAA5 levels were much higher in the chlorinated sample. Bromochloroacetic acid and bromodichloroacetic acid were also highest in the chlorinated sample. Despite the high levels of the regulated THM4 and HAA5, it was interesting that the chlorinated sample was still not as toxic as the UV-treated and chloraminated samples. The increased presence of cyanogen chloride in the UV-treated and chloraminated samples versus the other two iodide-spiked samples may have contributed to the higher cytotoxic response observed, but likely other compounds that were not measured in this study nor have been previously identified played a role. These results highlight the importance of studying the toxicity of DBPs as a complex mixture in which the identities of many DBPs are unknown.

Cytotoxicity curves for the 72 and 96 hour chloraminated samples are shown in Figure 14, with the curve for the 24 hour chloraminated sample (same as in Figure 8) also included for comparison. Of special note is that the DBP and TOX levels reported for the 72 and 96 hour samples in Tables 2, 3, 4, 6 and 7, are for samples disinfected under the same conditions as the samples run on the GIA, but on different dates. This differs from the chemical data for the 24 hour treated samples, in which the samples used for chemical analysis came from the same original sample as the sample used for the GIA. Looking at the two-way ANOVA values, the mean cell density values for these three samples were not statistically different at the 0.0001 significance level for any of the concentration levels. This result was somewhat surprising, given that the TOX value for the 72 hour sample (6.49 mg/L as Cl) was almost two times as high as the TOX value in the 24 hour sample (3.35 mg/L as Cl). It was also unusual that the TOX value for the 96 hour sample (5.38 mg/L as Cl) was lower than that of the 72 hour sample. Perhaps this indicates that

some volatile DBPs are lost over time. For example, cyanogen chloride was highest in the 24 hour sample, at 10.1 μg/L, but 8.8 μg/L in the 72 hour sample, and 5.6 μg/L in the 96 hour sample. THM4 levels were over six times higher in the 72 and 96 hour samples compared to the 24 hour sample, and dichloroacetonitrile levels were over 10 times higher. Other halogenated volatiles found at enhanced concentrations in the 72 and 96 hour treated samples were trichloroacetonitrile, chloral hydrate, 1,1-dichloropropanone (although to not as great an extent as the others), trichloronitromethane, bromochloroacetonitrile, 1,1,1-trichloropropanone, and tribromonitromethane. Although HAA5 and HAA9 were approximately three times as high in the 72 and 96 hour samples as compared to the 24 hour sample, bromochloroacetic acid was lower in the 72 and 96 hour samples (6.1 and 5.6 μg/L, respectively) versus the 24 hour sample (27 μg/L). Still, these high levels of THMs and HAAs did not appear to have a large effect on cytotoxicity.

**CHAPTER 4**

DISCUSSION

The goal of this study was to compare chlorinated and chloraminated controlled synthetic natural waters that had been dosed to leave equivalent disinfectant residuals, on the basis of cytotoxicity, DBP, and TOX levels. Using those waters without adding iodide or nitrate or using UV treatment, the chlorinated water was more cytotoxic than the chloraminated water, a result that corresponds to previously reported *Salmonella* assay results for mutagenicity [32]. Upon inclusion of an iodide spike and pre-treatment with medium pressure UV, however, the chloraminated water was the most cytotoxic of all disinfected samples evaluated. This is likely due to the formation of iodinated DBPs, possibly enhanced by degradation of the natural organic matter with UV, which have been shown individually to possess higher cytotoxicity than their bromine- or chlorine-containing counterparts [54]. This current research has shown that the growth inhibition assay in NCM460 cells is able to detect these differential cytotoxic responses. It was also shown that the two types of NOM, Nordic Lake and Suwannee River, at equivalent DOC levels, behaved differently upon disinfection, but these differences may have been a function of the much higher concentration factor over the original source water for Nordic Lake versus Suwannee River.

While the cytotoxic potency of single DBP compounds is typically reported as $\%C_{1/2}$ values [23], which are analogous to $IC_{50}$ values, in this study involving DBP mixtures generated from concentrated source waters, not all of the samples induced a

50% reduction in cell density in the concentration range tested. Therefore, $IC_{20}$ and $IC_{10}$ values were also calculated in order to make comparisons among more of the samples. However, the cytotoxic rank order based on $IC_{20}$ and $IC_{10}$ values was slightly different from the one based on $IC_{50}$ values, indicating that the cytotoxic responses observed at higher sample concentrations do not always correspond with those observed at lower concentrations. While lower DOC concentrations are of particular interest because they approximate DOC levels in natural waters and are therefore better representative of human exposure, for most samples the DOC levels needed to be much higher before a significant cytotoxic response was observed with this assay. Notable exceptions were the iodide-spiked UV-treated and chloraminated sample from January 16, 2012, and the nitrate-spiked chlorinated sample, which had lowest cytotoxic concentrations of 10.8 mg/L as C and 11.2 mg/L as C, respectively, close to the ~10.7 mg/L as C level in the Nordic Lake source water. However, while the DOC level in these samples may approximate the original source water, the iodide (0.48 mg/L as I) or nitrate (10 mg/L as N) levels present at these sample concentrations were still elevated as compared to most surface waters, as described in the Results chapter.

Indeed, by using these elevated iodide and nitrate spiking ratios, and not including a bromide spike, DBP formation took place at distorted ratios, so it is difficult to make conclusions about relevance to actual drinking water. Still, the data provide insight into the cytotoxic effects of using chloramine in the presence of iodide, and UV in the presence of nitrate.

While the $IC_{50}$ values indicated that the Nordic Lake samples that were iodide-spiked and UV-treated prior to chloramination, from both October 13, 2011 and January

16, 2012, were the most cytotoxic of the disinfected samples, it was surprising that these values varied so greatly between the two experiments. The $IC_{50}$ values were 42.9 mg/L as C for the January 16 sample, and 60.7 mg/L as C for the October 13 sample, with 95% confidence intervals of (39.2 mg/L as C, 46.7 mg/L as C) and (58.2 mg/L as C, 63.0 mg/L as C), respectively. The dose-response curve for the positive control (Figure 13) in the January 16, 2012 experiment was steeper than in the October 13, 2011 experiment, meaning that greater reductions in cell density were observed. While including a control column in every plate and expressing the cell density for the sample columns as percent control is supposed to account for any differences between plates, the importance of including a positive control with every experiment in order to monitor a known cell growth response was highlighted in this case.

TOX levels in chlorinated waters have previously been strongly correlated with mutagenic responses in *Salmonella* [33], but this study showed that the relationship between TOX and cytotoxicity in NCM460 cells is not straightforward when evaluating samples treated with two different disinfectants, chlorine and chloramine. A general concordance between TOX levels and cytotoxicity was observed among the various chloraminated Nordic Lake samples. However, TOX could not explain the higher cytotoxicity of the iodide-spiked, UV-treated and chloraminated sample compared to the iodide-spiked, chlorinated sample, which contained an over five times higher TOX level. TOI levels, which represent the amount of total iodinated DBPs, do provide insight into this observed difference in cytotoxicity, with 0.59 mg/L as I in the chlorinated sample and ~2.3 mg/L as I in the chloraminated sample. This difference was expected based on known reactions of iodide with chloramine to produce iodinated DBPs that do not occur

between iodide and free chlorine [51, 52]. The higher cytotoxic response and higher iodinated DBP levels in the chloraminated sample as compared to the chlorinated sample together correspond with current knowledge regarding the elevated cytotoxicity of iodinated DBPs [49, 53, 54]. The measured iodinated DBPs only represented ~12% of the TOI for the chloraminated sample, and 0% of the TOI for the chlorinated sample, highlighting the limitation of including only nine iodinated DBPs in the analysis and the difficulty in detecting iodinated DBPs at low levels.

It is also important to point out that the iodide-spiked chlorinated sample was less cytotoxic than the iodide-spiked, UV-treated, chloraminated sample, despite its containing much higher levels of regulated trihalomethanes and haloacetic acids. This result indicates that the regulated DBPs are not representative of overall toxic DBP content. In general, for all of the samples, it was difficult to identify specific DBPs that were consistently correlated with increased cytotoxicity. The DBP measurements did indicate that the presence of cyanogen chloride (CNCl) may be a major contributor of cytotoxicity in the chloraminated samples, but because less than 20% of the TOX was accounted for by the measured DBPs, it seems likely that other cytotoxic compounds are present in the chloraminated samples that are not being measured.

Another observation is that the nitrate-spiked, UV-treated and chlorinated sample exhibited reduced cytotoxicity as compared to its non-UV-treated counterpart, despite the former sample having an increased disinfectant demand. This result is surprising because a higher disinfectant demand is typically associated with increased DBP formation, which would be expected to lead to greater cytotoxicity. However, the TOX level is slightly lower in the UV-treated sample, indicating that the increased demand was not associated

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with increased formation of halogenated DBPs, and perhaps is associated with formation of nitrogenous DBPs that in this case may not be as cytotoxic. In addition, for the UV-treated and chlorinated sample, the cytotoxicity was reduced despite the increased reaction and higher TOX observed, indicating that the DBPs that were elevated in this case were likely not as cytotoxic. Further, while the chlorinated Nordic Lake sample was more cytotoxic than the chloraminated sample, the UV-treated and chlorinated sample was similar in cytotoxic potency to the UV-treated and chloraminated sample, even though the TOX level for the latter sample was much lower. Use of UV treatment prior to chlorine or chloramine disinfection seems to influence the overall sample cytotoxicity compared to treatment with chlorine or chloramine using doses that leave similar disinfectant residuals.

For the Nordic Lake samples that were chloraminated over longer treatment times, one surprising observation was that TOX levels, as well as CNCl levels, were lower in the 96 hour sample as compared to the 72 hour sample. This result suggests that some DBPs, like CNCl, may be lost through volatilization. However, this reduction in CNCl is below 0.01 mg/L as Cl, indicating that some other DBPs not measured in this study are contributing to the overall loss in TOX. Despite the expectation that the samples treated with chloramine over longer contact times, which are more representative of a distribution system, would be more cytotoxic than the 24 hour sample, the cytotoxicity data for these samples indicate that this was not the case.

This study took an interdisciplinary approach to assessing differences in water quality between chlorinated and chloraminated simulated source waters by simultaneously evaluating cytotoxicity, DBP occurrence and TOX levels. Chlorinated

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and chloraminated water concentrates containing non-volatile DBPs had previously been evaluated for mutagenicity in *Salmonella* [32], but this current work was novel in that the cytotoxic potency of both the volatile and non-volatile portions of DBP mixtures produced during chlorination and chloramination was evaluated using a normal human colon cell line. The results of this cytotoxicity analysis showed that in the absence of iodide and nitrate, and without UV-pretreatment, the chlorinated sample was more cytotoxic than the chloraminated samples, which corresponds with the results of the mutagenicity study. The advantages of using a cytotoxicity assay in addition to chemical analysis was highlighted by the iodide-spiked UV-treated and chloraminated sample, which had the highest cytotoxic response of the disinfected samples, despite low levels of TOX as compared to the iodide-spiked chlorinated sample, and similar levels of iodinated DBPs as compared to the iodide-spiked chloraminated sample. The difficulty in correlating specific DBP levels with cytotoxic responses in this study highlights the inherent complexity of DBP mixtures, as well as the challenge involved in regulating individual DBPs when so many DBPs are unidentified.

**CHAPTER 5**

LIMITATIONS OF THIS STUDY AND SUGGESTIONS FOR FUTURE WORK

One aspect of this work that remains to be investigated is whether the composition of the disinfected sample changes over the 72 hour treatment period in the growth inhibition assay, due to the presence of unquenched disinfectant residual which could possibly continue to react with the NOM. In addition, although filtering and vortexing of disinfected samples were avoided prior to placement on the cells, and Alumaseal was placed on the plates to further minimize loss of volatiles, it is unclear to what extent the cells are actually exposed to the volatile portion of the sample. If possible, the levels of volatile DBPs present in the media should be analyzed following addition of disinfected sample to dilution tubes.

The inclusion of a concurrent positive control, diiodoacetamide, with each set of experiments is a useful tool for monitoring unusual cell response patterns, but it is also important that steps be taken to improve the reproducibility of the growth inhibition assay. One action that might reduce differences in cell growth observed in the positive control between experiment sets would be to consistently use cell cultures that have been passaged two to three times rather than ones that have been growing for shorter or longer periods. In addition, the iodide-spiked, UV-treated and chloraminated Nordic Lake sample and the chloraminated Suwannee River sample were the only samples evaluated on separate days in this study, aside from the positive control. In order to confirm reproducibility, it is recommended that all of the disinfected samples be evaluated again

and a two-way ANOVA be carried out with experimental day as a factor. While the complexity of the sigmoidal model may make it difficult, another suggestion is to determine a way to adjust the sample cytotoxicity curves based on the positive control response. If such an adjustment could be made, perhaps the cytotoxicity curves obtained for the iodide-spiked, UV-treated and chloraminated Nordic Lake sample in two separate experiments in this study would no longer differ.

Once the reproducibility of the cytotoxicity assay is improved, samples that have been spiked with bromide prior to disinfection should also be evaluated because although iodinated DBPs have been found to be more cytotoxic than brominated DBPs [54], the bromide levels in surface waters are typically an order of magnitude higher than the iodide levels [49], and the levels of brominated DBPs that form during treatment of source waters may still be toxicologically significant. When doing so, a realistic bromide to iodide ratio that is scaled to the NOM concentration should be used in order to produce waters that are representative of those that utilities might encounter. In order to better understand the correlation between specific DBP levels and cytotoxicity, levels of known DBPs could be spiked into a disinfected sample to see if the cytotoxic response is additive. For the chloraminated samples evaluated in this study, the %known TOX was only 12-18%, indicating that many of the chloramination by-products are not being measured. If possible, more iodinated and nitrogenous DBPs, which are associated with chloramination, should be included in future chemical analysis.

It is also important to note that the highest concentration of Nordic Lake NOM was itself cytotoxic to the NCM460 cells, which may have affected the interpretation of the cytotoxicity data for the disinfected samples. One recommendation is for the

statistical analysis to be carried out with the cytotoxicity data at this highest NOM

concentration removed, in order to confirm that the trends described here hold.  Also,

while the sigmoidal regression model appears to be a good fit for the cytotoxicity curves,

as observed in the high $R^2$ values, another suggestion would be to explore other

regression models, such as a logistic model.

It must also be recognized that the concentrations of NOM used in this study were

much higher than is usually present in source waters.  In a nationwide DBP occurrence

study, the median TOC level in raw waters was 5.8 mg/L as C, whereas the TOC levels in

this study were in the 120 mg/L as C range [19]. While the cytotoxicity of the diluted

sample was already measured, the DBP levels may not scale down proportionately when

the sample is originally disinfected at a high DOC.  Although cytotoxicity would not be

detected due to method sensitivity limitations, in order to confirm that composition of

DBPs is similar between a dilution of a disinfected concentrated NOM and a NOM that is

diluted to more realistic levels and then disinfected to leave the same level of residual, 3

mg/L as $Cl_2$, the latter should be evaluated for DBPs to confirm proportional DBP

formation.  If the resources were available, it would be interesting to also evaluate the *in

vivo* effects of these samples, such as by using the developmental toxicity screen in

Sprague-Dawley rats described in the "Four-Lab Study" [39].

**Table 1. Experimental matrix**

| Date* | Sample |
|---|---|
| 2/21/2011 | Nordic Lake |
| | Suwannee River |
| 8/11/2011 | Phosphate Buffer, pH 7.1, in LGW |
| 9/1/2011 | Nordic Lake + Chlorine |
| | Suwannee River + Chlorine |
| 9/15/2011 | Nordic Lake + Chloramine |
| | Suwannee River + Chloramine |
| 9/23/2011 | Nordic Lake + Nitrate |
| | Nordic Lake + Iodide |
| 10/6/2011 | Nordic Lake + Nitrate + Chlorine |
| | Nordic Lake + Nitrate + UV + Chlorine |
| | Nordic Lake + UV + Chlorine |
| 10/13/2011 | Nordic Lake + Iodide + Chlorine |
| | Nordic Lake + Iodide + Chloramine |
| | Nordic Lake + Iodide + UV + Chloramine |
| 10/20/2011 | Chlorine Residual in LGW |
| | Chloramine Residual in LGW |
| | Suwannee River + Chloramine (repeat) |
| 1/16/2012 | Nordic Lake + Iodide + UV + Chloramine |
| | Nordic Lake + UV + Chloramine |
| 2/8/2012 | Suwannee River + Chloramine (repeat**) |
| 2/20/2012 | Nordic Lake + Chloramine, 72 h |
| | Nordic Lake + Chloramine, 96 h |

*Date listed is for the first day of the GIA
**with newly prepared NOM stock

**Table 2. Listing of disinfected samples prepared for GIA with organic halogen analysis. (Unless otherwise noted, disinfection carried out over 24 hours.)**

| Sample | Dose (mg/L Cl$_2$) | Residual (mg/L Cl$_2$) | Demand (mg/L Cl$_2$) | TOX (mg/L as Cl) | TOCl (mg/L as Cl) | TOI (mg/L as I) | %known[#] TOX | %known[#] TOCl | %known[#] TOI |
|---|---|---|---|---|---|---|---|---|---|
| SR + HOCl | 221 | 2.6 | 219 | 25.1 | 18.6 | <0.32 | 57% | 77% | NA |
| SR + NH$_2$Cl | 44 | 5.0 | 39 | 3.73 | 3.82 | <0.32 | 13% | 13% | NA |
| NL + HOCl | 225 | 5.4 | 214 | 24.2 | 22.7 | <0.32 | 64% | 68% | NA |
| NL + UV + HOCl | 234 | 5.4 | 229 | 30.5 | 19.1 | <0.48 | 61% | 97% | NA |
| NL + Nitrate + HOCl | 224 | 2.0 | 222 | 29.5 | 22.1 | <0.48 | 58% | 77% | NA |
| NL + Nitrate + UV + HOCl* | 262 | 2.4 | 260 | 27.1 | 20.9 | <0.48 | 69% | 90% | NA |
| NL + Iodide + HOCl | 236 | 3.6 | 232 | 27.8 | 24.4 | 0.59 | 67% | 76% | 0.0% |
| NL + NH$_2$Cl | 32 | 2.7 | 29 | 3.35 | 3.29 | <0.32 | 13% | 13% | NA |
| NL + UV + NH$_2$Cl (1/16/12) | 47 | 4.1 | 43 | 4.96 | 3.65 | <0.24 | 15%** | 21% | NA |
| NL + Iodide + NH$_2$Cl | 40 | 2.5 | 37 | 4.39 | 2.37 | 2.88 | 12% | 18% | 8.6% |
| NL + Iodide + UV +NH$_2$Cl(10/13/11) | 54 | 3.4 | 50 | 5.07 | 3.33 | 2.49 | 18% | 23% | 13% |
| NL + Iodide + UV + NH$_2$Cl (1/16/12) | 52 | 4.5 | 48 | 4.63 | 3.11 | 2.16 | 15%** | 21% | 11% |
| NL + NH$_2$Cl, 72 h | 99 | 4.6 | 94 | 6.49 | NM | NM | 23%** | NM | NM |
| NL + NH$_2$Cl, 96 h | 99 | 2.8 | 96 | 5.38 | NM | NM | 28%** | NM | NM |

SR=Suwannee River NOM; NL=Nordic Lake NOM

NM=not measured; NA=not applicable; *not pH adjusted prior to TOCl/Br/I adsorption; **not including haloacetamides

[#] values calculated as the difference between sum of the individual DBPs and the total halogen (both values converted to mg/L of the appropriate halogen atom).

**Table 3. Formation of THM10 halogenated volatile DBPs (µg/L) in treated SR and NL samples. Value shown is the average between duplicate samples. Br₂ClCH, BrClICH and Br₂ICH were below detection limit (<0.5-1.0 µg/L) in all samples.**

| Sample | [Cl3CH] | [BrCl2CH] | [Cl2ICH] | [Br3CH] | [ClI2CH] | [BrI2CH] | [I3CH] | ∑THM4 | ∑THM10 |
|---|---|---|---|---|---|---|---|---|---|
| SR+HOCl | 7362 | 11 | <1.0 | 4.1 | <1.0 | <1.0 | <1.0 | 7377 | 7377 |
| SR+NH₂Cl | 132 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | 132 | 132 |
| NL+HOCl | 8002 | 40 | <1.0 | 3.6 | <1.0 | <1.0 | <1.0 | 8046 | 8046 |
| NL+UV+HOCl | 10652 | 43 | <1.0 | 2.0 | <1.0 | <1.0 | <1.0 | 10697 | 10697 |
| NL+Nitrate +HOCl | 9826 | 43 | <1.0 | 2.0 | <1.0 | <1.0 | <1.0 | 9872 | 9872 |
| NL+Nitrate +UV+HOCl | 9356 | 41 | <1.0 | 1.2 | <1.0 | <1.0 | <1.0 | 9398 | 9398 |
| NL+Iodide +HOCl | 10197 | 41 | <0.5 | 2.7 | <0.5 | <0.5 | <0.5 | 10241 | 10241 |
| NL + NH₂Cl | 95 | 0.8 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | 96 | 96 |
| NL+UV+NH₂Cl (1/16/12) | 152 | 0.9 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | 153 | 153 |
| NL+Iodide+ NH₂Cl | 75 | 1.3 | <0.5 | <0.5 | 91 | <0.5 | 137 | 77 | 305 |
| NL+Iodide+UV +NH₂Cl (10/13/11) | 123 | 1.6 | <0.5 | <0.5 | 119 | 1.1 | 182 | 125 | 427 |
| NL+Iodide+UV +NH₂Cl (1/16/12) | 125 | 0.9 | 22 | <0.5 | 96 | <0.5 | 157 | 126 | 401 |
| NL+NH₂Cl,72h | 614 | 8.0 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | 622 | 622 |
| NL+NH₂Cl,96h | 649 | 8.3 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | 657 | 657 |

| | |
|---|---|
| Cl₃CH | trichloromethane |
| BrCl₂CH | bromodichloromethane |
| Br₂ClCH | dibromochloromethane |
| Cl₂ICH | dichloroiodomethane |
| Br₃CH | tribromomethane |
| BrClICH | bromochloroiodomethane |
| Br₂ICH | dibromoiodomethane |
| ClI₂CH | chlorodiiodomethane |
| BrI₂CH | bromodiiodomethane |
| I₃CH | triiodomethane |

**Table 4. Formation of other halogenated volatile DBPs (µg/L) in treated SR and NL samples. Value shown is the average between duplicate samples.**

| Sample | [TCAN] | [DCAN] | [CH] | [11DCP] | [TCNM] | [BCAN] | [111TCP] | [DBAN] | [TBNM] |
|---|---|---|---|---|---|---|---|---|---|
| SR+HOCl | 2.4 | 80 | 968 | 3.5 | 2.8 | <1.0 | 157 | <1.0 | <1.0 |
| SR+NH$_2$Cl | <1.0 | 18 | <0.5 | 64 | <0.5 | <0.5 | 4.3 | <0.5 | <0.5 |
| NL+HOCl | 3.7 | 88 | 1108 | 3.8 | 15 | <1.0 | 174 | <1.0 | <1.0 |
| NL+UV+HOCl | 3.7 | 82 | 1857 | 4.5 | 18 | <1.0 | 195 | <1.0 | <1.0 |
| NL+Nitrate +HOCl | 4.1 | 101 | 1060 | 4.3 | 17 | <1.0 | 161 | <1.0 | <1.0 |
| NL+Nitrate +UV+HOCl | 4.8 | 124 | 1981 | 4.7 | 2512 | <1.0 | 219 | 3.8 | 1.2 |
| NL+Iodide +HOCl | 3.9 | 82 | 952 | 3.0 | 19 | <0.5 | 139 | <0.5 | <0.5 |
| NL + NH$_2$Cl | <1.0 | 13 | <0.5 | 75 | 1.2 | <0.5 | 2.8 | <0.5 | <0.5 |
| NL+UV+NH$_2$Cl (1/16/12) | <0.5 | 23 | 11 | 158 | 1.9 | <0.5 | 8.5 | <0.5 | <0.5 |
| NL+Iodide+ NH$_2$Cl | <0.5 | 19 | <0.5 | 51 | 1.8 | <0.5 | 1.3 | <0.5 | <0.5 |
| NL+Iodide+UV+NH$_2$Cl (10/13/11) | <0.5 | 31 | 3.3 | 94 | 3.5 | <0.5 | 5.6 | <0.5 | <0.5 |
| NL+Iodide+UV+NH$_2$Cl (1/16/12) | <0.5 | 24 | 4.5 | 96 | 2.5 | <0.5 | 4.9 | <0.5 | <0.5 |
| NL+NH$_2$Cl,72h | 2.4 | 140 | 20.4 | 87.9 | 11.5 | 1.3 | 32.9 | <0.5 | 1.4 |
| NL+NH$_2$Cl,96h | 1.8 | 140 | 19.4 | 87.3 | 10.6 | 1.4 | 27.3 | <0.5 | 0.6 |

| | |
|---|---|
| TCAN | trichloroacetonitrile |
| DCAN | dichloroacetonitrile |
| CH | chloral hydrate |
| 11DCP | 1,1-dichloropropanone |
| TCNM | trichloronitromethane |
| BCAN | bromochloroacetonitrile |
| 111TCP | 1,1,1-trichloropropanone |
| DBAN | dibromoacetonitrile |
| TBNM | tribromonitromethane |

**Table 5. Formation of haloacetamides (µg/L) in treated SR and NL samples.**

| Sample | *Average concentration between duplicates (µg/L)* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | [DCAM] | [BCAM] | [TCAM] | [DBAM] | [CIAM] | [BDCAM] | [BIAM] | [DBCAM] | [TBAM] | [DIAM] |
| SR + HOCl | 27 | <1 | 64 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| SR + NH$_2$Cl | 44 | <0.5 | 1.9 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| NL + HOCl | 39 | <1 | 101 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| NL + UV + HOCl | 38 | <0.5 | 114 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| NL + Nitrate + HOCl | 59 | <1 | 102 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| NL + Nitrate + UV + HOCl | 65 | <1 | 140 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| NL + Iodide + HOCl | 50 | <1 | 92 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| NL + NH$_2$Cl | 54 | <0.5 | 1.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| NL + UV + NH$_2$Cl (1/16/12) | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| NL + Iodide + NH$_2$Cl | 50 | <0.5 | 1.2 | <0.5 | 29 | <0.5 | <0.5 | <0.5 | <0.5 | 26 |
| NL + Iodide + UV + NH$_2$Cl (10/13/11) | 91 | <0.5 | 2.2 | <0.5 | 45 | <0.5 | <0.5 | <0.5 | <0.5 | 34 |
| NL + Iodide + UV + NH$_2$Cl (1/16/12) | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| NL + NH$_2$Cl, 72 h | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| NL + NH$_2$Cl, 96 h | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |

**Not Available

| | |
|---|---|
| DCAM | dichloroacetamide |
| BCAM | bromochloroacetamide |
| TCAM | trichloroacetamide |
| DBAM | dibromoacetamide |
| CIAM | chloroiodoacetamide |
| BDCAM | bromodichloroacetamide |
| BIAM | bromoiodoacetamide |
| DBCAM | dibromochloroacetamide |
| TBAM | tribromoacetamide |
| DIAM | diiodoacetamide |

**Table 6. Formation of haloacetic acids (μg/L) in treated SR and NL samples.**

| Sample | [ClAA] | [BrAA] | [Cl2AA] | [BrClAA] | [Cl3AA] | [Br2AA] | [BrCl2AA] | [Br2ClAA] | [Br3AA] | HAA5 | HAA9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Average concentration between duplicate samples (μg/L)* | | | | | | | |
| SR + HOCl | 142 | <2 | 2467 | 31 | 8467 | <0.5 | 20 | <5 | <1 | 11076 | 11127 |
| SR + NH2Cl | 52 | <1 | 434 | 8 | 9 | <1 | <1 | <2.5 | <0.5 | 495 | 503 |
| NL + HOCl | 149 | <2 | 2575 | 85 | 8968 | <0.5 | 59 | <5 | <1 | 11691 | 11835 |
| NL + UV + HOCl | 189 | <1 | 3108 | 10 | 8925 | <1 | 63 | <5 | <1 | 12222 | 12295 |
| NL + Nitrate + HOCl | 167 | <1 | 2557 | 9 | 8805 | <1 | 60 | <5 | <1 | 11529 | 11598 |
| NL + Nitrate + UV + HOCl | 194 | <1 | 3033 | 12 | 8153 | <1 | 60 | <5 | <1 | 11380 | 11452 |
| NL + Iodide + HOCl | 192 | <1 | 3138 | 11 | 10484 | <0.5 | 79 | <2.5 | <0.5 | 13814 | 13904 |
| NL + NH2Cl | 46 | <1 | 381 | 27 | 5 | <1 | <1 | <2.5 | <0.5 | 431 | 458 |
| NL + UV + NH2Cl (1/16/12) | 131 | <0.5 | 710 | 2 | 8 | <0.5 | <0.5 | <2.5 | <2.5 | 850 | 852 |
| NL + Iodide + NH2Cl | <7.5 | <1 | 430 | 4 | 6 | <0.5 | <1 | <2.5 | <0.5 | 435 | 439 |
| NL + Iodide + UV + NH2Cl (10/13/11) | <7.5 | <1 | 797 | 6 | 11 | <0.5 | <1 | <2.5 | <0.5 | 808 | 813 |
| NL + Iodide + UV + NH2Cl (1/16/12) | <15 | <0.5 | 611 | 3 | 9 | <0.5 | <0.5 | <2.5 | <2.5 | 620 | 623 |
| NL + NH2Cl, 72 h | 117 | 8.1 | 1171 | 6.1 | 60.7 | <0.5 | <0.5 | <2.5 | <2.5 | 1356 | 1362 |
| NL + NH2Cl, 96 h | 121 | 7.8 | 1161 | 5.6 | 59.3 | <0.5 | <0.5 | <2.5 | <2.5 | 1349 | 1355 |

| ClAA | chloroacetic acid |
|---|---|
| BrAA | bromoacetic acid |
| Cl2AA | dichloroacetic acid |
| BrClAA | bromochloroacetic acid |
| Cl3AA | trichloroacetic acid |
| Br2AA | dibromoacetic acid |
| BrCl2AA | bromodichloroacetic acid |
| Br2ClAA | dibromochloroacetic acid |
| Br3AA | tribromoacetic acid |

**Table 7. Formation of cyanogen chloride (CNCl) in treated SR and NL samples.**

| Sample | [CNCl] (μg/L) |
|---|---|
| SR + HOCl | 3.3 |
| SR + NH$_2$Cl | 15.9 |
| NL + HOCl | 4.2 |
| NL + UV + HOCl | 4.2 |
| NL + Nitrate + HOCl | 4.3 |
| NL + Nitrate + UV + HOCl | 4.2 |
| NL + Iodide + HOCl | 1.2 |
| NL + NH$_2$Cl | 10.1 |
| NL + UV + NH$_2$Cl (1/16/12) | 37.7 |
| NL + Iodide + NH$_2$Cl | 12.5 |
| NL + Iodide + UV + NH$_2$Cl (10/13/11) | 35.7 |
| NL + Iodide + UV + NH$_2$Cl (1/16/12) | 50.8 |
| NL + NH$_2$Cl, 72 h | 8.8 |
| NL + NH$_2$Cl, 96 h | 5.6 |

SR=Suwannee River NOM; NL=Nordic Lake NOM

**Table 8. Lowest significant cytotoxic concentration, $IC_{10}$, $IC_{20}$, and $IC_{50}$ values (in mg/L as C) for each treated SR and NL sample.**

| Sample | Lowest Cytotox. Conc.[a] | $IC_{10}$[b] | $IC_{20}$[b] | $IC_{50}$[b] | ANOVA test p-value |
|---|---|---|---|---|---|
| SR + HOCl | 92.0 | 73.1 (66.4, 79.2) | 83.4 (78.9, 90.1) | NA | <0.001 |
| SR + NH$_2$Cl | NA | NA | NA | NA | <0.001 |
| NL + HOCl | 23.0 | 14.7 (10.9, 18.5) | 29.4 (26.1, 32.7) | 65.8 (63.4, 68.3) | <0.001 |
| NL + UV + HOCl | 33.6 | 18.6 (13.4, 23.5) | 34.1 (29.0, 38.5) | 72.2 (68.7, 75.9) | <0.001 |
| NL + Nitrate + HOCl | 11.2 | 9.8 (6.4, 13.1) | 26.3 (22.1, 30.4) | 75.0 (70.7, 80.3) | <0.001 |
| NL + Nitrate + UV + HOCl | 22.4 | 17.4 (13.2, 21.7) | 29.8 (25.3, 34.6) | 61.8 (58.3, 65.8) | <0.001 |
| NL + Iodide + HOCl | 44.8 | 32.0 (26.9, 36.9) | 46.4 (42.0, 50.9) | 80.6 (76.2, 83.9) | <0.001 |
| NL + NH$_2$Cl | 89.9 | 83.9 (76.8, 89.3) | NA | NA | <0.001 |
| NL + UV + NH$_2$Cl (1/16/12) | 32.3 | 12.1 (6.4, 17.7) | 28.4 (22.8, 34.1) | 66.8 (62.9, 70.2) | <0.001 |
| NL + Iodide + NH$_2$Cl | 43.5 | 36.3 (31.1, 45.2) | 58.3 (53.6, 66.6) | NA | <0.001 |
| NL + Iodide + UV + NH$_2$Cl (10/13/11) | 32.3 | 27.1 (22.7, 31.2) | 37.8 (34.0, 41.7) | 60.7 (58.2, 63.0) | <0.001 |
| NL + Iodide + UV + NH$_2$Cl (1/16/12) | 10.8 | 8.0 (4.5, 11.4) | 17.8 (14.2, 21.7) | 42.9 (39.2, 46.7) | <0.001 |
| NL + NH$_2$Cl, 72 h | 82.9 | 56.3* | NA | NA | <0.001 |
| NL + NH$_2$Cl, 96 h | 83.1 | NC | NC | NC | <0.001 |

Values in () are 95% confidence intervals calculated using bootstrapping in the statistical program, R.

*Could not calculate 95% confidence interval due to error that occurred during bootstrapping.

NA=Not applicable because this level of growth inhibition was not observed, either due to apparent stimulation of cell growth (SR+NH$_2$Cl) or due to concentrations that were too low to induce this level of cytotoxic response.

NC=Not calculated because data did not fit the sigmoidal 3 parameter model used for the others.

[a]Lowest concentration at which a significant reduction in mean cell density as compared to the control was detected using a Holm-Sidak multiple comparison versus the control group analysis.

[b]The $IC_x$ is the concentration at which the cell density is reduced by x% as compared to the control. This value is calculated using the sigmoidal regression equation fit to each curve.

**Table 9. Model coefficients and $R^2$ values for cytotoxicity curves**

| Sample | a | b | $x_0$ | $R^{2\ a}$ |
|---|---|---|---|---|
| SR+HOCl | 106.7264 | -17.4945 | 102.5585 | 0.87 |
| SR+NH$_2$Cl | 226.5184 | 124.5601 | 35.4105 | 0.90 |
| NL+HOCl | 117.6493 | -34.4168 | 55.3606 | 0.99 |
| NL+UV+HOCl | 117.3322 | -36.0286 | 61.5225 | 0.99 |
| NL+Nitrate +HOCl | 143.3213 | -56.7847 | 39.5548 | 0.98 |
| NL+Nitrate +UV+HOCl | 121.5811 | -31.6143 | 50.4772 | 0.99 |
| NL+Iodide +HOCl | 114.0784 | -30.9879 | 72.891 | 0.99 |
| NL + NH$_2$Cl | 106.3612 | -20.2044 | 118.3884 | 0.78 |
| NL+UV+NH$_2$Cl (1/16/12) | 113.6885 | -34.6684 | 58.4002 | 0.98 |
| NL+Iodide+ NH$_2$Cl | 138.514 | -72.1482 | 80.9014 | 0.93 |
| NL+Iodide+UV+NH$_2$Cl (10/13/11) | 107.6967 | -19.011 | 57.9778 | 0.99 |
| NL+Iodide+UV+NH$_2$Cl (1/16/12) | 121.216 | -24.7546 | 34.1828 | 0.99 |
| NL+NH$_2$Cl,72h | 107.5749 | -53.1083 | 143.0748 | 0.84 |
| NL+NH$_2$Cl,96h | NA | NA | NA | NA |

NA=Not available because data did not fit the sigmoidal 3 parameter model used for the others.

[a] Value indicative of how well the sigmoidal regression model, $f = \dfrac{a}{1 + e^{-(x-x_o)/b}}$ , fits the data, where f=cell density as percent control, x=concentration, and a, b, and $x_0$ are coefficients that are determined using a series of iterations that minimize the error between the observed f value and the predicted f value.

**Table 10. Two-way analysis of variance (ANOVA)**

| | Testing Results (p-values) for Difference between Means [a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Concentration Level* [b] | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Figure 8 | 0.278 | 0.0009 | 0.0005 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| Figure 9 | 0.004 | 0.0407 | 0.0134 | 0.6315 | 0.4031 | 0.0041 | <.0001 | <.0001 |
| Figure 10 | 0. 221 | 0. 0724 | 0.0074 | 0.0183 | 0.0376 | 0.0136 | <.0001 | <.0001 |
| Figure 11 | 0.076 | 0.054 | 0.0008 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| Figure 12 | 0.264 | 0.197 | 0.0022 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| Figure 14 | 0.482 | 0.0868 | 0.0698 | 0.0225 | 0.0006 | 0.1431 | 0.0522 | 0.1109 |

[a] Results comparing cell density means at nearby DOC concentrations, lowest to highest.  The zero concentration (negative control) is excluded from this ANOVA analysis because the mean cell density for these wells is set at one hundred percent during data analysis so there is no difference between means (p=1.000) in this case.

[b] DOC concentrations varied slightly among the different samples.  #1~8 refer to the first through eighth concentration levels used in the experiments (the negative control is considered the zero level, as described above).  The exact DOC concentrations are listed in Appendix B, but concentration level #1 ≈ 0.5 mg/L as C, #2 ≈ 1 mg/L as C, #3 ≈ 10 mg/L as C, #4 ≈ 20 mg/L as C, #5 ≈ 30 mg/L as C, #6 ≈ 40 mg/L as C, #7 ≈ 70 mg/L as C, #8 ≈ 90 mg/L as C.
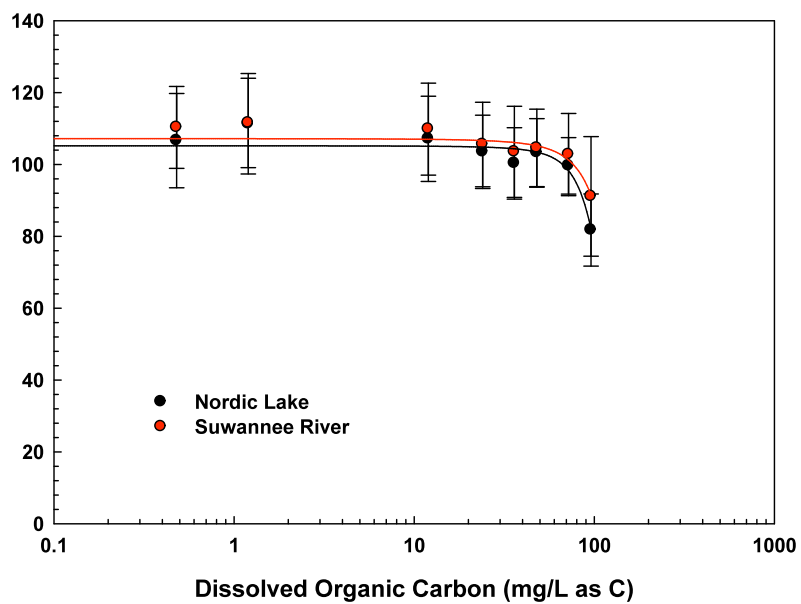
**Figure 1. Cytotoxicity Dose-Response Curves for Two Types of Natural Organic Matter (NOM); Error bars represent standard deviation for n=16 replicates.**
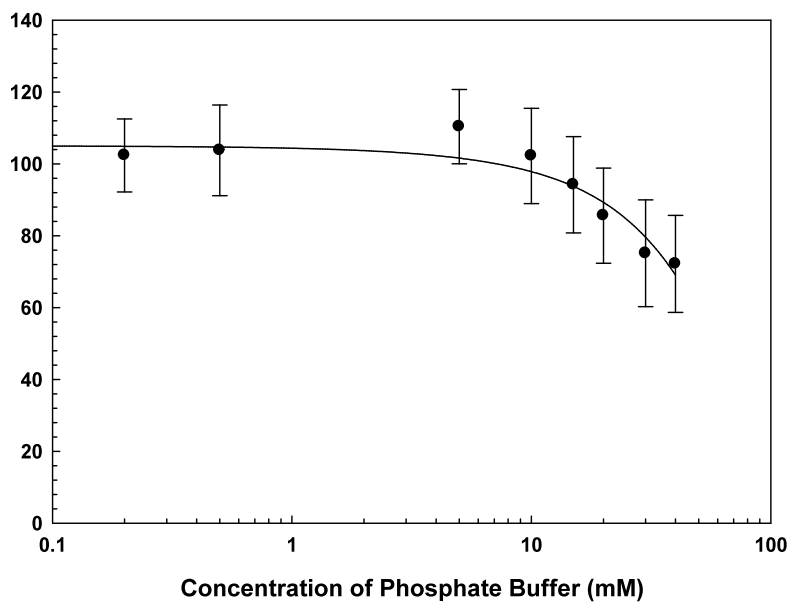


**Figure 2. Cytotoxicity Dose-Response Curve for Phosphate Buffer, pH 7.1, in LGW; Error bars represent standard deviation for n=16 replicates.**
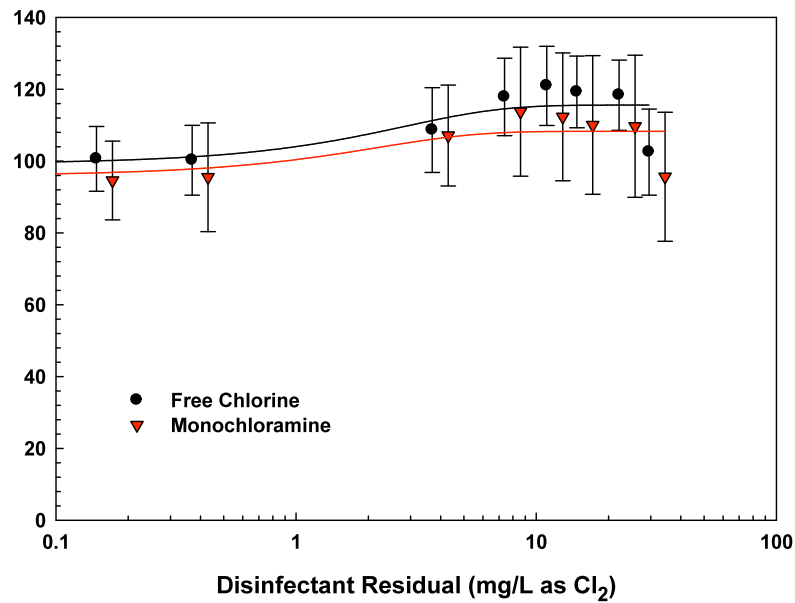
**Figure 3. Cytotoxicity Dose-Response Curves for Chlorine and Chloramine Residuals in LGW, pH 7.1; Error bars represent standard deviation for n=16 replicates.**
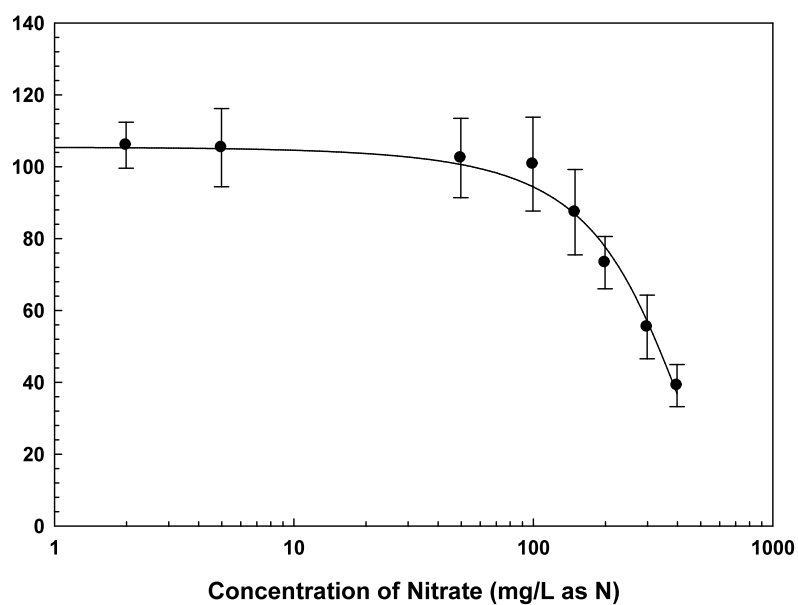
**Figure 4. Cytotoxicity Dose-Response Curve for Nitrate-spiked Nordic Lake NOM; Error bars represent standard deviation for n=16 replicates.**
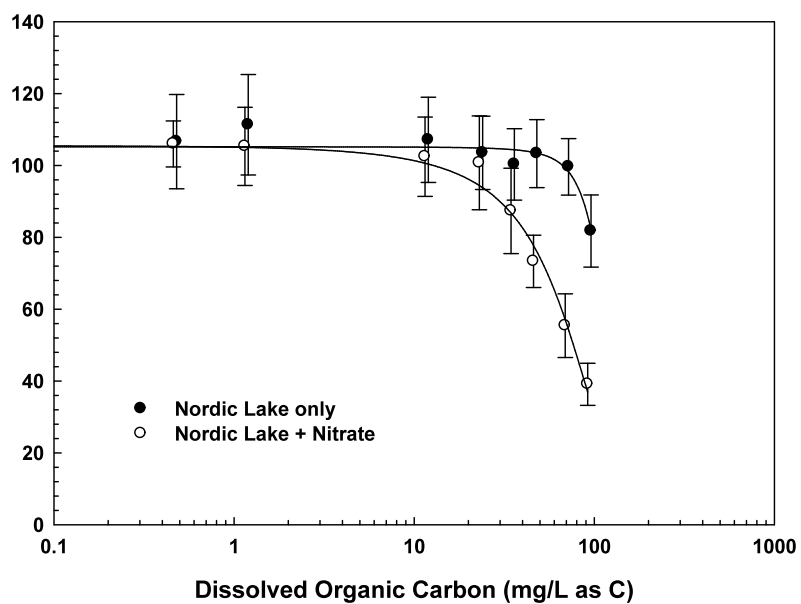


**Figure 5. Cytotoxicity Dose-Response Curves for Unspiked and Nitrate-spiked Nordic Lake NOM; Error bars represent standard deviation for n=16 replicates.**
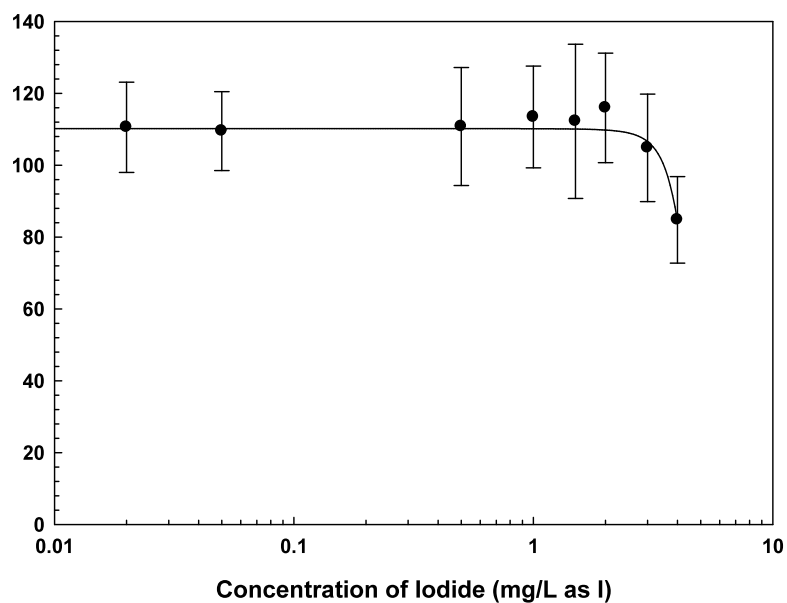
**Figure 6. Cytotoxicity Dose-Response Curve for Iodide-spiked Nordic Lake NOM; Error bars represent standard deviation for n=16 replicates.**
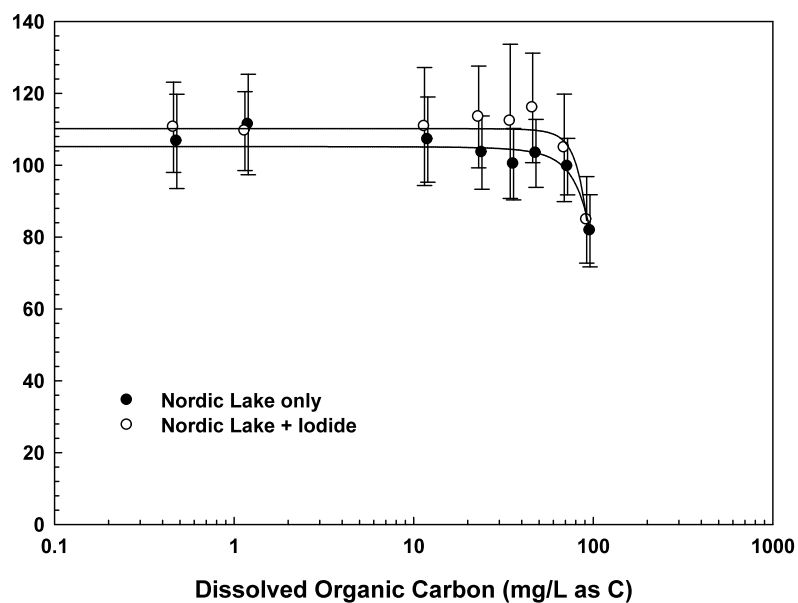


**Figure 7. Cytotoxicity Dose-Response Curve for Unspiked and Iodide-spiked Nordic Lake NOM; Error bars represent standard deviation for n=16 replicates.**

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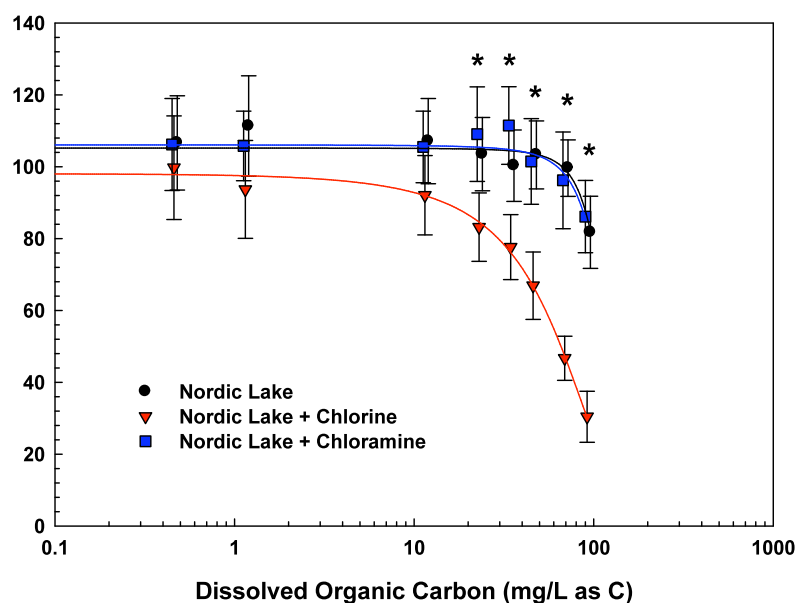
**Figure 8. Cytotoxicity Dose-Response Curves for Untreated, Chlorinated, and Chloraminated Nordic Lake NOM; Error bars represent standard deviation for n=16 replicates. Asterisks show statistically significant differences among samples (p<0.0001, Two-Way ANOVA).**
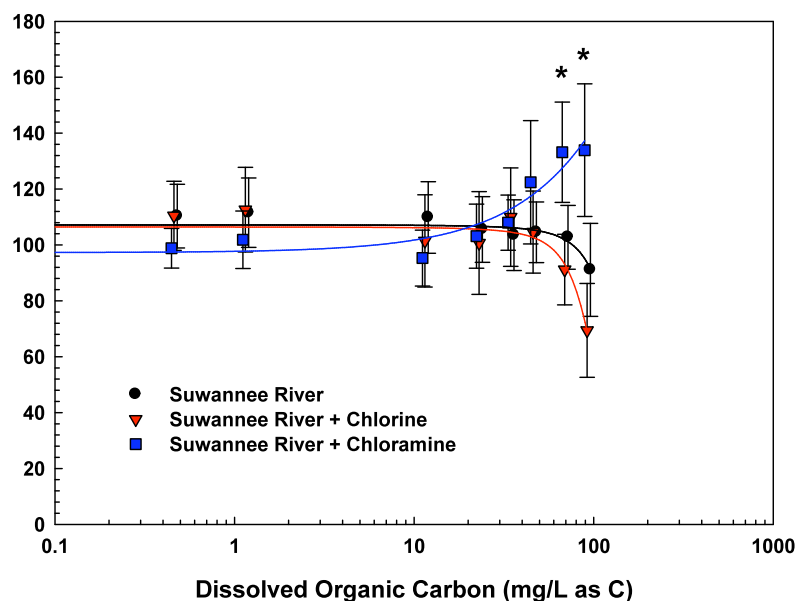


**Figure 9. Cytotoxicity Dose-Response Curves for Untreated, Chlorinated, and Chloraminated Suwannee River NOM; Error bars represent standard deviation for n=16 replicates. Asterisks show statistically significant differences among samples (p<0.0001, Two-Way ANOVA).**
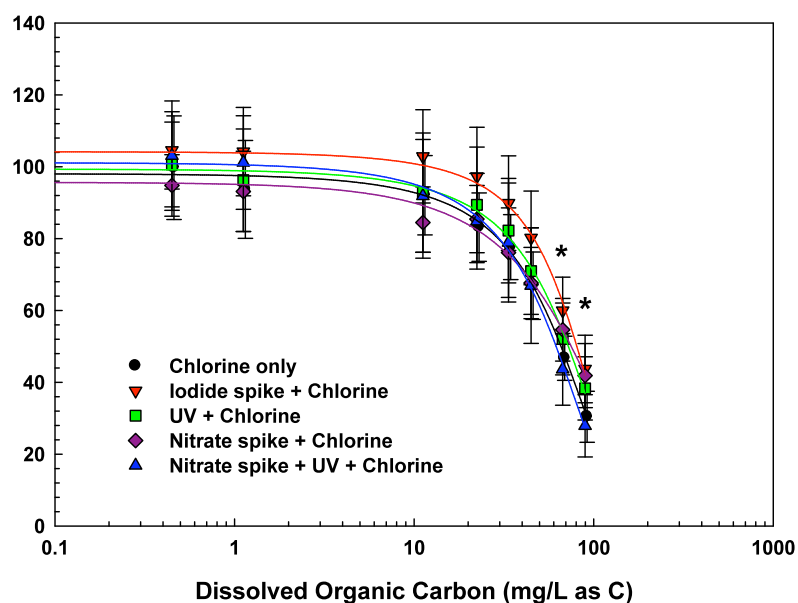
**Figure 10. Cytotoxicity Dose-Response Curves for Chlorinated Nordic Lake NOM under different spiking conditions and with or without UV treatment; Error bars represent standard deviation for n=16 replicates. Asterisks show statistically significant differences among samples (p<0.0001, Two-Way ANOVA).**
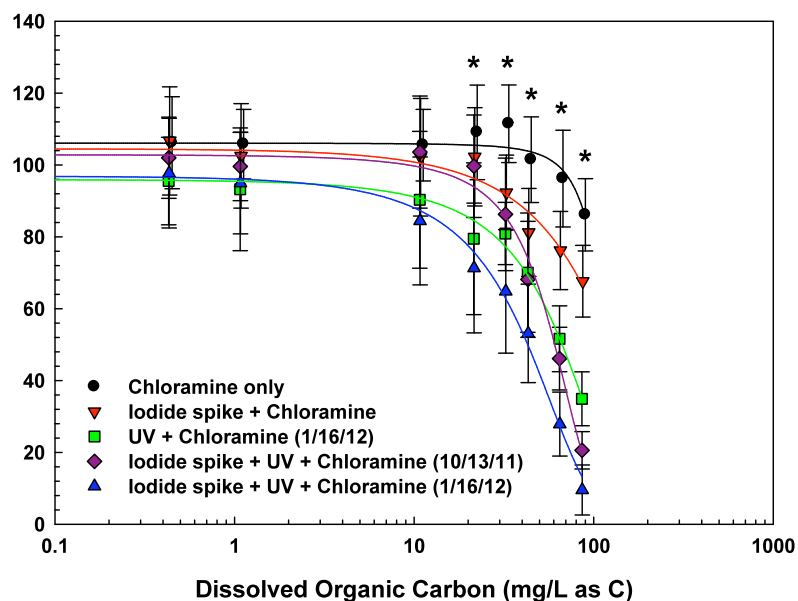


**Figure 11. Cytotoxicity Dose-Response Curves for Chloraminated Nordic Lake NOM with or without Iodide spike and with or without UV treatment; Error bars represent standard deviation for n=16 replicates. Asterisks show statistically significant differences among samples (p<0.0001, Two-Way ANOVA).**
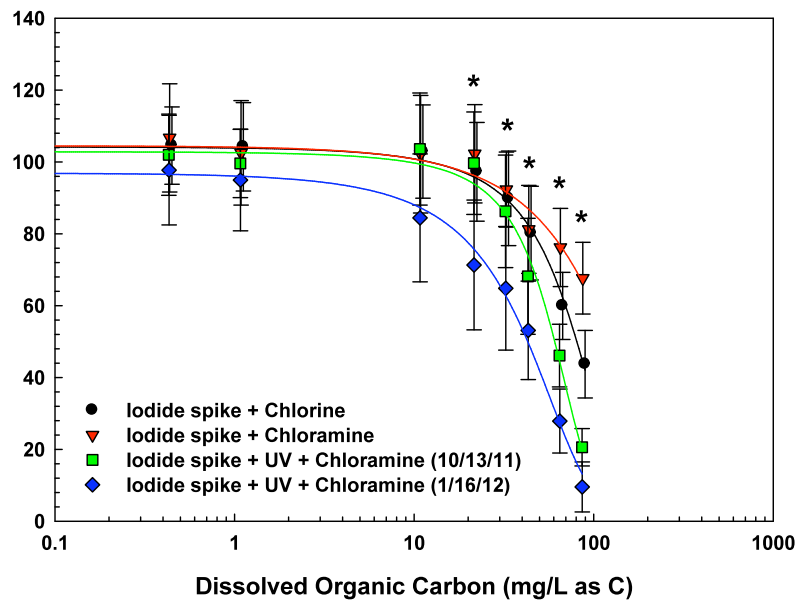
**Figure 12. Cytotoxicity Dose-Response Curves for Iodide-spiked Nordic Lake NOM with different disinfection conditions; Error bars represent standard deviation for n=16 replicates. Asterisks show statistically significant differences among samples (p<0.0001, Two-Way ANOVA).**
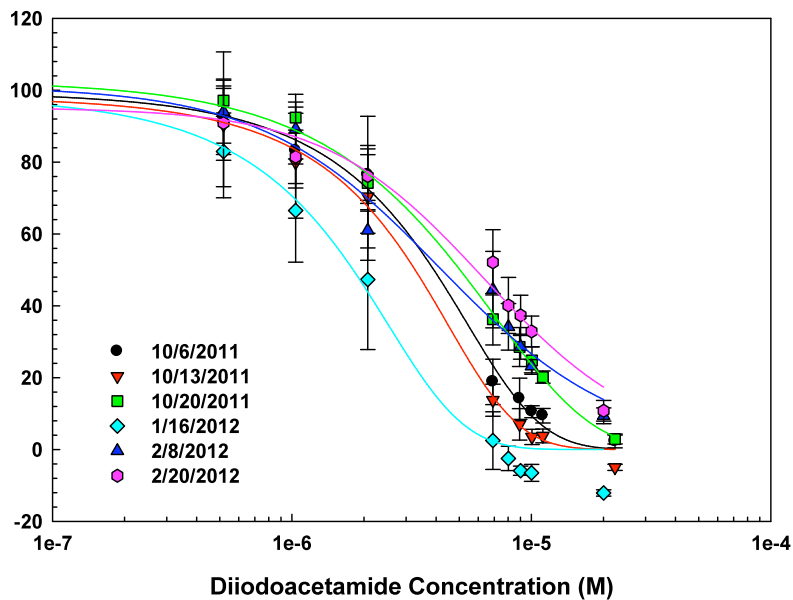


**Figure 13. Cytotoxicity Dose-Response Curves for the Positive Control, Diiodoacetamide, in different experiment sets; Error bars represent standard deviation for n=8 replicates.**
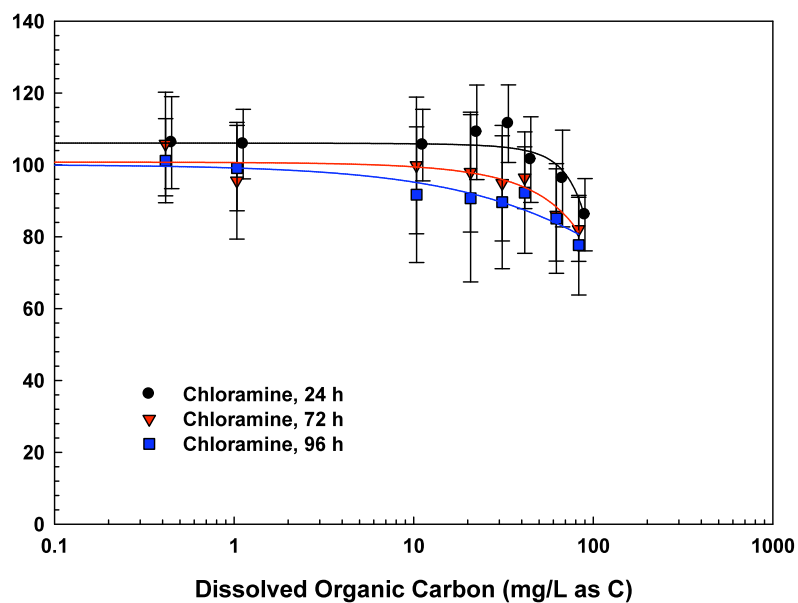
**Figure 14. Cytotoxicity Dose-Response Curves for Nordic Lake NOM Chloraminated for 24, 72, and 96 h; Error bars represent standard deviation for n=16 replicates.**

Standard Operating Procedures

**Procedure for Total/Dissolved Organic Carbon (TOC/DOC) and Total Nitrogen (TN) analysis in water samples**

*Standards Preparation*

**Dissolved Organic Carbon (DOC) Stock Standard** (1000 mg/L as C)

- Dissolve 2.125 g Potassium Hydrogen Phthalate in 1-L lab grade water (LGW); mix with a magnetic stir bar

- Store in fridge in amber bottle with PTFE-lined septa/cap. Good for 2 months

**Total Nitrogen (TN) Stock Standard** (1000 mg/L as N)

- Dissolve 7.219 g Potassium nitrate in 1-L LGW; mix with a magnetic stir bar

- Store in fridge in amber bottle with PTFE-lined septa/cap. Good for 2 months

**HCl solution (2 N)**

- Carefully add 41 mL concentrated HCl (12.1 N) to LGW in a 250 mL volumetric flask.

- Fill to line with LGW and carefully invert 3 times. Store in amber bottle with PTFE-lined septa/cap.

-----------------------------------------------------------------------------------------------------------

**DOC Working Solution** (100 mg/L as C)

- Pipette 10-mL of DOC Stock Standard into a 100 mL volumetric flask; fill to line with LGW; invert stoppered flask three times

- Store in fridge in amber bottle with PTFE-lined septa/cap. Good for 1 week

**DOC/TN Working Solution** (100 mg/L as C, 100 mg/L as N, 0.05 M HCl)

- Pipette 10-mL of DOC Stock Standard, 10 mL of TN Stock Standard, and 2.5 mL of 2 M HCl into a 100 mL volumetric flask; fill to line with LGW; invert stoppered flask three times

- Store in fridge in amber bottle with PTFE-lined septa/cap. Good for 1 week

-----------------------------------------------------------------------------------------------------

Calibration points should be made fresh for every run

- To make 0.5 mg-C/L calibration point, pipette 0.5 mL of DOC Working Solution into a 100-mL volumetric flask; fill to line with LGW; invert stoppered flask three times

- Additional calibration points are made in an analogous fashion

*Procedure*

Notes:

*The concentrations of the samples need to be **less than 10 mg/L** as C or N – you should first test a highly diluted sample to make sure you will be in the correct range.

*If you do not plan to analyze your water samples soon after you collect them, adjust to pH 4.5 and store them in the fridge.

*Before you start running samples, you need to talk to person in charge of the TOC/TN about what type of samples you will be running – to make sure they will not compromise the instrument

1. Prepare calibrations (for example: 0, 0.5, 5, 10 mg C and N/L) and samples (dilute if necessary – concentration needs to be less than 10 mg/L as C or N).

2. Pour your samples and calibrations into acid-washed TOC vials.

3. Acidify all samples and calibrations to pH 2-2.5 using 2 N HCl. A typical surface water requires about 2-4 drops of 2 N HCl if using 24 mL sample vials, but you need to test your actual sample matrix using a pH meter to be sure you adjust the pH to this value. Cover each vial with aluminum foil. Calibration points prepared using LGW from Weinberg lab typically require ~6 drops of 2 N HCl (but you should check the pH using an extra aliquot with the pH meter).

4. Start the system: Before using the instrument, check a day or two in advance that the head pressure on the air tank is above 500psi by opening the regulator attached to the air tank and reading the pressure. If it is not, consult with whoever is responsible for the instrument so that a new gas tank can be ordered. Use only UHP air ("air grade zero"). On the day of use turn on computer, turn on TOC analyzer, and open the air tank at the regulator.

5. Check the system:

   Open Software (TOC ControlV)

   Sample table (User = TOC; password = UNC)

   File → New → sample run → (TC/IC-TN 24mL system (default) or use TC/IC-TN 40 mL if using 40 mL sample vials)

   Instrument → connect

   Check the following on the instrument:

   (a)    Carrier gas flow = 150 (TOC analyzer)

   (b)    Pressure = 200 (TOC analyzer)

   (c)    Continuous bubbles in the plastic bottle (TOC analyzer)

   (d)    N flow ~ 0.5 (Nitrogen unit)

(e) Fill the humidifier tank with laboratory grade water (LGW) of TOC < 0.5ppm water if it is empty or almost empty.

Instrument → Background monitor → run and wait for all points to be checked and green (about 20 mins)

6. Create your calibration curve

For TOC/DOC:

File → New → Calibration curve → 24mL system (default) → NPOC (for Non Purgeable Organic Carbon)

Standard

TOC

Linear Regression (uncheck the 'zero shift')

Check 'multiple injections'

Put the number of standards and the range of the concentrations

Adjust the concentrations of each standard and save

For TN:

File → New → Calibration curve → 24mL system (default) → TN

Standard

TN

Linear Regression (uncheck the 'zero shift')

Check 'multiple injections'

Put the number of standard and the range of the concentrations

Adjust the concentrations of each standard and save

7. Create your sequence

(a) First excel cell → insert autogenerate→ choose your method → put 3-4 blank LGW vials to rinse the system

(b) Run a 5 mg/L as C and N standard after LGWs. You will record the area counts for these in the logbook and do the same for a 5 mg/L as C and N standard at the end of your run.

(c) Click on next excel cell → insert calib curve NPOC → enter the vial #s in the ASI vial view

(d) Next excel cell → insert calib curve TN → enter the vial #s

(e) Next excel cell → insert auto generate → choose your method → enter the number of samples and start vial # (only after the standards) → Enter your sample name in the excel cells→ Save as your sequence

*be sure to run another 5 mg/L as C and N standard after all of your samples and a LGW blank. Run 3 LGW blanks after this standard too.

8. Check the system: Recheck the previous signals, if all lights are green, Maintenance → replace flow line content (cleans the syringe)

9. Run the sequence

Instrument → Start → Shut down → make sure external acid addition is checked → run

10. Instrument will shut down once sample run is finished, but you need to come in and manually turn off the gas tank at the regulator when run is done.

11. In the notebook by the instrument, record the method and calibration you used next to your name and the date. When your samples have finished running, record the calibration curve information: slope, y-intercept, $R^2$, and the area counts for

the first non-zero calibration point area. Also record the area counts for the 5 mg/L standards at the start and end of your run.

12. After running your samples, remove vials from instrument immediately and clean them. Any vial containing environmental samples (tap water or dirtier) needs to be rinsed and put in the 10% nitric acid bath overnight. Then rinse at least 3x with LGW and dry in 180$^o$C oven overnight. Any vial containing LGW or standards made up in LGW can be rinsed 3x with LGW and dried in 180$^o$C oven overnight.

13. Maintenance – All users are expected to contribute their time in maintaining the instrument, troubleshooting problems, and providing resources to replace consumables.

Prepared by: Bonnie Lyon, 10/20/08

**Standard Operating Procedure for Halogenated Volatile and Haloacetamide**

**Analysis**

<u>Halogenated Volatiles</u>

| Abbrev. | Compound | CAS # | mol. wt. (g/mol) |
|---|---|---|---|
| CHCl$_3$ | Chloroform | 67-66-3 | 119.38 |
| TCAN | trichloroacetonitrile | 545-06-2 | 144.39 |
| DCAN | dichloroacetonitrile | 3018-12-0 | 109.94 |
| BrCl$_2$CH | bromodichloromethane | 75-27-4 | 163.83 |
| TCA | chloral hydrate | 302-17-0 | 165.40 |
| 11 DCP | 1,1-dichloropropanone | 513-88-2 | 126.97 |
| TCNM | Trichloronitromethane (chloropicrin) | 76-06-2 | 164.38 |
| Br$_2$ClCH | dibromochloromethane | 124-48-1 | 208.28 |
| BCAN | bromochloroacetonitrile | 83463-62-1 | 154.39 |
| 111TCP | 1,1,1-trichloropropanone | 918-00-3 | 161.42 |
| CHBr$_3$ | bromoform | 75-25-2 | 252.73 |
| DBAN | dibromoacetonitrile | 3252-43-5 | 198.85 |

<u>Haloacetamides</u>

| Abbrev. | Compound | CAS # | mol. wt. (g/mol) |
|---|---|---|---|
| DIAM | diiodoacetamide | 5875–23–0 | 310.85 |
| BIAM | bromoiodoacetamide | 62872–36–0 | 263.86 |
| CIAM | chloroiodoacetamide | 62872–35–9 | 219.41 |
| DBAM | dibromoacetamide | 598–70–9 | 216.86 |
| TBAM | tribromoacetamide | 594–47–8 | 295.75 |
| BCAM | bromochloroacetamide | 62872–34–8 | 172.41 |
| DBCAM | dibromochloroacetamide | 855878–13–6 | 251.31 |
| BDCAM | bromodichloroacetamide | 98137–00–9 | 206.85 |
| BAM | bromoacetamide | 683–57–8 | 137.96 |
| DCAM | dichloroacetamide | 683–72–7 | 127.96 |
| TCAM | trichloroacetamide | 594–65–0 | 162.40 |

*Equipment*

- Clear 60-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, 250 µL

- 50-250 µL Dade Model J micropipetter fitted with clean glass capillary tips

- Eight 100-mL glass volumetric flasks with glass stoppers

- 1-L amber bottle mounted with 10-mL pump pipetting dispenser containing PFTE transfer line

- 23-cm disposable glass Pasteur pipettes

- Rubber Pasteur pipette bulb

- pH indicator strips pH 0-6 – colorpHast, EMD Chemicals, (Fisher Scientific – catalog #M95863)

- GC vials - 12x32 mm 1.8-mL Amber glass vials, Laboratory Supply Distributors, (catalog #20211ASRS-1232)

- GC Caps - 11 mm seal w/ Red Teflon® faced silicone septa, 40 Mils thick, Supelco (catalog #27360-U)

- GC vial inserts – 5x30 mm Flat Bottom LVI, Laboratory Supply Distributors, (catalog #20870-530)

- Hand crimper for sealing gas chromatography autosampler vials

- Vortexer

- Teflon tape

- Stainless steel scupula

*Instrumentation*

Gas Chromatograph

- Hewlett-Packard GC5890 Series II 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: OmniSolv Methyl-t-Butyl Ether, EMD Chemicals, (Fisher Scientific – catalog # MMX08266)

- Sodium sulfate ($Na_2SO_4$), Mallinckrodt, granular, ACS grade (catalog #8024) from Scientific Storeroom. Bake at $400^{\circ}C$ in muffle furnace for 24 hours in a shallow, porcelain dish covered with aluminum foil. Store in glass-stoppered bottle in desiccator.

- Solvent for dilution of standards: OmniSolv Methyl-t-Butyl Ether (EMD Chemicals, Fisher Scientific – catalog # MMX08266)

- Methanol (for rinsing glassware) HPLC grade

- L-Ascorbic Acid (for quenching residual $Cl_2$) Certified ACS grade (Fisher Scientific – catalog #A61-25)

- Sulfuric Acid (for pH adjustment) Certified ACS Plus (Fisher Scientific – catalog #A300-212)

*Standards*

- THM Calibration Mix, 2000 μg/mL each in methanol. (Supelco, Bellafonte - catalog # 48140-U)

- EPA 551B Halogenated Volatiles Mix, 2000 μg/mL each in methanol (Supelco, Bellafonte - catalog # 4-8046)

- Chloral Hydrate, 1000 μg/mL in acetonitrile (Supelco, Bellafonte - catalog # 47335-U)

- Internal Standard (IS): Aldrich (Milwaukee, WI) 1,2-dibromopropane neat standard, 99+% (catalog #14,096-1)

- Bromoacetamide (98%), Acros Organics (catalog # 291100050)

- Dichloroacetamide (98%), Acros Organics (catalog # 113050100)

- Trichloroacetamide (99%), Acros Organics (catalog # 202920250)

- Bromochloroacetamide, CanSyn Chemical Corporation

- Bromodichloroacetamide, CanSyn Chemical Corporation

- Tribromoacetamide, CanSyn Chemical Corporation

- Chloroiodoacetamide, CanSyn Chemical Corporation

- Dibromochloroacetamide, CanSyn Chemical Corporation

- Dibromoacetamide, CanSyn Chemical Corporation

- Diiodoacetamide, CanSyn Chemical Corporation

- Bromoiodoacetamide, CanSyn Chemical Corporation

*Samples*

Samples should be collected headspace-free in pre-cleaned 60 mL glass vials with screw caps and PTFE-lined silicone septa containing 1.4 mg ascorbic acid. Samples should be filled head-space free and holding vial at an angle so halogenated volatiles do not escape through volatilization. Store samples in fridge at $4^{o}$C. Samples should be extracted within 24 hours of quenching.

*Procedure*

**Internal Standard**

Stock solution of Internal Standard (IS) at ~2000 µg/mL in MtBE - prepared by injecting 10mL of the neat standard and injecting into a 5 mL volumetric flask containing MtBE, fill to line with MtBE.

Primary dilution at 100µg/mL: prepared by injecting 250µL of IS stock solution using a micropipette into a 5 mL volumetric flask containing MtBE, fill to line with MtBE.

Extracting solution at 50 µg/L or 100 µg/L (depending on what expected concentration of analytes in samples): calculate how much extracting solvent will be needed for all of your samples and calibrations (3 mL for each sample and calibration). Make from primary dilution, and 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.

**Halogenated Volatiles Calibration Standards**

These are prepared as a mix of THM4, 551B Halogenated Volatiles and chloral hydrate.

Calibration Standard #1: 100 µg/mL, 100 µL of each THM4 and EPA551B stock calibration mix and 200 µL of chloral hydrate to 2 mL volumetric flask containing MtBE, fill to line with MtBE.

Calibration Standard #2: 1 µg/mL, 20 µL of Calibration Standard #1 into 2 mL volumetric flask containing MtBE, fill to line with MtBE.

**Haloacetamide Stock & Calibration Standards**

Primary dilution stock: 2000 µg/mL. Prepared from solid standards of each haloacetamide. Weigh out 20 mg of each compound, dissolve in 10 mL high purity MtBE.

Calibration Standard #1: 20 µg/mL, 20 µL of primary dilution stock into 2 mL volumetric flask containing MtBE, fill to line with MtBE.

Calibration Standard #2: 1 µg/mL, 100 µL of Calibration standard #1 into 2 mL volumetric flask containing MtBE, fill to line with MtBE.

Transfer standards to a 2-mL amber glass vial and store in laboratory standards freezer at -15$^{\circ}$C.

14. Check calibration standards a few days before extraction. Make up two dilutions (50μg/L and 1μg/L) in MtBE containing internal standard. 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 for instrument detector drift and the values for each compound 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.

15. 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.

16. Prepare calibration standards in 100 mL LGW according to the range of concentrations expected in the samples. Examples for halogenated volatiles and haloacetamides are shown below.

**Example of Halogenated Volatile Calibrations**

| Level | Calibration standard | Volume Cal. std. added (µL) to 100 mL LGW | Analyte conc. (µg/L) |
|-------|---------------------|-------------------------------------------|----------------------|
| 1 | -- | 0 | 0 |
| 2 | 1 µg/mL | 10 | 0.1 |
| 3 | 1 µg/mL | 100 | 1 |
| 4 | 100 µg/mL | 10 | 10 |
| 5 | 100 µg/mL | 20 | 20 |
| 6 | 100 µg/mL | 50 | 50 |
| 7 | 100 µg/mL | 100 | 100 |
| 8 | 100 µg/mL | 200 | 200 |

**Example of Haloacetamide Calibrations**

| Level | Calibration standard | Volume Cal. std. added (µL) to 100 mL LGW | Analyte conc. (µg/L) |
|-------|---------------------|-------------------------------------------|----------------------|
| 1 | -- | 0 | 0 |
| 2 | 1 µg/mL | 10 | 0.1 |
| 3 | 1 µg/mL | 50 | 0.5 |
| 4 | 20 µg/mL | 25 | 5 |
| 5 | 20 µg/mL | 50 | 10 |
| 6 | 20 µg/mL | 125 | 25 |
| 7 | 20 µg/mL | 250 | 50 |

17. Prepare matrix spike (MS) and matrix spike duplicate (MSD) in 30 mL samples
→ should be ~2-3 times halogenated volatile levels in samples.

18. Measure 30 mL from all calibration standards using a 50 mL measuring cylinder starting from lowest to highest concentration and then follow with the samples all in duplicate and transfer into 60 mL vials.  Rinse cylinder 3 times with LGW and once with sample to be measured next between each.  Pour at an angle so halogenated volatiles are not lost through volatilization.

19. Adjust all samples and calibrations to approximately pH 3.5 with 0.2 N $H_2SO_4$. (Amount required for pH adjustment will likely be different for calibrations compared to samples. Use remaining 30 mL aliquot from 60 mL vial to determine how much $H_2SO_4$ will be needed.)

20. Add 3 mL extracting solvent from a solvent dispenser bottle to each 30 mL aliquot. Make sure there are no bubbles in the dispenser addition line.

21. Add ~6 g pre-baked sodium sulfate to each 30 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.

22. 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. Fill three GC vials for each sample (one for halogenated volatile analysis, one for haloacetamide analysis, and one backup), and two GC vials with each calibration (since you will have separate halogenated volatile and haloacetamides calibrations – need one for analysis and one backup). Use GC vial inserts. Store in the laboratory freezer at -$15^{o}$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 containing MtBE + IS. Analyze within 4 weeks.

23. Analyze according to specified GC method (see GC temperature programs below) 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 the sample identity based on received samples and shipping list information. If a reasonable determination can be made, the sample will be analyzed and those data will be qualified in reports and future data analyses.

*GC-ECD analysis on Hewlett-Packard GC5890 Series II:*

Injector:

Syringe size = 10μL; Injection volume = 2μL

Wash solvent = MtBE; Pre-injection washes = 3; Post-injection washes = 3; Pumps = 3

Injector Temperature = 200°C; Injection splitless (split after 0.5 min)

Oven/Column:

Oven equilibration time = 3 min; Oven max °C = 300°C

Gas = He; Column flow = 1mL/min

Column type = DB1 (Agilent), 30.0m length, 0.25mm inner diameter, 1μm film thickness

Split flow = 1mL/min; Split ratio = 1:1

**Halogenated Volatiles Oven Temperature program (Total time = 55.75 min)**

|  | Velocity (°C/min) | Temp. (°C) | Time (min) |
|---|---|---|---|
| **Initial** | - | 35 | 22 |
| **Level 1** | 10 | 145 | 2 |
| **Level 2** | 20 | 225 | 10 |
| **Level 3** | 20 | 260 | 5 |

Electron Capture Detector (ECD), Detector temperature = 290°C, Injector temp: 117°C

**Haloacetamides Oven Temperature program (Total time = 59.60 min)**

|  | Velocity (°C/min) | Temperature (°C) | Time (min) |
|---|---|---|---|
| **Initial** | - | 37 | 1 |
| **Level 1** | 5 | 110 | 10 |
| **Level 2** | 5 | 280 | 0 |

Electron Capture Detector (ECD), Detector temperature = 300°C

Prepared by Bonnie Lyon, 08/2009

**Analysis of Cyanogen Chloride by Liquid-Liquid Extraction and Gas Chromatography with Electron Capture Detector**

<u>Materials</u>

*Equipment*

- Clear 60 mL clean glass screw cap sample vials with PTFE-lined silicone septa.

- 10-50 μL and 50-250 μL micropipetter and glass capillary tips

- 100 mL and 10 mL volumetric flasks with glass stoppers, 2 mL volumetric flask with screw cap and PTFE-lined silicone septa

- 50 mL graduated cylinder

- 1-L amber bottle mounted with 10 mL pump pipetting dispenser

- 23 cm disposable glass Pasteur pipettes and rubber bulb

- pH indicator strips pH 0-6: colorpHast (HC781889 catalog # 9586)

- 2 mL amber glass autosampler vials with rubber/TFE aluminum seals

- Hand crimper for sealing autosampler vials

- Vortexer

- Teflon tape

- Stainless steel scupula

*Instrumentation*

Gas Chromatograph

- Hewlett-Packard GC6890 Series with autosampler/autoinjector tower

- Column: Zebron 1701 (Phenomenex)

- Electron Capture Detector (ECD): Hewlett-Packard Model ECD

- Data System: Hewlett Packard ChemStation

GC Gases

- Carrier Gas-Ultra High Purity (UHP) helium (He) through UNC Scientific Storerooms (catalog #SG62350)

- Makeup Gas-Ultra High Purity (UHP) nitrogen ($N_2$) through UNC Scientific Storerooms (catalog #SG62750)

*Reagents*

- Cyanogen chloride stock standard 2000 μg/mL SPEX CertiPrep

- Internal Standard (IS): Aldrich (Milwaukee, WI) 1,2-dibromopropane neat standard, 99+% (catalog #14,096-1)

- Sodium sulfate (Mallinckrodt, Paris KY) granular ACS grade (catalog #8024) baked at 400° for 24 hours, stored in desiccator

- Extraction solvent: OmniSolv Methyl-t-Butyl Ether (Fisher Scientific catalog #MX08266)

- Solvent for dilution of standards and working solutions: Sigma-Aldrich Purge & Trap Methanol (Sigma catalog # 414816)

- Methanol (for rinsing glassware) HPLC grade

- L-ascorbic acid (for quenching residuals) Certified ACS grade (Sigma catalog #10K0256)

- Sulfuric acid (for pH adjustment) Certified ACS Plus (Fischer Scientific catalog #A300-212)

- Lab Grade Water (LGW)

<u>Procedure</u>

**Internal Standard – 1,2 dibromopropane**

Stock solution of IS at ~2000 μg/mL in MtBE – prepared by injecting 10 μL of neat standard into a 5 mL volumetric flask containing 5 mL MtBE.

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

Extracting Solution at 100 μg/L in MtBE. Volume depends on number of samples (4 mL x # samples, be sure to make extra, as some will be used to clear bubbles in dispensing line)

**Sample collection**

Collect samples in 60-mL glass vials with PTFE-lined septa and screw caps, containing ascorbic acid for quenching. The amount of ascorbic acid will depend on expected chlorine residual – the stoichiometric ratio is 2.48 mg ascorbic acid/mg $Cl_2$, and a safety factor of 2 is typically used. Use 1M sulfuric acid to adjust the sample pH to 2-3 to stabilize cyanogen chloride. Fill vials headspace-free and store at 4$^o$C until analysis.

**Working Solutions**

Prepare one or two intermediate concentrations in high-purity methanol from the 2000 μg/mL stock solutions in order to make calibrations.

**Calibration Standards**

Prepare a range of calibrations based on levels expected in samples. Working solutions and calibration standards are kept on ice at all times when not being used.

**Extraction procedure**

**\*Samples and Calibrations must be kept on ice during extraction procedure. CNCl and CNBr have very low boiling points and can volatilize easily. \***

1. Measure out 30 mL of each calibration and sample using a graduated cylinder, tilting the cylinder to prevent volatilization.

2. Adjust the pH of all standards to pH 2-2.5 pH using 2 drops of 1M sulfuric acid (amount may vary for different types of samples, so test with pH strips)

3. Add 4 mL of MtBE with IS extracting solution via pump dispenser to each vial.

4. Add ~10 g of baked sodium sulfate, cap, and vortex for one minute.

5. Transfer the organic layer via disposable Pasteur pipette to 2 mL amber autosampler vials.  Use two autosampler vials per sample for backup.  Store in freezer until ready for analysis.

*GC-ECD analysis on Hewlett-Packard GC6890:*

Injector:

Syringe size = 10μL; Injection volume = 1μL

Wash solvent = MtBE; Pre-injection washes = 3; Post-injection washes = 3; Pumps = 3

Injector Temperature = 120°C; Splitless injection

Oven/Column:

Oven equilibration time = 3 min; Oven max °C = 300°C

Gas = He; Flow column = 1mL/min; Column Pressure= 11.3 psi

Column type = 1701 (Zebron), 30.0m length, 0.25mm diameter, 1.0 μm film thickness

**CNX Oven Temperature program (Total time = 38.5 min)**

|         | Velocity (°C/min) | Temp. (°C) | Time (min) |
|---------|-------------------|------------|------------|
| **Initial** | -             | 35         | 9          |
| **Level 1** | 10            | 200        | 10         |

Electron Capture Detector (ECD), Detector temperature = 300°C

BL – updated 10/27/11

*(Semi-)Quantification of CNCl*

The current stock standards available in our lab have passed their expiration date and

CNCl standards are not readily commercially available.  SPEX Certiprep sent us all of

their remaining CNCl standards because they no longer make or sell this compound.  As

a result, we have CNCl stocks in different solvents at a range of concentrations, but many

are degraded. We are currently quantifying CNCl in samples by comparing to the

response of a fresh standard which was run on the 6890 GC-ECD on 04/14/10.  When

this standard was opened, it was measured on the total organic halogen (TOX) analyzer

and total nitrogen (TN) analyzer.  These analyses estimated that the stock concentration

was 1488 µg/mL, compared to the 2000 µg/mL advertised concentration. The calibration

response of this standard in LGW is used to monitor the concentration of a CNCl

standard when it is first opened and before each extraction.  Below is a table and resulting

plot of the calibration curve obtained using the reference CNCl standard.  If the detector

or column in the 6890 GC-ECD is changed, this comparison will no longer be valid.

| Conc. spiked into LGW (µg/L) | CNCl peak area |
|---|---|
| 0.4 | 8.6 |
| 0.4 | 8.3 |
| 0.7 | 19.8 |
| 0.7 | 20.0 |
| 3.7 | 99.1 |
| 3.7 | 96.7 |
| 7.4 | 255.0 |
| 7.4 | 253.5 |
| 18.6 | 598.1 |
| 18.6 | 596.7 |
| 37.2 | 1087.8 |
| 37.2 | 1041.6 |

$y = 29.013x + 11.108$

$R^2 = 0.99436$
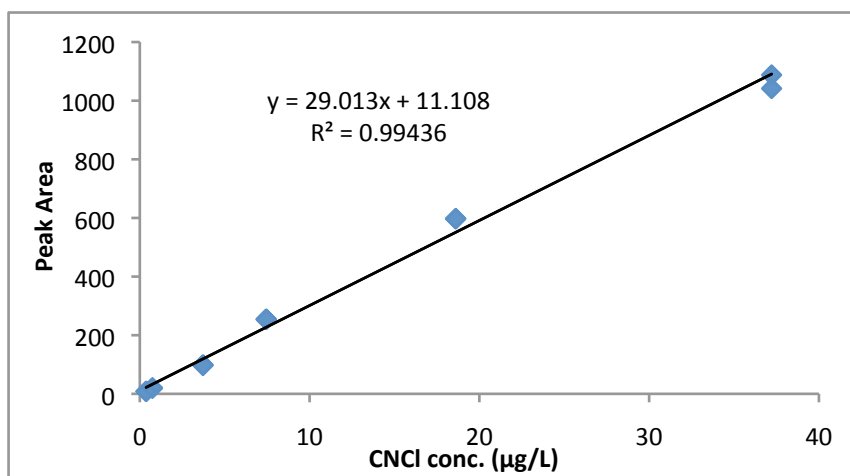
Prepared by: Jennifer Chu, January 2008

Adapted from EPA Method 552.2

**Analysis of Haloacetic Acids (HAAs)**

This entire procedure should also be performed under a well-ventilated hood. Hazardous

reagents will again be used during HAA extraction.

Materials

1) Several clear 40 mL glass vials with open-top screw caps and Teflon-lined septa

2) 10-50 μL micropipetter with clean glass capillary tips

3) 50-250 μL micropipetter with clean glass capillary tips

4) Several 23 cm disposable glass Pasteur pipettes

5) Rubber Pasteur pipette bulb

6) Volumetric flasks & glass toppers: three 25 mL, eight 100 mL, several 2 mL (number

depends on number of samples and calibration standards)

7) Two 25 mL glass graduated cylinder

8) 10mL glass beaker

9) 1 L glass bottle with 10mL pump pipetting dispenser containing PTFE transfer line

10) 500 mL amber bottle mounted with 5 mL pump pipetting dispenser containing PTFE

transfer line

11) 5 mL amber glass standard storage vials with open top screw caps and PTFE-lined

septa

12) Several 12x32 1.8 mL 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 ($SiO_2 \times nH_2O$): JT Baker

7) Methanol for rinsing glassware: (HPLC grade or equivalent)

8) Sodium Azide ($NaN_3$): Aldrich, 99.99+%. Prepared as preservation agent at 80mg/L by adding 400mg solid $NaN_3$ to a 5mL volumetric flask containing just under 5mL of LGW. Fill flask to 5mL mark, invert 3 times. Transfer solution to 40mL amber vial, capped with an open top screw cap and PTFE-lined septa. Seal cap with Teflon tape and store in lab refrigerator. This solution should be prepared every 3 months.

9) Ammonium Sulfate (($NH_4)_2SO_4$): Mallinckrodt granular, AR grade

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 laboratory standards freezer at -15°C. Standards should be monitored frequently for degradation by comparing standard area values to the initial calibration of the standard. Fresh standards should be prepared if this check exceeds a 20% drift. Stock standards should not be used more than 6 months after opening of sealed ampule.

1) Supelco EPA 552.2 Acids Calibration Mix (BrAA, BrClAA, BrCl$_2$AA, ClAA, Br$_2$ClAA, Br$_2$AA, Cl$_2$AA, Br$_3$AA, Cl$_3$AA)

Stock standards should be stored in 5mL amber vials fitted with screw cap and PFTE-lined silicone septa in freezer with screw caps sealed with Teflon tape for a maximum of 3 months or until significant degradation or contamination occurs.

1) Internal Standard Stock Solution: Aldrich; 1,2-dibromopropane neat standard, 99+%

2) Haloester Standard Stock Solution: Supelco; EPA 552.2 Esters Calibration Mix at 200-2000 µg/mL in MtBE

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

Daily Working Standards: Primary Calibration Standards

1) HAA Standard Primary Dilution prepare at 20 mg/L (or 20 µg/mL).

a. 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 600 µg/mL.

b. Fill a 2 mL volumetric flask to just below the 2 mL mark with MtBE.

c. With a micropipetter, inject 67 µL of the EPA 552.2 Acids Calibration Mix into the volumetric flask. Make sure that it is injected below the MtBE surface.

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

e. Transfer this standard to a 5 mL 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 area values to the initial calibration of the standard. Fresh standards should be prepared if this check exceeds a 20% drift.

2) Internal Standard Primary Dilution prepare at 2000 µg/mL

a. Weigh out 10 mg of Aldrich 1,2-dibromopropane neat standard 99+%.

b. Inject with micropipetter into a 5mL volumetric flask containing 5 mL of Aldrich 99+% MtBE. Fill to the line with MtBE, cap, and invert 3 times.

c. Transfer immediately to 5 mL 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.

3) MtBE and Internal Standard Extraction Solution prepare at approximately 50 µg/L

a. Using a micropipetter, directly inject 250 µL of internal standard secondary dilution standard (100 µg/mL) into a 500 mL volumetric flask containing just under 500 mL of Aldrich 99+% MtBE.

b. Fill volumetric flask to the 500 mL mark with MtBE. Cap and invert 3 times.

c. Transfer this standard solution and store it in 1L amber bottle with PTFE pipetting

dispenser screw top assembly.

d. Store in lab refrigerator at 4-5°C.

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) * 4 mL + 100 mL MtBE

4) Multicomponent haloester reference standard prepare at 50 µg/L – 500 µg/L

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

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

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

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

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

5) Acid Surrogate Additive Standard prepare at 20 µg/mL

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

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

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

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

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

a. 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 600 µg/mL. The final concentration of this dilution is 6 µg/mL of ClAA.*

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

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

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

e. 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, but 6µg/mL was the concentration that at which we prepared this solution for HAA analysis on 11/3/07.

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) 30 m length x 0.30 mm inner diameter, 1.0 µm 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 11 mm diameter Thermolite Septa

b. Injector Liner Sleeves Supelco Split/Splitless Injector Sleeve with deactivated
glass wool, 4 mm inner diameter

c. Column Ferrules J&W graphite/vespel 0.5 mm ferrules

d. Autosampler Syringes 10 µL Agilent tapered needle syringe

Samples

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

Test Mixes: Preparation & Procedure

1) Prepare a dilution of MtBE + IS stock solution in a 25 mL volumetric flask.

2) Add a dilution of MtBE stock solution to a small GC vial. Cap and label the vial.

3) Add the MtBE + IS from step 1 to a small GC vial. Cap and label the vial.

4) Add a dilution of HAA9 ester mix to a third GC vial. Cap and label the vial.

5) Run all three vials on a GC prior to extracting samples to ensure the purity and
cleanliness of these reagents.

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

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

Calibration Standards

1) Prepare all standards in 100 mL of LGW.

2) Label 6 separate 100 mL volumetric flasks with the concentrations to be prepared. The lowest concentration should not be below 0.1 μg/L.

3) Fill each 100 mL 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 40 mL clear glass vials with 20 mL LGW.

a. Measure 20 mL LGW with a clean glass 25 mL graduated cylinder.

b. Label, then cap vials using open top screw caps with PTFE-lined septa.

7) Rinse the 25 mL graduated cylinder three times with LGW.

8) Using the cleaned 25 mL graduated cylinder, transfer 20 mL of the secondary calibration standards in the 100 mL 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 20 mL 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 25 µL of the HAA9 Matrix Spike Standard to a 25 mL of the matrix spike sample.

3) Make a duplicate 25 mL aliquot of sample.

4) Measure 20 mL 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 40 mL glass vials) from refrigerator and let them warm to

room temperature while preparing calibration standards.

2) Use a clean, glass 25 mL graduated cylinder to measure out 20 mL of each sample.

3) Dispose remaining amount of sample into a waste beaker.

4) Pour the measured 20 mL sample back into its 40 mL 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 20 µL of the acid surrogate additive standard at 20 µL/mL to all 20 mL

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 1.5 mL of concentrated sulfuric acid ($H_2SO_4$) to all 20 mL of calibration standards, samples, and matrix spike samples.

2) Let vials cool in an ice bath for 20-30 min.

3) Swirl these vials gently to mix water and acid.

Internal Standard Addition

1) Using a pump pipette dispenser, add 4 mL of MtBE + IS to each 20 mL 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 10 g 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 2 mL Volumetric Flasks

1) For each sample and calibration standard: With a clean, glass, 23 cm Pasteur pipette, transfer 2 mL of the top layer (MtBE + IS layer) to a clear, glass 2 mL 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.

Derivitization

To all MtBE extracts in 2 mL 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 **225 µL** 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 2 mL 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.

*GC-ECD analysis on Hewlett-Packard GC-ECD 5890:*

Injector:

Syringe size = 10 µL; Injection volume = 1 µL

Wash solvent = MtBE; Pre-injection washes = 3; Post-injection washes = 3; Pumps = 3

Injector Temperature = 180°C; Splitless injection

Oven/Column:

Oven equilibration time = 3 min; Oven max °C = 300°C

Gas = He; Flow column = 1 mL/min; Column Pressure= 11.3 psi

Column type = ZB-1 (Zebron), 30.0 m length, 0.25 mm diameter, 1.0 µm film thickness

**Oven Temperature program (total time = 52.5 min)**

|          | **Velocity (°C/min)** | **Temperature (°C)** | **Time (min)** |
|----------|-----------------------|----------------------|----------------|
| **Initial** | -                   | 37                   | 21             |
| **Level 1** | 5                   | 136                  | 3              |
| **Level 2** | 20                  | 250                  | 3              |

Electron Capture Detector (ECD), Detector temperature = 300°C

**Updated 10/17/11 by Bonnie Lyon**

**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 no: 890-161

   Serial no: 99292009

   For 100 mL 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 no: 890-162

   Serial no: 99292009

   For 100 mL sample size: Range: 4-1000 μg TOX/L; Precision: ±2 μg/L or ±2%

   For 10 mL 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)

- Silver acetate (≥99% purity, Sigma-Aldrich)

- $Na_2SO_3$ (anhydrous, ACS Grade, Fisher Scientific)

- 1.13 g/L $KNO_3$/L (as N) (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)

- Glass-packed Carbon Columns 2mm ID (CPI International)

- UHP Helium, 220ft$^3$

- Oxygen, 99% purity, 220ft$^3$

**Sample Collection & Dechlorination**

Samples for TOX analysis should be collected in amber vials/bottles with open-top PTFE-lined septa (bottle/vial size will depend on sample adsorption volume used and whether you are running duplicates, triplicates, etc.). To quench about 3 mg/L of free

chlorine, 40 μL of a 40 mg/mL solution of sodium sulfite ($Na_2SO_3$) should be added to the 125 mL sample bottles prior to collecting the samples, for example. Adjust this amount based on the collection volume and expected chlorine residual. The stoichiometric quenching amount is 1.8 mg $Na_2SO_3$/1 mg $Cl_2$ (we typically use a safety factor of 2 (i.e. Multiply stoichiometric amount by 2)).

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$^o$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. Concentrated sulfuric acid (A.C.S. Plus) should be used to adjust the pH of the sample to approximately pH 2 with a glass Pasteur pipette. Use pH paper to verify the adjusted pH, or if you have extra sample that will not be analyzed, you can test the pH adjustment and measure with the pH meter.

**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):

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Sample volume: 100 mL (can also use 50, 25 or 10-mL, depending on range of TOX expected)

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, rinse each sample channel with LGW. 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, carefully pierce the endcaps of two glass carbon columns (if holes are too big or you go through the yellowish filling hold the carbon in, carbon will come out during adsorption), 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.

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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 (1.13 g $KNO_3$/L (as N)) at a rate of 1 mL/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 above 500 psi. Change gas tanks when pressure of the gas tanks reaches 500 psi.
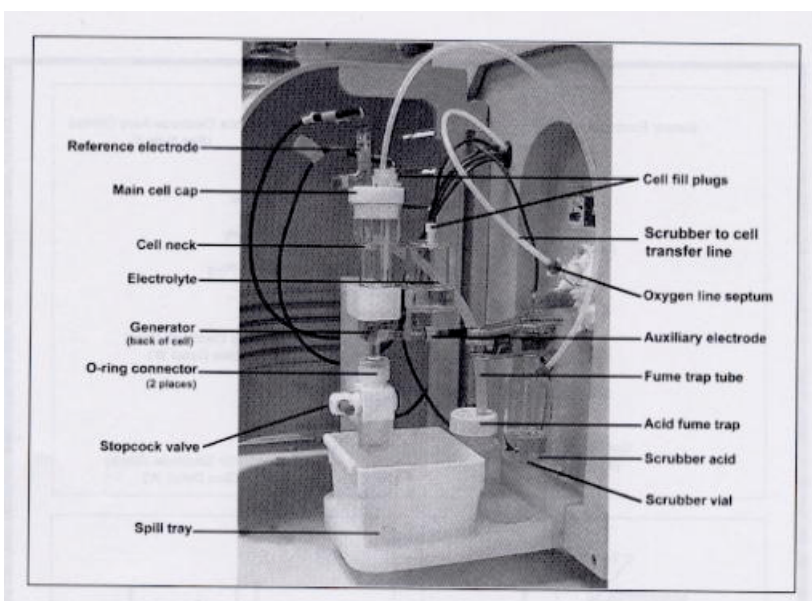


Figure 2: The titration cell parts.

**Scrubber vial**

When the instrument is not in use, 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 every day of TOX use and may need to be changed again during the day if the TOX is used for more than 6-7 hours at a time.

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**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 add sodium bicarbonate to about 1/4 full and LGW to about 1/2 full as shown in Figure 3.
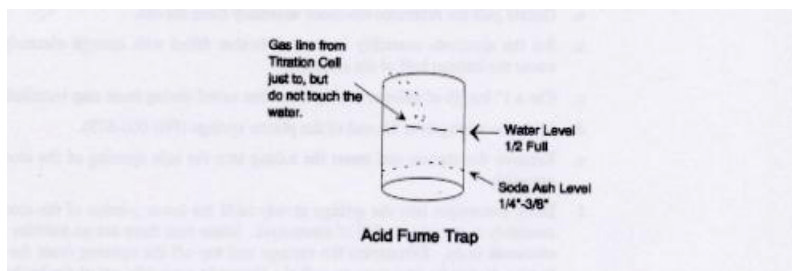


Figure 3: Acid fume trap.

**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.

Make sure that the reference electrode (Figure 4) has no bubbles in it.
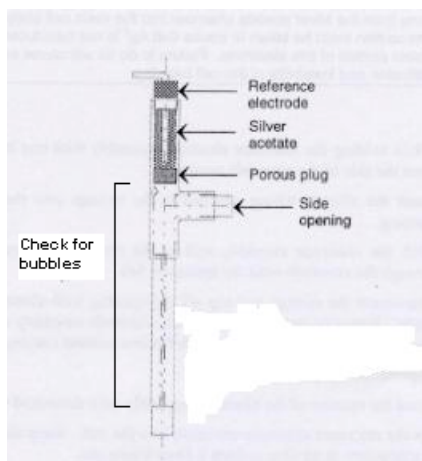
Figure 4: Reference electrode.

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 bring to the hood (fill clean beaker with 70% acetic acid and place reference electrode in beaker so no bubbles are introduced into reference electrode). Remove the side-opening plug and insert a syringe filled with 70% acetic acid in the side opening. Repeat this procedure three times making sure that all the bubbles are removed by moving the electrode.

To replace silver acetate solution in the top of the reference electrode, first prepare a slurry of solid silver acetate in 70% acetic acid. Use glass stir rod to stir slurry. Empty out old silver acetate/acetic acid mixture from reference electrode using a Pasteur pipet (can use a kimwipe to break off tip of pipet for easier use) and rinse bottle of acetic acid. **Be very careful not to get any silver acetate into bottom compartment of reference electrode.** This will require rinsing and refilling the entire electrode. Hold a kimwipe around top of electrode so nothing drips down. Use another broken-tip Pasteur

pipet to add slurry to now-empty top compartment of reference electrode. Allow to settle

for a few minutes and fill to top with 70% acetic acid, making sure there are no bubbles.

The silver acetate should be about 2/3 of the top compartment, with 70% acetic acid

filling the rest. Cover with aluminum foil while slurry is settling. Reconnect reference

electrode to cell.

**DX-2000 Organic Halide Analyzer Settings**

To begin sample analysis in the computer program, choose the icon "AOX/TOX

by Column" and click "OK."

- Gas & Temperature Settings

    In the "System Setup" menu bar click "Open" and turn the system to

"Standby." Slowly increase temperature in $50^{\mathrm{o}}$C increments to $550^{\mathrm{o}}$C. Make sure

to press tab or click in another box each time you increase the temperature. Once

system reaches $550^{\mathrm{o}}$C, make sure temperature in "Ready" box is set at $550^{\mathrm{o}}$C.

Open both gas tank main valves (keep fly valves shut for now). Turn system to

"Ready." Slowly open the fly valves of both gas tanks. You should see vigorous

bubbling in the scrubber vial and cell. Make sure the injection port hatch door is

completely closed. On the front panel of the TOX, check that the gas gauge for

oxygen is around 50 and helium is around 25. Adjust the flow up and down by

turning the knobs on gauges, if necessary.

    Increase the furnace temperature to $850^{\mathrm{o}}$C from its standby temperature of

$550^{\mathrm{o}}$C in increments of $50^{\mathrm{o}}$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.

- 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 around 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 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

    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." Inject 5 µL of a 200ng Cl/µL NaCl solution and check the recovery obtained. If the resulting percentage recovery is between 90-110%, then the cell is working properly. Perform this check three times for consistent results. **Clean this syringe immediately with LGW and then Methanol so that salt does not clog up syringe.**

- Clean Boat

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

- Combustion Check

    First, run two blank carbons to obtain an average background level that you will subtract from the TCP spike value. Then, to verify the furnace performance, inject 5 µL of a 500 ng/µL solution of 2,4,6-trichlorophenol (TCP) into the boat on top of a dry carbon column) and check the recovery obtained.

    Carefully the dry carbon 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 pyrolyzed carbon is a light orange color. If the carbon is not completely pyrolyzing, 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 pyrolyzed 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 μg Cl/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.

**Shutdown**

First check that there are no runs in progress. Next, clean the boat as necessary. Save your results and convert to spreadsheet so you can look at the data on another computer. Turn off the cell. Set the system into Standby mode and set temperature to $35^{o}C$. Close the fly valves and main valves of the gas tanks.

**Power Off**

Let system reach $35^{o}C$ – this will take several hours. Shut down software and turn off TOX power.

**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**

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] (µg/L) | [std] (µg as Cl/L) | Final result (µg 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 mg/L to mg 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.

Updated from 10/23/08 document by Bonnie Lyon 10/17/11

**Total Organic Chloride (TOCl), Total Organic Bromide (TOBr), and Total Organic**

**Iodide (TOI) Detection on the Dionex Ion Chromatograph**

**Instrumentation**

1)  Dionex Ion Chromatograph

    Conductivity Detector, Serial #911003E930302 XTC: uses a Dionex AS22

    analytical column, a Dionex AG22 guard column, 250 μL sample loop, Dionex

    AMMS III-4mm ion suppressor

    Gradient Pump: set to use 100% of eluent at 1.0 mL/minute

    Eluent Gas Module: uses a mobile phase (eluent) at 5 mM sodium carbonate

    ($Na_2CO_3$) & 1.4 mM sodium bicarbonate ($NaHCO_3$)

    Column Storage Solution: 100 mM $NaHCO_3$

    Regenerant Pump: uses 25 mN $H_2SO_4$ at ~3-5mL/minute

2)  Adsorption Module (Tekmar-Dohrmann)

    Model: AD-2000 Adsorption Module

    Model n$^o$: 890-161

    Serial n$^o$: 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$^o$: 890-162

    Serial n$^o$: 99292009

    For 100mL sample size: Range: 4-1000μg TOX/L; Precision: ±2μg/L or ±2%

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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)

- $Na_2CO_3$ (anhydrous, granular, ACS Grade, Mallinckrodt)

- $NaHCO_3$ (ACS Grade, Mallinckrodt)

- Concentrated $H_2SO_4$ (ACS Plus Grade, Fisher Scientific)

- NaCl (ACS Grade, Fisher Scientific)

- NaBr (ACS Grade, Fisher Scientific)

- KI (ACS Grade, Fisher Scientific)

- Methanol (halogen free, highest purity, LCMS Grade, Fisher Scientific)

- Helium, 99.9+% purity, $220ft^3$

- 47 mm 0.45 µm 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)

- 40 mg/mL $Na_2SO_3$ (anhydrous, ACS Grade, Fisher Scientific) in LGW

- 1.13 g $KNO_3$/L (as N) (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)

- Pentachloroacetone (85% PW, Aldrich)

- (±)1,2-dibromopropane (97%, Aldrich)

- Methanol (halogen free, highest purity, LCMS Grade, Fisher Scientific)

- Glass-packed Carbon Columns 2 mm ID (CPI International)

- Helium, 99.9+% purity, 220 ft$^3$

- Oxygen, 99% purity, 220 ft$^3$

**Wear gloves, goggles, close-toed shoes and a lab coat for all laboratory work.**

**Sample Collection & Dechlorination**

Samples for TOX analysis should be collected in amber vials/bottles with open-top PTFE-lined septa (bottle/vial size will depend on sample adsorption volume used and whether you are running duplicates, triplicates, etc.). To quench about 3 mg/L of free chlorine, 40 μL of a 40 mg/mL solution of sodium sulfite ($Na_2SO_3$) should be added to a 125 mL sample bottles prior to collecting the samples, for example. Adjust this amount based on the collection volume and expected chlorine residual. The stoichiometric quenching amount is 1.8 mg $Na_2SO_3$/mg $Cl_2$ (we typically use a safety factor of 2 (i.e. multiply stoichiometric amount by 2)).

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 septa 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 $4^{o}C$ until analysis, which should take place within 14 days of collection.

**Sample Pre-Treatment**

Before the analysis, allow sample bottles to achieve room temperature. Concentrated sulfuric acid (A.C.S. Plus) should be used to adjust the pH of the sample to approximately pH 2 with a glass Pasteur pipette. Use pH paper to verify the adjusted pH, or if you have extra sample that won't be analyzed, you can test the pH adjustment and measure with the pH meter.

**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: 50 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. Drain the old nitrate wash solution into a waste container. Fill the nitrate channel with fresh nitrate wash solution (1.13 g $KNO_3$/L (as N)). 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 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/TOI sample collection. Also prepare three LGW/nitrate blanks (pass same volume of LGW you are using for samples through one carbon column, and use nitrate wash). You will use the average of this value to subtract from the sample and correct for any background TOCl/TOBr/TOI.

*Instrument Preparation of Total Organic Halides (TOX) Analyzer*

Before using the DX-2000 Organic Halide Analyzer module for sample analysis, make sure that the helium and oxygen are above 500 psi. Do not use the instrument if the pressure of either gas is at or below 500 psi. Replace gas tanks if necessary.

Set the coulometric cell setup to the side. Even though the cell is not needed for detection in this method, check for bubbles in the reference electrode and make sure that the level of acetic acid in the cell covers the reference cell. If not, refill with 70% acetic acid. Check if there are any bubbles in the electrode. If bubbles are present, follow directions in TOX SOP to get rid of them to protect the electrode.

Fill a clean, dry scrubber vial (not the same one used for the 80% $H_2SO_4$) with ~10 mL of LGW. Connect this to the end of pyrolysis tube where the acid scrubber vial is usually connected during TOX analysis. **Be very careful when connecting and disconnecting this vial, do not put too much pressure on the pyrolysis tube or it will break.**

In the "System Setup" menu bar click "Open" and set system to "Standby." Slowly increase temperature in 50$^o$C increments to 550$^o$C. Make sure to press tab or click in another box each time you increase the temperature. Once system reaches 550$^o$C, make sure temperature in "Ready" box is set at 550$^o$C. Open both gas tank main valves (keep fly valves shut for now). Turn system to "Ready." Slowly open the fly valves of both gas

tanks. You should see vigorous bubbling in the scrubber vial. Make sure the injection port hatch door is completely closed. On the front panel of the TOX, check that the gas gauge for oxygen is around 50 and helium is around 25. Adjust the flow up and down by turning the knobs on gauges, if necessary.

Go to "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 preparing samples. Fill the scrubber vial with 10 mL of LGW (measure this out volumetrically with a graduated cylinder). Connect vial to furnace tube and begin timer (6 minutes). 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" and "Yes" when it asks if you want to run with the cell turned off. The boat will start to move into the furnace and sample collection will begin. After 6 minutes, remove the sample scrubber vial and bring to the hood. Pour the collected sample into a 25 mL glass vial, labeled appropriately, with an open-top cap and PTFE-lined septa. Store this sample at 4°C. Rinse the sample vial and removable glass insert 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 25 mL 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 and set the temperature to 35°C. Close the gas valves and clean up the space around the instrument.

**Matrix & Scrubber Spikes**

Matrix spike samples are aqueous samples into which a known amount of organic halogen has been added into the matrix or the actual water sample. Pentachloroacetone and 1,2-dibromopropane can be used for this check.

Scrubber spikes are samples into which a known amount of inorganic halogen ($Cl^-$, $Br^-$, $I^-$) has been spiked into the collected 10 mL sample in LGW. Use the same calibration stock solutions that are analyzed on the IC for this check.

*Calibration Standards*

Separate stock solutions of inorganic chloride, bromide and iodide are prepared at 1000 mg/L for each ion species. These solutions are stored in amber glass bottles with open-top caps and PTFE-lined septa at 4°C and are good for about six months. Using these stock solutions, working solutions are prepared, from which the calibration points are made. Prepare calibrations in the range that you expect your samples to fall. For example, prepare working solutions of 100 mg/L and 10 mg/L as a mixture of chloride, bromide, and iodide.  An example of a calibration set is 20, 40, 80, 200, 400, 1000, 2000 and 4000 µg/L.

All of the calibration points should be made in 50 mL volumetric flasks. They should be filled to three-fourths of the way to the line with LGW, the appropriate amount of working solution added, LGW added to the fill line, and inverted three times. These

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calibration solutions should be placed in appropriately labeled 40 mL glass vials at 4°C until IC analysis. Calibration points should be run for every new set of samples.  If sample analysis takes place over several days, inject one or two calibration check points (for example 100 and 400 µg/L) and be sure they have similar responses for each ion to the calibration standard injected the previous day before proceeding with samples. After every 10 samples, a calibration check should be injected to as a performance check. Calibration solutions should be made fresh weekly.

*Dionex Ion Chromatograph Preparation*

The mobile phase for this procedure is 5 mM $Na_2CO_3$/1.4 mM $NaHCO_3$ and should be filtered using a 47mm, 0.45µm hydrophilic polyether sulfone filter. The column storage solution is 100 mM $NaHCO_3$. The regenerant for this procedure is 25 mN $H_2SO_4$.

**IC operating procedure** (from document Prepared July 2009 by Ryan Kingsbury)

**Initial Startup:**

1. Fill all eluent bottles and the regenerant bottle with the appropriate solutions.

2. Turn on pressure at the Helium tank

3. Turn the eluent degas module ON.  Set all bottles to SPARGE and turn each individual bottle to ON.  Loosen the cap on each bottle.

4. Verify that gas is flowing out of each sparge line that is turned on. Connect the sparge lines to the bottles. Allow the eluents to sparge for 20 minutes

5. While the eluents are sparging, take out the suppressor and remove the  caps on all four ports

6. Hydrate the suppressor membranes by using a syringe and the luer-lok adapter to push ~5 mL LGW through the REGEN IN port and ~3 mL through the ELUENT OUT port.  Be careful not to push through the ELUENT OUT port too fast or you may damage the membrane

7. Uncap the ends of the regenerant and eluent lines in the sink

8. Install the suppressor in the cabinet and connect the REGEN OUT port of the suppressor to the appropriate line

9. Connect the column and guard column to the injection assembly

10. When sparging is complete, remove the sparge lines.  Tighten the caps on the bottles and switch them to PRESSURIZE.  Adjust the regulator on the degas module to 7 +/- 2 psi. Re-cap the sparge lines.

11. Turn the pump ON

12. Prime the pump.  For each eluent bottle, set the flow to 100% and 1.0 mL/min. Turn the silver bar on the pump perpendicular to the pump face and attach a 3 mL syringe.  Press START and draw about 3 mL from the port into the syringe. Discard.  Repeat two more times or until no air bubbles are seen.  On the third time, loosen the black knob and push the syringe contents back into the pump while tapping on the clear tube to remove any air bubbles.  Re-tighten the knob.

13. Begin pumping eluent through the system at 1.0 mL/min.  As soon as you see eluent dripping out of the column line, connect it to the ELUENT IN port of the suppressor.

14. When you see eluent emerge from the ELUENT OUT port of the suppressor, connect it to the detector.

15. Tighten the cap and the gas line connection on the regenerant bottle. Carefully turn on pressure to the regenerant bottle at the regulator, watching to see when regenerant begins to flow in the line. When regenerant begins to flow, connect the line to the REGEN IN port of the suppressor.

16. Adjust the pressure until the desired regenerant flow rate is achieved (measure flow out of the REGEN OUT line in the sink with a graduated cylinder and a watch). Consult the suppressor manual for optimal regenerant flow rates for each eluent strength.

17. Turn the ACI and Detector ON. Turn the cell OFF.

18. Allow the system to equilibrate for 30 minutes

19. Turn the cell ON.

20. Switch the detector and pump to "Remote" and open the Run ACI on the PeakNet main menu. Record the baseline conductivity and the pump back-pressure in the log book.

21. Run samples: Close the "IC 2" window. Click LOAD at the top of the screen and select "Method." Select the appropriate method and click "OK."

22. Inject about 1 mL of your sample with a syringe and then on the computer, click "Run," "Start," enter the sample name, make sure it is saving to the correct directory, and then click "Start Run."

**Short Term (Daily operation) Shutdown:**

1. Flush the system with LGW at 1.0 mL/min for 10 minutes.

2. Turn the cell OFF. Turn the detector OFF.

3. STOP and turn off the pump

4. Turn off pressure to the regenerant bottle at the regulator.  Loosen the regenerant bottle cap to relieve the pressure.  Re-tighten the cap.

5. Cap the ends of the eluent and regenerant lines in the sink to keep them from drying out.

6. Leave the Eluent Degas Module ON with pressure to the eluent and LGW bottles.

**Short Term (Daily operation) Startup:**

1. Uncap the ends of the regenerant and eluent lines in the sink

2. Turn on pressure to the regenerant bottle at the regulator.  Adjust until the desired regenerant flow rate is achieved (measure flow with a graduated cylinder in the sink)

3. Turn the pump ON

4. Prime the pump.  For each eluent bottle, set the flow to 100% and 1.0 mL/min.  Turn the silver bar on the pump perpendicular to the pump face and attach a 3 mL syringe.  Press START and draw about 3 mL from the port into the syringe.  Discard.  Repeat two more times or until no air bubbles are seen.  On the third time, loosen the black knob and push the syringe contents back into the pump while tapping on the clear tube to remove any air bubbles.  Re-tighten the knob.

5. Begin pumping eluent through the system at 1.0 mL/min

6. Turn the ACI and Detector ON.  Turn the cell OFF.

7. Allow the system to equilibrate for 30 minutes

8. Turn the cell ON.  Record the baseline conductivity and the pump back-pressure in the log book.

9. Run samples.

**Long Term (> 1 week) Shutdown:**

1. Flush the system with LGW at 1.0 mL/min for 10 minutes

2. Turn the cell OFF.  Turn the detector OFF.

3. STOP the pump.

4. Turn off pressure to the regenerant bottle at the regulator.  Loosen the regenerant bottle cap to relieve the pressure.

5. Remove the suppressor.  Cap both ends of the regenerant out line.  Cap the ELUENT IN and ELUENT OUT ports with the original plugs.

6. Using a disposable syringe and the luer-lok adapter in the drawer, push 5-6 mL of LGW through the REGEN IN port on the suppressor.

7. Cap the REGEN IN and REGEN OUT ports with the original plugs.

8. Connect the column directly to the detector.  Flush the system with operating eluent for 10 minutes.

9. Remove the column and guard column.  Cap the ends with the original caps and place in their respective boxes.  Be careful not to tap, drop, or otherwise shock the columns as this will disturb the packing.

10. Connect the detector directly to the injection assembly.  Flush the system with LGW at 9.9 mL/min for 10 minutes.

11. STOP and turn OFF the pump.

12. On the eluent degas module, switch all bottles to SPARGE.  Loosen the caps to relieve the pressure.  Switch the entire module OFF.  Switch each bottle OFF.  Switch all bottles to PRESSURIZE.  Turn off the gas supply.

*Data Analysis*

To view the chromatograms, click on "Optimize" in the main menu and then open the desired file. Click on "Manual Integration" to view the peak areas. Check every chromatogram and make sure that the integration is uniform. If not, manually adjust the integrations so that the peaks are integrated at baseline. Save the file after adjustment.

To organize and export your data, click "Batch" in the main menu. Save this batch file. Click "Processing" and choose "Input." Click "Build" to set up your sample list. Go to the correct data folder, highlight your calibrations and samples and press "Add," then "Exit." Select "From Data Files" under the "Process Methods" section. Next, click to the "Output" tab. If you are just outputting a file for excel, you do not need to select any of these output options. You will make your own calibration curves in excel, so you also do not need to select any of the options under "Update Options." Click on the "Export" tab. Click "Browse" and enter a file name for your output file. Select "Ask" where it says "If the file already Exists." Under Report Type, select "Summary." Under Summary options, select "One line Per Injection." Do not check "Include Unknown Peaks." Click "Fields" and select which fields you want to be in your exported file. I typically choose only Sample Name in "Header Fields," and Peak Area, Peak Height and Peak Retention Time under "Data Fields." Click Ok. Make sure file is saved, and press Start.

Prepare calibration curves for chloride, bromide and iodide in Excel from your exported file. Use the calibration curves to determine the chloride, bromide, and iodide concentrations in the samples and subtract the amounts from the average nitrate blank. Back-calculate the actual concentration in the original samples by taking into account the 10 mL collection volume and adsorption volume used.

# APPENDIX B:

## Cytotoxicity Data

2/21/2011

Nordic Lake

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±13 |
| 0.48 | 107±13 |
| 1.2 | 111±14 |
| 12.0 | 107±12 |
| 24.0 | 103±10 |
| 36.0 | 100±10 |
| 48.0 | 103±9 |
| 72.0 | 100±8 |
| 96.0 | 82±10 |

Suwannee River

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±9 |
| 0.48 | 110±11 |
| 1.2 | 112±12 |
| 12.0 | 110±13 |
| 24.0 | 106±12 |
| 36.0 | 104±13 |
| 48.0 | 105±11 |
| 72.0 | 103±11 |
| 96.0 | 91±17 |

8/11/2011

Phosphate Buffer, pH 7.1, in LGW

| Phosphate Buffer (mM) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±8 |
| 0.2 | 102±10 |
| 0.5 | 104±13 |
| 5 | 110±10 |
| 10 | 102±13 |
| 15 | 94±13 |
| 20 | 86±13 |
| 30 | 75±15 |
| 40 | 72±14 |

9/1/2011

Nordic Lake+Chlorine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±13 |
| 0.46 | 100±14 |
| 1.15 | 94±14 |
| 11.5 | 92±11 |
| 23.0 | 83±10 |
| 34.5 | 78±9 |
| 46.0 | 67±9 |
| 69.0 | 47±6 |
| 92.0 | 30±7 |

Suwannee River+Chlorine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±22 |
| 0.46 | 110±12 |
| 1.15 | 113±15 |
| 11.5 | 101±17 |
| 23.0 | 101±18 |
| 34.5 | 110±18 |
| 46.0 | 105±15 |
| 69.0 | 91±13 |
| 92.0 | 69±17 |

9/15/2011

Nordic Lake+Chloramine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±11 |
| 0.45 | 106±13 |
| 1.12 | 106±10 |
| 11.2 | 106±10 |
| 22.5 | 109±13 |
| 33.7 | 111±11 |
| 44.9 | 101±12 |
| 67.4 | 96±13 |
| 89.9 | 86±10 |

Suwannee River+Chloramine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±13 |
| 0.45 | 99±7 |
| 1.11 | 102±10 |
| 11.1 | 95±10 |
| 22.3 | 103±11 |
| 33.4 | 108±10 |
| 44.6 | 122±22 |
| 66.8 | 133±18 |
| 89.1 | 134±24 |

9/23/2011

Nordic Lake+Nitrate

| Nitrate (mg/L as N) | Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|---|
| 0 | 0.0 | 100±15 |
| 2 | 0.4 | 106±6 |
| 5 | 1.1 | 105±11 |
| 50 | 11.2 | 102±11 |
| 100 | 22.4 | 101±13 |
| 149 | 33.6 | 87±12 |
| 199 | 44.8 | 73±7 |
| 299 | 67.2 | 55±9 |
| 398 | 89.6 | 39±6 |

Nordic Lake+Iodide

| Iodide (mg/L as N) | Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|---|
| 0.0 | 0.0 | 100±18 |
| 0.02 | 0.4 | 111±13 |
| 0.05 | 1.1 | 109±11 |
| 0.50 | 11.1 | 111±16 |
| 1.0 | 22.3 | 113±14 |
| 1.5 | 33.4 | 112±21 |
| 2.0 | 44.6 | 116±15 |
| 3.0 | 66.9 | 105±15 |
| 4.0 | 89.2 | 85±12 |

10/6/2011

Nordic Lake+Nitrate+Chlorine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±10 |
| 0.45 | 95±9 |
| 1.12 | 93±11 |
| 11.2 | 84±10 |
| 22.4 | 85±9 |
| 33.6 | 76±12 |
| 44.8 | 68±10 |
| 67.2 | 55±9 |
| 89.6 | 42±9 |

Nordic Lake+Nitrate+UV+Chlorine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±10 |
| 0.45 | 103±15 |
| 1.12 | 101±13 |
| 11.2 | 92±16 |
| 22.4 | 85±13 |
| 33.6 | 79±17 |
| 44.8 | 67±16 |
| 67.2 | 44±10 |
| 89.6 | 28±9 |

Nordic Lake+UV+Chlorine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±18 |
| 0.45 | 101±12 |
| 1.12 | 96±14 |
| 11.2 | 93±17 |
| 22.4 | 89±16 |
| 33.6 | 82±15 |
| 44.8 | 71±12 |
| 67.2 | 52±10 |
| 89.6 | 38±9 |

10/13/2011

Nordic Lake+Iodide+Chlorine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±12 |
| 0.45 | 105±11 |
| 1.12 | 104±12 |
| 11.2 | 103±13 |
| 22.4 | 97±14 |
| 33.6 | 90±13 |
| 44.8 | 80±13 |
| 67.2 | 60±9 |
| 89.6 | 44±9 |

Nordic Lake+Iodide+Chloramine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±11 |
| 0.44 | 107±15 |
| 1.09 | 103±15 |
| 10.9 | 102±16 |
| 21.8 | 102±14 |
| 32.6 | 92±10 |
| 43.5 | 81±12 |
| 65.3 | 76±11 |
| 87.0 | 68±10 |

Nordic Lake+Iodide+UV+Chloramine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±13 |
| 0.43 | 102±11 |
| 1.08 | 100±10 |
| 10.8 | 104±16 |
| 21.5 | 100±14 |
| 32.3 | 86±16 |
| 43.1 | 68±16 |
| 64.6 | 46±9 |
| 86.1 | 21±5 |

10/20/2011

Chlorine Residual in LGW

| Chlorine Residual (mg/L as $Cl_2$) | Cell Density as Percent Control ($\pm$SD) |
|---|---|
| 0.0 | 100$\pm$7 |
| 0.15 | 101$\pm$9 |
| 0.37 | 100$\pm$10 |
| 3.69 | 109$\pm$12 |
| 7.38 | 118$\pm$11 |
| 11.07 | 121$\pm$11 |
| 14.76 | 119$\pm$10 |
| 22.14 | 118$\pm$10 |
| 29.52 | 102$\pm$12 |

Chloramine Residual in LGW

| Chloramine Residual (mg/L as $Cl_2$) | Cell Density as Percent Control ($\pm$SD) |
|---|---|
| 0.0 | 100$\pm$15 |
| 0.17 | 95$\pm$11 |
| 0.43 | 95$\pm$15 |
| 4.30 | 107$\pm$14 |
| 8.60 | 114$\pm$18 |
| 12.90 | 112$\pm$18 |
| 17.20 | 110$\pm$19 |
| 25.80 | 110$\pm$20 |
| 34.40 | 96$\pm$18 |

Suwannee River+Chloramine (repeat)

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control ($\pm$SD) |
|---|---|
| 0.0 | 100$\pm$9 |
| 0.44 | 100$\pm$9 |
| 1.11 | 102$\pm$9 |
| 11.1 | 106$\pm$10 |
| 22.2 | 110$\pm$8 |
| 33.2 | 122$\pm$9 |
| 44.3 | 124$\pm$10 |
| 66.5 | 127$\pm$9 |
| 88.7 | 125$\pm$18 |

1/16/2012

Nordic Lake+Iodide+UV+Chloramine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±7 |
| 0.43 | 98±15 |
| 1.08 | 95±14 |
| 10.8 | 84±18 |
| 21.6 | 71±18 |
| 32.4 | 65±17 |
| 43.2 | 53±14 |
| 64.8 | 28±9 |
| 86.4 | 10±7 |

Nordic Lake+UV+Chloramine

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±11 |
| 0.43 | 96±12 |
| 1.08 | 93±17 |
| 10.8 | 90±19 |
| 21.5 | 79±21 |
| 32.3 | 81±9 |
| 43.0 | 70±17 |
| 64.5 | 52±9 |
| 86.0 | 35±8 |

2/8/2012

Suwannee River+Chloramine (repeat with newly prepared NOM stock)

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±7 |
| 0.50 | 104±7 |
| 1.25 | 103±9 |
| 12.5 | 104±10 |
| 25.0 | 102±12 |
| 37.6 | 105±9 |
| 50.1 | 108±9 |
| 75.1 | 104±9 |
| 100.1 | 99±8 |

2/20/2012

Nordic Lake+Chloramine, 72h

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±17 |
| 0.41 | 106±14 |
| 1.04 | 96±16 |
| 10.4 | 100±19 |
| 20.7 | 98±17 |
| 31.1 | 95±16 |
| 41.5 | 96±9 |
| 62.2 | 86±13 |
| 82.9 | 82±9 |

Nordic Lake+Chloramine, 96h

| Dissolved Organic Carbon (mg/L as C) | Cell Density as Percent Control (±SD) |
|---|---|
| 0.0 | 100±12 |
| 0.42 | 101±12 |
| 1.04 | 99±12 |
| 10.4 | 92±19 |
| 20.8 | 91±23 |
| 31.2 | 90±18 |
| 41.5 | 92±17 |
| 62.3 | 85±15 |
| 83.1 | 78±14 |

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