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Cytotoxicity and Mutagenicity of Disinfection Byproduct Mixtures Formulated Using Free Chlorine and Monochloramine

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Abstract

Bioassays have been used extensively to assess various toxicity endpoints of drinking water disinfection byproducts (DBPs), but an emphasis on single compounds prevails. In this research, DBP mixtures were assessed using a cytotoxicity test with Chinese Hamster Ovary (CHO) cells and an Ames fluctuation test with *Salmonella typhimurium* TA-98 and TA-100 with and without S9 rat liver homogenate. Seven whole mixture DBP concentrates were formulated using reconstituted natural organic matter (NOM) extracts from the Upper Mississippi River using scaled disinfectant dosing protocols with free chlorine and monochloramine in the presence and absence of added bromide. DBPs were identified by gas chromatography-mass spectrometry (GC-MS) and quantified with an electron capture detector (GC-ECD). Four trihalomethanes, three dihaloacetonitriles, 1,1-dichloro-2-propanone, chloropicrin, nine haloacetic acids, and dalapon were identified and quantified. Exogenous *N*-nitrosodimethylamine (NDMA) was added to two monochloramine-derived concentrates because the NOM did not include precursors for this known human carcinogen that forms in chloraminated waters. Five synthetic DBP analog mixtures were formulated based on the profiles of the whole mixture DBP concentrates. DBP mixture concentrates exerted mild cytotoxicity to CHO cells, approaching or reaching the LD50 in all seven concentrates, a result which could be only partially explained by the identified DBPs. In the Ames tests, no revertant wells were observed with the TA-98 bacterial strain, indicating the DBP-concentrates did not induce frameshift mutations in *his*D3052. However, statistically significant increases in the number of revertant wells were observed in TA-100 in all seven DBP mixture concentrates, indicating base-substitution mutations in *his*G46. Mutagenicity was greater with the DBP mixtures formulated with free chlorine and bromide, suggesting that bromine-substituted DBPs were important contributors. Exogenous NDMA had a synergistic effect in the concentrate formulated with monochloramine and bromide in the presence of S9 only, indicating NDMA was important in mammalian mutagenicity. However, none of the observed

mutagenicity in TA-100 was explained by the nineteen identified DBPs and/or the associated interactions. Future work should focus on high resolution mass spectrometry techniques aimed at identifying additional DBPs in the mixture concentrates and assessing the mutagenicity of these DBPs.

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1 Introduction and Motivation

In drinking water treatment, source water natural organic matter (NOM) reacts with disinfectants such as chlorine and chloramines to form disinfection byproducts (DBPs). Halogenated DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs) form in the greatest abundance on a mass basis in most drinking waters and are regulated by the USEPA in finished drinking waters under the Stage 2 Disinfectant/DBP Rule (USEPA, 2006). Bromide and iodide naturally occur in some source waters and, if present, are oxidized by disinfectants and react with NOM form bromine- and iodine-substituted DBPs (Richardson et al., 2003; Plewa et al., 2004). Compliance with the Stage 2 Rule has driven many drinking water utilities to switch to chloramines as a secondary disinfectant (Seidel et al., 2005). Chloramines are associated with depressed formation of THMs and HAAs but enhanced formation of nitrogeneous DBPs, including non-halogenated species such as *N*-nitrosamines, of which *N*-nitrosodimethylamine (NDMA) is the most commonly detected species in drinking water systems (Russell et al., 2012). Among all the identifiable DBPs to date, NDMA is the only proven carcinogen *in vivo* (Tricker and Preussmann, 1991), but drinking water is estimated to contribute less than 1% of total NDMA exposure due to high endogenous formation in humans (Hrudey et al., 2013). DBP formation and speciation can vary based on the NOM quantity and character, disinfectant type and dose, and water quality parameters such as pH, temperature, bromide, and iodide. In the nearly 50 years of DBP research, over 700 DBPs have been identified, formed using various disinfection schemes in a wide range of source waters (Richardson et al., 2007). Despite these important advances, the drivers of toxicity in drinking waters have yet to be identified.

Epidemiological and studies suggest that treated drinking water leads to increased risks of certain cancers (Villanueva et al., 2004; Jeong et al., 2012). Toxicological studies offer weight-of-evidence to support the conclusion that DBPs exert carcinogenicity and teratogenic effects in humans (Plewa et al., 2002; Plewa et al., 2004). Much of the current understanding of DBP

toxicity stems from *in vitro* studies with single compounds or a small number of compounds unlike what is present in treated drinking waters. Individual and collective toxicological DBP properties have been shown to provide the best information available on potential adverse health consequences resulting from exposure (Rice et al., 2009).

There is a glaring lack of *in vitro* bioassay data for well-characterized drinking water DBP mixtures. This stems from the need to enrich DBPs formed in treated drinking waters by several orders of magnitude to induce a toxic response in a bioassay. Enrichment typically occurs by passing ca. 10 liters of treated drinking water through columns containing resins, followed by back-elution of the DBP-loaded resins with an organic solvent, such as methanol. The solvent can be volume reduced by evaporation to further enrich the DBPs and is then spiked into a small volume of water. The goal of this process is to generate DBP mixtures enriched by up to 10^5-fold (Liviac et al., 2010). However, enrichment efficiencies are rarely reported in the literature and few studies include characterization of the DBP mixtures that are applied in subsequent bioassays. Rather, DBP characterization often occurs before enrichment only (Neale et al., 2012). This is an important limitation as DBPs with low affinities for the resins are enriched at lower levels or not at all. These shortcomings prevail in the vast majority of *in vitro* bioassays with DBP mixtures (Le Roux et al., 2017). An alternative approach to generate enriched DBP mixtures suitable for bioassays is to first concentrate the NOM from a source water prior to a scaled application of oxidant (e.g., free chlorine or chloramines) to maintain similitude in DBP speciation relative to the original water. This approach was used in the EPA's Four Lab Study for *in vivo* bioassays (Pressman et al., 2010), but only once for *in vitro* testing and never with scaled oxidant dosing protocols.

Cell-based bioassays targeting health-relevant biological endpoints can complement chemical analyses of drinking water quality. Previous research has evaluated bioassays that cover multiple toxicity pathways to benchmark water quality and assess treatment processes (Escher et al., 2013). Bioassays cover a wide variety of toxicity pathways such as induction of xenobiotic

metabolism, specific and reactive modes of toxic action, activation of adaptive stress response pathways and system responses. A battery of bioassays is recommended to assess water quality comprehensively with cytotoxicity, genotoxicity, and mutagenicity the most suitable for drinking water DBPs (Plewa et al., 2002; Mestankova et al., 2014).

Cytotoxicity tests are used to evaluate the impact of a chemical or mixture of chemicals on cell viability and ability for cellular growth. Cytotoxicity tests using mammalian cells are used to investigate the effect of single chemicals and chemical mixtures by measuring the cell density reduction in a prescribed manner. Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. The Chinese Hamster Ovary (CHO) cells are used for assessing DBP cytotoxicity because of their stability, sensitivity and high resistance to contamination (Plewa et al., 2002; Wagner and Plewa, 2017). The assay is performed in sterile 96-well flat-bottomed microplates containing growth media, CHO cells, a blank (no toxicant), and serial dilutions of the toxicant, usually spanning three to six orders of magnitude. The microplates are sealed and incubated for 72 hours at 37 °C in a humidified atmosphere of 5% carbon dioxide, which corresponds to three growth cycles for the CHO cells. Following this incubation period, the growth medium is discarded and the CHO cells are fixed in methanol and stained with crystal violet with the stain adhering only to intact cell membranes (i.e., viable CHO cells). A microplate reader is used to measure the absorbance at 595 nm in each well and the percentage of viable cells is calculated relative to the blank to develop a dose-response curve and estimate the LD50, the lethal dose at which 50% of the population is killed.

The Ames test with *Salmonella typhimurium* is a sensitive tool in screening for potential genotoxic carcinogens using mutagenicity in bacteria as an endpoint (Hengstler and Oesch, 2001). Ames tests are a cost-effective approach to identify substances that can cause genotoxic damage leading to mutations. The Ames test detects mutations in a gene of a histidine-requiring bacterial strain that produces a histidine-independent strain (Ames et al., 1975). TA-98 and TA-

100 are histidine-requiring tester strains with a frameshift mutation in *his*D3052 and base-substitution mutation in *his*G46, respectively. Because a mutation restores the histidine-independent strain, the Ames test is classified as a reverse mutation assay. Approximately 10^9 bacteria are incubated with a known dose of the chemical or chemical mixture being assessed. The large number of bacteria results in a high probability that a mutation will cause a reverse mutation. As bacteria lack most of the enzymes required for the activation of promutagens to mammalian carcinogens, a metabolically-active fraction of rat liver homogenate – known as S9 – is added. A new version of the Ames test, referred to as *Ames II* has been developed as a microwell fluctuation test in contrast to the more laborious plate incubation test. The Ames II or Ames fluctuation test is performed entirely in liquid culture and allows for high-throughput screening and requires less test chemical. An Ames-positive chemical is not necessarily harmful in humans and, therefore, often triggers additional mutagenicity tests, both *in vitro* and *in vivo*, to eliminate false-positives caused by differences in the rat and human liver function.

The objectives of this research are to (1) investigate the cytotoxicity and mutagenicity of DBP mixtures formulated with either free chlorine or monochloramine with and without added bromide and exogenous NDMA, and (2) assess the contribution of the cytotoxicity and mutagenicity attributable to known and identifiable DBPs. A cytotoxicity test with CHO cells and the Ames mutagenicity assay with TA-98 and TA-100 were used in the presence and absence of the S9 rat liver enzyme. DBP-mixture concentrates were formulated with reconstituted NOM obtained from Upper Mississippi River dosed with free chlorine and monochloramine with and without added bromide using procedures developed elsewhere (Pressman et al., 2010; Pressman et al., 2012; Do et al., 2015). The cytotoxicity and mutagenicity of these DBP-mixture concentrates were assessed and the DBPs identified by GC-MS and quantified by GC-ECD. Next, synthetic DBP-mixture analogs were formulated with the identifiable and commercially-available DBPs in accordance to their respective concentration profiles in the DBP-mixture concentrates and tested in the bioassays. The DBP-mixture concentrates and synthetic DBP-mixture analogs

were compared using frequentist statistical analyses (Berthouex and Brown, 2002) to identify toxicity relative to the controls. The findings of this study could be leveraged to improve DBP toxicity methodologies and help prioritize *in vivo* toxicity studies.

2 Materials and Methods

2.1 Overview

This chapter details the materials and methods used in the *in vitro* toxicity bioassays with disinfection byproduct (DBP) mixtures and include the formulation of the DBP mixture-concentrates, DBP identification and quantification, and the formulation of the synthetic DBP mixture-analogs. Both the DBP mixture-concentrates and synthetic analogs were assessed for toxicity using *in vitro* bioassays, which included a cytotoxicity test with Chinese Hamster Ovary (CHO) cells and Ames mutagenicity test with two strains of *Salmonella typhimurium* bacteria, TA98 and TA100, in the presence and absence of the S9 rat liver enzyme. Tukey's tests at the $\alpha = 0.01$ significance-level were applied to the bioassay data to compare conditions and identify toxicity relative to the control conditions.

2.2 Sterile Procedures for Contamination Prevention

Milli-Q water (18.2 MΩ-cm) generated from Millipore Integral 3 Milli-Q water system (Billerica, MA) was used for all aqueous phase preparations for the *in vitro* toxicity bioassays. CHO cells and bacteria strains were grown in a CO_2 incubator and a benchtop orbital shaker, respectively. The CHO cells and bacteria strains were handled in a biosafety cabinet (BSL-2A) and checked to ensure they were contamination-free before use. Prior to each experiment, the biosafety cabinet was disinfected using UV light for fifteen minutes and work surfaces wiped periodically with 70% ethanol to suppress contamination to the extent possible.

2.3 Disinfection Byproduct Mixtures

2.3.1 Formulation of DBP Mixture Concentrates

To induce toxicity in short-term bioassays (i.e., less than one week), DBPs must be present at higher concentrations than in treated drinking waters, often by five to seven orders of magnitude (Plewa et al., 2002). The technique developed by Pressman et al. (2010) was used to formulate concentrated DBP mixtures with speciation profiles proportionally scaled-up from

the disinfection of a natural water. Here, DBP mixture-concentrates were formulated using free chlorine and monochloramine dosed to reconstituted natural organic matter (NOM) in the presence and absence of purposely-added bromide. Freeze-dried NOM from the Upper Mississippi River (UMR) was acquired from the International Humic Substances Society and reconstituted under acidic conditions (i.e., pH less than 3) at a target dissolved organic carbon (DOC) ca. 100 $mg \cdot L^{-1}$. Batches of concentrate were formulated by dissolving 200 mg UMR-NOM in 600 mL Milli-Q water and stirring continuously for five days. The NOM concentrate was adjusted to pH 7 with NaOH and twice-filtered through 0.45 µm poly(ether)-sulfone membranes prior to measurement of DOC and dilution with Milli-Q water to a DOC of ca. 100 $mg \cdot L^{-1}$. The DOC was measured with a Sievers 5310C Portable TOC Analyzer (Sievers, Boulder, CO) and the UV absorbance was measured in a 1 cm pathlength quartz cuvette. The specific UV absorbance (SUVA) was calculated by dividing the UV_{254} by the DOC and reported in units of $L \cdot mg^{-1} \cdot m^{-1}$. Half of the reconstituted UMR-NOM concentrate (i.e., a 300 mL aliquot) was spiked with bromide at a final concentration of 3.96 $mg \cdot L^{-1}$ to form brominated-DBPs upon chlorination or chloramination, which are generally considered to be more toxic (Richardson et al., 2003; Plewa et al., 2004). The other 300-mL aliquot was left unamended without any added bromide. Each 300-mL aliquot was halved again to produce a total of four, 150-mL, aliquots for dosing with either free chlorine or monochloramine as described next.

The DOC in the UMR-NOM concentrates (ca. 100 $mg \cdot L^{-1}$) was considered to be a fifty-fold-enrichment (i.e., 50X) by assuming the DOC in the 1X sample was 2 $mg \cdot L^{-1}$. The free chlorine dose applied to the UMR-NOM concentrate was 218 $mg \cdot L^{-1}$-Cl_2, a dose that scaled based on the 24-hour free chlorine dose of the 1X sample that resulted in a residual of 1.0 $mg \cdot L^{-1}$-Cl_2 (Pressman et al., 2010). A second free chlorine dose of 60 $mg \cdot L^{-1}$-Cl_2 was used to formulate an additional concentrate for comparison purposes. For the monochloramine-based DBP concentrate-mixtures, the monochloramine dose was 250 $mg \cdot L^{-1}$-Cl_2 and a hold-time of seven days, a

protocol that was based on previously work by Do et al. (2015). Following their respective hold-times, the residual free chlorine or monochloramine was quenched using ascorbic acid such that the mixtures contained DBPs but no residual disinfectant. These samples were assessed in the cytotoxicity and mutagenicity bioassays as described in Sections 2.4 and 2.5.

2.3.2 Commercially-available and Identifiable DBPs

Table 2-1 shows the commercially-available and identifiable DBPs that formed in the DBP-concentrates, which include the four regulated trihalomethanes, one haloketone, one halonitromethane, and two dihaloacetonitriles, nine haloacetic acids, and dalapon (see Table 2-1). The eighteen DBPs in Table 2-1 were quantified by two separate analyses, one for haloacetic acids and dalapon and the other for trihalomethanes, haloketones, halonitromethanes, and dihaloacetonitriles.

Previous work by this lab group has shown that the DBPs listed in Table 2-1 with the exception of the HAAs and dalapon could be quantified by a single GC analysis following a liquid-liquid extraction into a 1:1 v/v mixture of hexane and dichloromethane. Here, a 10 mL sample of the DBP mixture-concentrate was fortified with 0.5 mL of 0.5 mg/L trichloroethane internal standard. Next, 0.5 mL hexane:dichloromethane (1:1 v/v) was added and the sample shaken for 15 minutes on a back-and-forth shaker table at high-speed; after a 5-minute quiescent settling period, the organic solvent layer was decanted with a Pasteur pipette. This extraction process was then repeated in an attempt to achieve a more complete DBP extraction and the ca. 1 mL extract was put into a glass GC vial and stored for at 4 °C prior to DBP analyses. DBPs in the mixture-concentrates were identified and quantified GC-ECD by matching the retention times of analytical DBP standards injected one at a time. Splitless injections of 1 µL were used with an injector temperature of 250 °C. The separation column used was a RESTEK DB-1 with a length of 30 m, inner diameter 0.25 mm, and a stationary phase film thickness of 1 µm. Six-point DBP standard curves (1-1,000 µg/L) were used to quantify the unknowns and correlation coefficients,

R^2 exceeded 0.99 for all compounds. Blanks and check standards were run after every ten injections. DBP-standards were extracted following the same procedures as the samples.

Table 2-1. Commercially-available and Identifiable DBPs in the Concentrate-Mixtures

Name	Abbreviation	Class	Cas No	Molecular Formula
Trichloromethane	TCM	Trihalomethane	67-66-3	$CHCl_3$
Bromodichloromethane	BDCM		75-27-4	$CHBrCl_2$
Chlorodibromomethane	CDBM		124-48-1	$CHBr_2Cl$
Tribromomethane	TBM		75-25-2	$CHBr_3$
1,1-dichloro-2-propanone	DCP	Haloketone	513-88-2	$C_3H_4Cl_2O$
Trichloronitromethane (Chloropicrin)	TCNM	Halonitromethane	76-06-2	CCl_3NO_2
Dichloroacetonitrile	DCAN	Dihaloacetonitrile	3018-12-0	Cl_2CHCN
Bromochloroacetonitrile	BCAN		83463-62-1	$C_2HBrClN$
Monochloroacetic acid	MCAA	Haloacetic acid	79-11-8	$ClCH_2COOH$
Monobromoacetic acid	MBAA		79-08-3	$BrCH_2COOH$
Dichloroacetic acid	DCAA		79-43-6	$Cl_2CHCOOH$
Bromochloroacetic acid	BCAA		5589-96-3	$BrClCHCOOH$
Trichloroacetic acid	TCAA		76-03-9	Cl_3CCOOH
Dibromoacetic acid	DBAA		631-41-1	$Br_2CHCOOH$
Bromodichloroacetic acid	BDCAA		7113-314-7	$BrCl_2CCOOH$
Chlorodibromoacetic acid	CDBAA		5278-95-5	$Br_2ClCCOOH$
Tribromoacetic acid	TBAA		75-96-7	Br_3CCOOH
2,2-dichloropropanoic acid	Dalapon	Herbicide	75-99-0	$C_3H_4Cl_2O_2$

HAAs were quantified by EPA Method 552.2 in which compounds are alkylated with methanol to methyl esters prior to liquid-liquid extraction into MtBE and analysis by GC-ECD. DBP-standards were extracted following the same procedures as the samples. Trichloropropane was used as the internal standard and bromobutanoic acid was used as the surrogate standard. The DBPs in the concentrate mixtures were brought to a pH lower than 2.0 by addition of concentrated sulfuric acid. Addition of anhydrous sodium sulfate and MTBE with internal standard allowed the target analytes to transition out of the aqueous phase and into the solvent phase. This transition was aided by the use of a back-and-forth shaker table at high speed for 3 minutes. After a 2-minute settling period, the upper MTBE layer was decanted with a Pasteur pipette into a glass conical vial. Acidic methanol was added to the extract prior to the 2-hour heating period at 50 °C. After the heating period, an aqueous solution of sodium sulfate was added to the conical vials followed by a brief vortex and 2-minute settling period. The acidic aqueous methanol layer was removed before neutralization with saturated sodium bicarbonate.

After neutralization, the upper ether layer was extracted with a Pasteur pipette into a glass GC vial and stored at 4 °C prior to DBP analyses. DBPs were identified by gas chromatography with a mass-selective detector and quantified GC-ECD. Splitless injections of 1 µL were used with an injector temperature of 200 °C. The separation column used was an Agilent DB-1 with a length of 30 m, inner diameter 0.25 mm, and a stationary phase thickness of 1 µm. Twelve-point HAA standard curves (0.01-500 µg/L) were used to quantify the unknowns and correlation coefficients, R^2, exceeded 0.99 for all compounds. Blanks were run after every six injections and check standards were run after every nine injections.

2.3.3 Synthetic Analog DBP Mixtures

The commercially-available DBPs identified in mixture-concentrates (Table 2-1) were acquired as single compounds from AccuStandard (New Haven, CT) and used to quantify DBPs in the mixture-concentrates and formulate synthetic analog mixture for testing in the CHO-cytotoxicity and Ames mutagenicity bioassays. A total of five synthetic mixtures were formulated based on the whole-mixture DBP concentrates, which included two with free chlorine (added bromide/no bromide), two with monochloramine (added bromide/no bromide), and NDMA only. Using the approach, differences in cytotoxicity and/or mutagenicity between the DBP mixture-concentrates and synthetic DBP-analogs are attributable to identified DBPs that are not commercially-available, non-identified DBPs, or interaction effects (synergistic and antagonistic) amongst these various groups of DBPs.

2.4 Cytotoxicity Bioassay

2.4.1 Test Preparation

Chinese hamster ovary (CHO) mammalian cells were used for the *in vitro* cytotoxicity tests with cell line AS52, clone 11-4-8.39-41. The live culture of CHO cells were obtained from Dr. Michael Plewa at the University of Illinois Urbana-Champaign and preserved in Ham's F-12 Fetal Bovine Serum (FBS) and dimethyl sulfoxide (DMSO) at -80 °C. To prepare the CHO cells

for use in the bioassays, an aliquot was removed from the freezer and centrifuged to remove the FBS and DMSO prior to transferring into Ham's F12 nutrient medium containing 5% FBS, 1% antibiotics (100 Units·mL^{-1} sodium penicillin G, 100 µg·mL^{-1} streptomycin sulfate, and 0.25 µg·mL^{-1} amphotericin B in 0.85% saline), and 1% glutamine. The CHO cells were grown to confluency over three-to-four days at 37 °C in a humidified atmosphere of 5% carbon dioxide in Ham's F12 medium. Confluency was approximated as 70-90% plate coverage when checked visually under the microscope. Before harvesting, the attached CHO cells were washed with Hank's Balanced Salt Solution (HBSS) twice, and detached from the petri-dishes using 1 mL of trypsin. Ham's F12 medium was used to stop the reaction with trypsin and resuspend the cells. The cell concentration was measured using a Beckman Coulter Multisizer-4 and reported in number of cells per mL. This concentration was used to determine the aliquot volume required to achieve three-thousand cells in each microplate well as detailed next in Section 2.4.2.

2.4.2 DBP Testing with Chinese Hamster Ovary Cells

The *in vitro* cytotoxicity bioassay with CHO cells is a measure of cell density reduction from a DBP or DBP-mixture over an exposure time of 72 hours. In this bioassay, there is a direct relationship between the absorbance of crystal violet dye and the number of attached (or viable) CHO cells (Plewa et al., 2002). A sterile-flat-bottom 96-well-microplate was used to test serial dilutions of the DBP mixtures. The first column in the microplate serves as blank (contains no CHO cells) consisting of 120 µL Milli-Q water and 80 µL F12-FBS – these wells should not retain any of the crystal violet staining. The second column serves as the negative control (or optimum growth condition), with each well containing ca. 3,000 CHO cells added in a 100 µL aliquot, 80 µL of F12-FBS medium, and 20 µL Milli-Q water – these wells should retain a maximum amount of the crystal violet staining. The remaining columns contain 3,000 CHO cells added in a 100 µL aliquot, 80 µL of F12-FBS medium, and a known concentration of the toxicant mixture added in a 20 µL aliquot. Following dosing, each plate was sealed with a sterile

aluminum sheet to prevent cross and outside contamination. The plates were agitated on a back-and-forth shaker for five minutes to ensure even distribution of the CHO cells on the flat bottom of each well, and then the plate was incubated at 37 °C under a 5% CO_2 atmosphere for 72 hours. Next, the liquid within each well was disposed and 50 μL of methanol was added for 10 minutes for cell fixation. Then the cells were stained using 1% crystal violet solution in 50% methanol for 10 min. The microplates were washed twice before 50 μL DMSO/methanol (3/1 v/v) was added and placed in darkness for 10 min. The microplate was analyzed at an absorbance of 595 nm using a Bio-Tek microplate reader. The absorbance readings for each plate were collected and stored in a MS Excel spreadsheet prior to analysis as described next in Section 2.4.3.

2.4.3 Data Analysis

The median absorbance of the blank wells was subtracted from all the wells to yield blank-corrected readings. The median blank-corrected absorbance of the negative-control was set at 100% and the absorbance for each treatment-well was calculated based on the percentage of the negative control. These data were used to generate a dose-response profile for each DBP mixture. In cases in which the LD50 was surpassed, regression analysis was applied to estimate the LD50 value, which is the concentration that resulted in a cell density that was 50% of the negative control. The lowest concentration that induced a significant level of cytotoxicity was determined using a Tukey's test at an $\alpha = 0.01$ significance-level.

2.5 Ames Mutagencity Bioassay

2.5.1 Test Preparation

Salmonella typhimurium bacteria strains TA98 and TA100 and S9 rat liver enzyme were obtained from Moltox Inc. and used for the Ames mutagenicity bioassay. The mutagenicity mechanism differs between these two bacteria strains: TA98 has a frameshift sensitivity and TA100 has a base-pair-substitution sensitivity. Four test conditions were evaluated: TA98

without S9 (TA98-S9), TA98 with S9 (TA98+S9), TA100 without S9 (TA100-S9), and TA100 with S9 (TA100+S9). Both bacteria strains were inoculated on a temperature controlled shaker table at 150 rpm and 37 °C in autoclaved Nutrient Broth #2 media (10 $mg \cdot L^{-1}$ beef and yeast extracts, 10 $mg \cdot L^{-1}$ peptone, 5 $mg \cdot L^{-1}$ sodium chloride, adjusted to pH 7.5 $\pm$ 0.2 with added ampicillin) for approximately 12 hours before Ames tests.

2.5.2 *DBP Testing with Bacteria Strains*

The Ames fluctuation assay (ISO 11350) was used to assess the mutagenicity of the DBP mixture-concentrates. The abundances of TA98 and TA100 bacteria in their respective stock cultures were estimated by measuring the optical density with a spectrophotometer at 595 nm; midway through the research, this measurement was replaced with a coulter counter estimate taken from particle diameters between 0.7 and 1.7 microns.

The Ames bioassay begins in sterile 24-well flat plates. Each well contained 100 μL of the TA98 or TA100 bacteria stock culture, 100 μL of exposure medium (0.81 mM magnesium sulfate heptahydrate, 10.41 mM citric acid, 57.4 mM dipotassium hydrogen phosphate, 16.74 mM sodium ammonium hydrogen phosphate tetrahydrate, and 22.2 mM D-glucose), and 34 μL of either Milli-Q water or S9 rat liver enzyme solution (3.3 μM potassium chloride, 8.0 μM magnesium chloride hexahydrate, 5.0 μM D-glucose-6-phosphate, 4.0 μM nicotinamide-adenine-dinucleotide phosphate, 30% (v/v) S9-fraction in 100 mM sodium dihydrogen phosphate buffer at pH 7.4). For each Test Condition (TA98-S9, TA98+S9, TA100-S9, and TA100+S9), there are four variables – a negative control, a positive control, and two toxicant dosing levels designated as D1 for the full-strength DBP mixture-concentrate and D10 for that diluted by a factor of ten. For each variable, an additional 800 μL (called the dosing volume) was added to the respective wells such that the total volume in each well is 1034 μL. For the negative control, the dosing volume is 800 μL of Milli-Q water; for the positive control, the dosing volume is 780 μL of Milli-Q

water and 20 μL of positive control stock solution, which includes a different agent for each Test Condition (see Table 2-2).

Table 2-2. Positive control chemicals for the Ames Bioassay

Test Condition	Positive Control Agent	Concentration in Well ($mg \cdot L^{-1}$)
TA98-S9	4-nitro-*o*-phenylenediamine (4-NOPD)	10
TA98+S9	2-aminoanthracene (2-AA)	0.1
TA100-S9	Nitrofurantoin (NF)	0.25
TA100+S9	2-aminoanthracene (2-AA)	0.4

For D1, the dosing volume is 800 μL of the DBP mixture-concentrate and for D10 the dosing volume is 80 μL of the DBP mixture-concentrate and 720 μL of Milli-Q water. The negative and positive controls were performed in triplicate and D1 and D10 each had nine observations.

The 24-well plates were shaken at 150 rpm at 37 °C for 100-minutes in the dark. Next, 500 μL from each well was transferred and mixed with 2.5 mL of histidine-deficient reversion-indicator medium (magnesium sulfate heptahydrate 0.21 $g \cdot L^{-1}$, citric acid monohydrate 2.1 $g \cdot L^{-1}$, di-potassium hydrogen phosphate 10.7 $g \cdot L^{-1}$, sodium ammonium hydrogen phosphate tetrahydrate 3.74 $g \cdot L^{-1}$, D-glucose-anhydrous 4.28 $g \cdot L^{-1}$, D-biotin 26 $mg \cdot L^{-1}$ and 26.4 $mg \cdot L^{-1}$ of the pH indicator dye bromocresol purple). Sample volume from each well in the 24-well-plate was transferred to 48-wells in a 384-well-microplate in 50 μL aliquots. Each 384-well-microplate was placed in sterile Ziploc plastic bags in the 37 °C incubator for an exposure time of 72-hours in the dark without shaking. During the exposure, the histidine auxotrophic test bacteria can revert to the prototrophic phenotype by a mutation event. This reversion is detected by a color shift of the histidine-free bromocresol purple reversion indicator medium from purple to yellow, caused by the acidification of the medium resulting the pH to drop due to the metabolic activity of the revertants. Subsequently, reverent wells are yellow and non-reverent wells remain purple, each of which are counted and recorded. Tukey's test at an $\alpha = 0.01$ significance-level was applied to the number of revertant wells for each DBP mixture-concentrate and test condition (TA98 - S9, TA98 + S9, TA100 - S9, and TA100 + S9).

3 Results

3.1 DBP Mixture Characterizations

Table 3-1 shows the DBP profiles in the concentrate-mixtures and Table 3-2 shows the DBP profiles in the synthetic DBP-mixture analogs.

Table 3-1. Oxidant doses, precursors, and disinfection byproduct profiles in the concentrate-mixtures.

Reactants* ($mg \cdot L^{-1}$)	Free Chlorine ($mg \cdot L^{-1}$ as Cl_2)			Monochloramine ($mg \cdot L^{-1}$ as Cl_2)	
Oxidant Dose	60	218	218	250	250
Bromide	0	0	4	0	4
Dissolved organic carbon	99.4	100.8	96.1	99.9	96.1
Disinfection byproducts ($\mu g \cdot L^{-1}$)					
Trichloromethane (TCM)	2,213	2,311	3,404	1,195	690
Dichlorobromomethane (DCBM)	26	45	1,643	16	1,126
Dibromochloromethane (DBCM)	ND	ND	249	ND	403
Tribromomethane (TBM)	ND	ND	ND	ND	44
Dichloroacetonitrile (DCAN)	117	41	42	193	167
Bromochloroacetonitrile (BCAN)	ND	ND	ND	ND	11
Dibromoacetonitrile (DBAN)	ND	ND	ND	ND	5
Propanone	35	35	26	35	15
Chloropicrin	ND	ND	ND	2	ND
Monochloroacetic acid (MCAA)	93	113	117	87	74
Monobromoacetic acid (MBAA)	ND	6	26	1	29
Dichloroacetic acid (DCAA)	522	1,910	1,737	1,167	860
Bromochloroacetic acid (BCAA)	5	9	368	10	371
Trichloroacetic acid (TCAA)	889	4,336	3,843	289	187
Dibromoacetic acid (DBAA)	ND	ND	38	ND	88
Bromodichloroacetic acid (BDCAA)	1	5	385	ND	29
Chlorodibromoacetic acid (CDBAA)	ND	ND	49	ND	5
Tribromoacetic acid (TBAA)	9	47	34	3	3
Dalapon	19	130	117	20	10

*samples dosed at pH 7 in 20 mM bicarbonate buffer following Do et al., (2015)
ND – not detected

The impact of NDMA was assessed by addition of this compound to an aliquot of each of mixture concentrates formulated with monochloramine (Table 3-1). Therefore, a total of seven whole-water DBP mixture concentrates were formulated and tested in the bioassays.

Table 3-2. Disinfection byproduct profiles in the synthetic mixture analogs.

Reactants* ($mg \cdot L^{-1}$)	Free Chlorine ($mg \cdot L^{-1}$ as Cl_2)			Monochloramine ($mg \cdot L^{-1}$ as Cl_2)	
Oxidant Dose	60	218	218	250	250
Bromide	0	0	4	0	4
Dissolved organic carbon	99.4	100.8	96.1	99.9	96.1
Disinfection byproducts ($\mu g \cdot L^{-1}$)					
Trichloromethane (TCM)	NF	2,400	3,600	NF	800
Dichlorobromomethane (DCBM)	NF	50	1,700	NF	1,200
Dibromochloromethane (DBCM)	NF	ND	300	NF	500
Tribromomethane (TBM)	NF	ND	ND	NF	50
Dichloroacetonitrile (DCAN)	NF	50	50	NF	200
Bromochloroacetonitrile (BCAN)	NF	ND	ND	NF	25
Dibromoacetonitrile (DBAN)	NF	ND	ND	NF	5
Propanone	NF	50	25	NF	25
Chloropicrin	NF	ND	ND	NF	ND
Monochloroacetic acid (MCAA)	NF	120	120	NF	74
Monobromoacetic acid (MBAA)	NF	8	32	NF	32
Dichloroacetic acid (DCAA)	NF	2,000	1,800	NF	900
Bromochloroacetic acid (BCAA)	NF	10	400	NF	400
Trichloroacetic acid (TCAA)	NF	5,000	4,000	NF	200
Dibromoacetic acid (DBAA)	NF	ND	40	NF	88
Bromodichloroacetic acid (BDCAA)	NF	5	389	NF	32
Chlorodibromoacetic acid (CDBAA)	NF	ND	52	NF	6
Tribromoacetic acid (TBAA)	NF	48	40	NF	4
Dalapon	NF	160	125	NF	10

*Profiles formulated based on these conditions and values in Table 3-1
NF – not formulated; ND – not detected

A total of five synthetic analog mixtures were tested in the bioassays, which included the three in Table 3-2, NDMA alone, and exogenous NDMA added to the synthetic analog formulated based on the DBP profile produced with monochloramine and bromide (Table 3-2).

3.2 Bioassays

Results of the cytotoxicity tests with CHO cells and Ames mutagenicity tests with TA-98 and TA100 in the presence and absence of S9 rat liver enzyme are shown in Figures 3-1 to 3-12. Chapter 4 includes a discussion of these data and their respective DBP profiles (Tables 3-1 and 3-2).

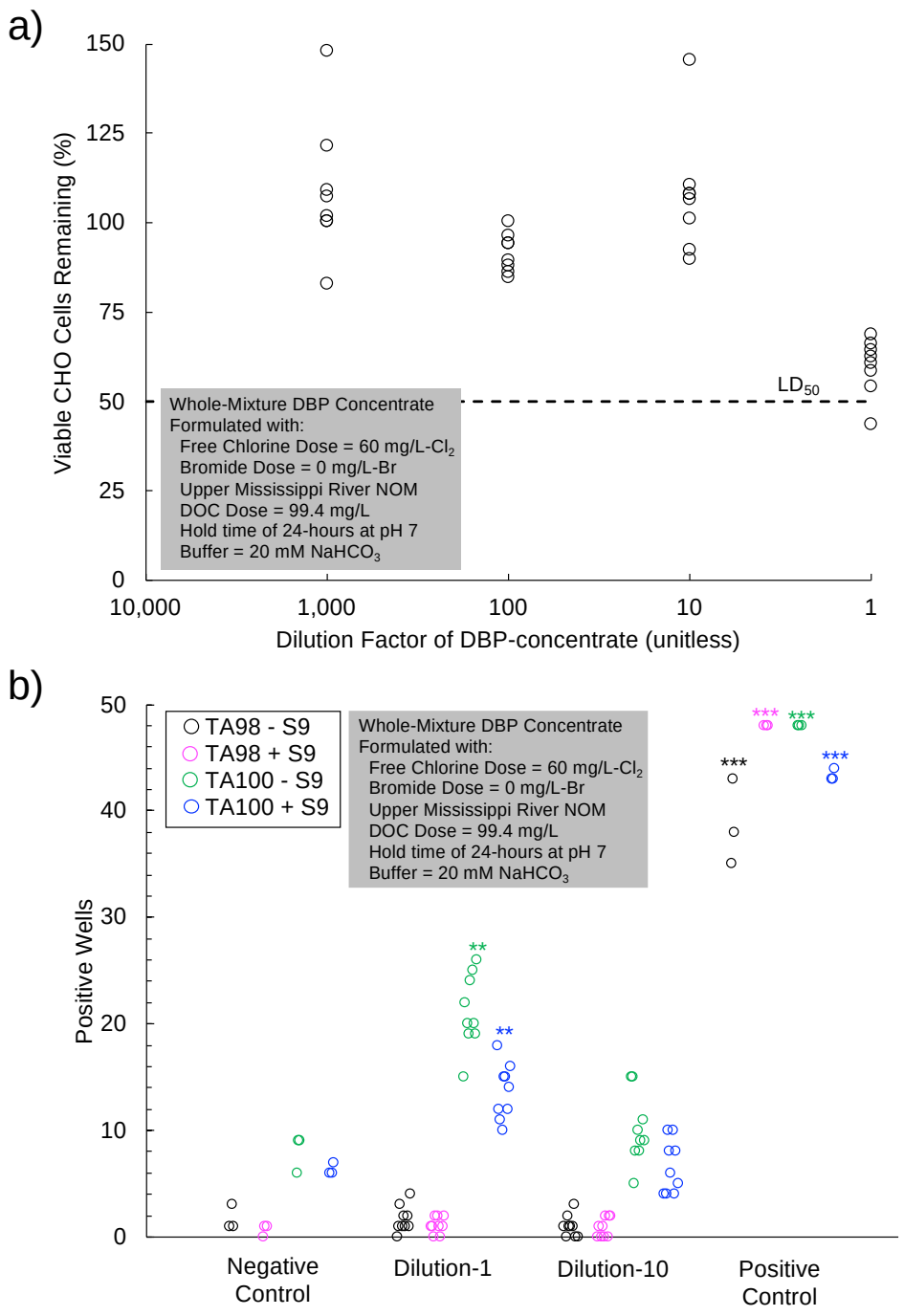

Figure 3-1. Bioassay results for a disinfection byproduct mixture-concentrate formulated by re-acting 60 mg/L-Cl_2 free chlorine with 99.4 mg/L dissolved organic carbon for 24-hours at pH 7. (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

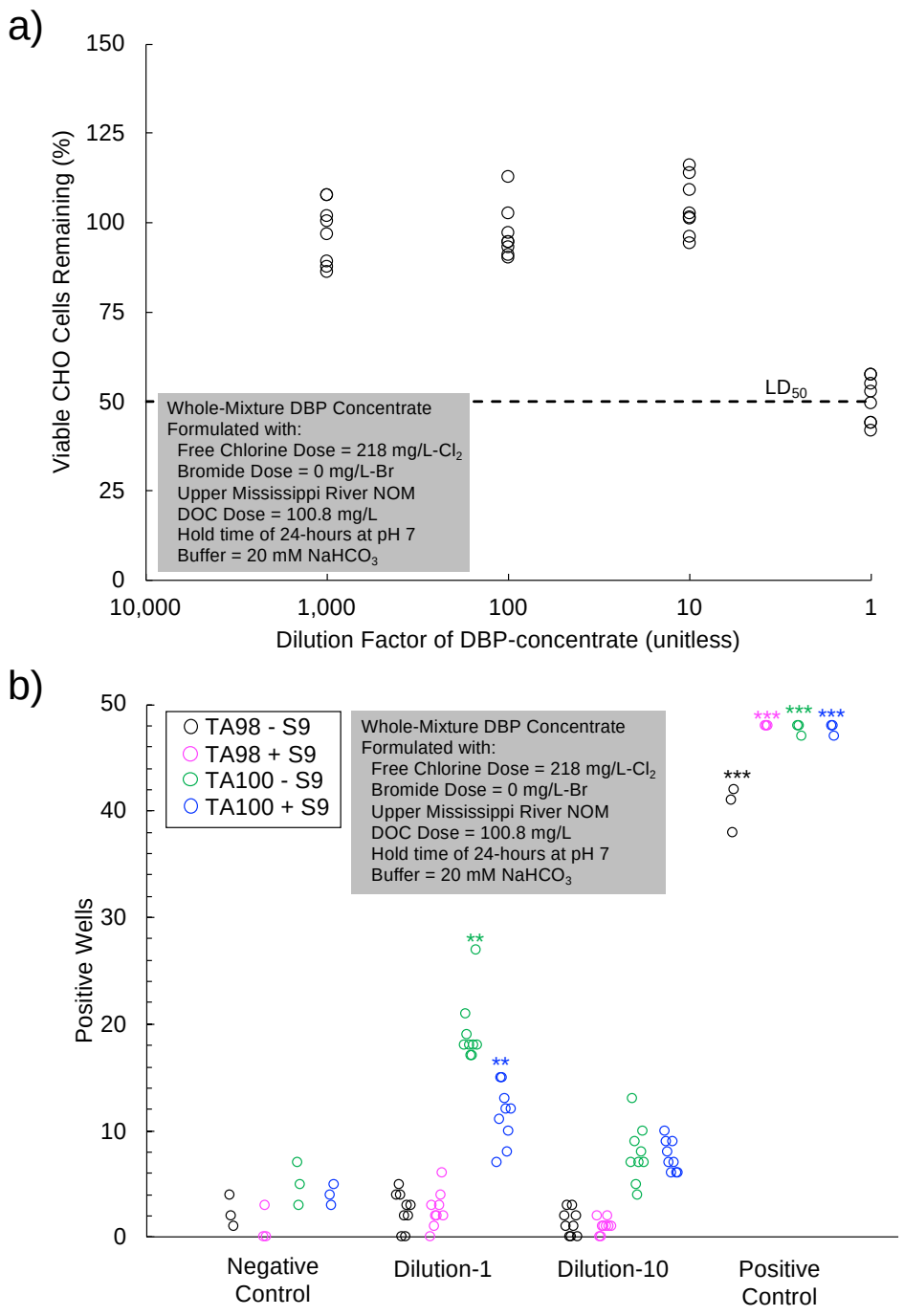

Figure 3-2. Bioassay results for a disinfection byproduct mixture-concentrate formulated by re-acting 218 mg/L-Cl_2 free chlorine with 100.8 mg/L dissolved organic carbon for 24-hours at pH 7. (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

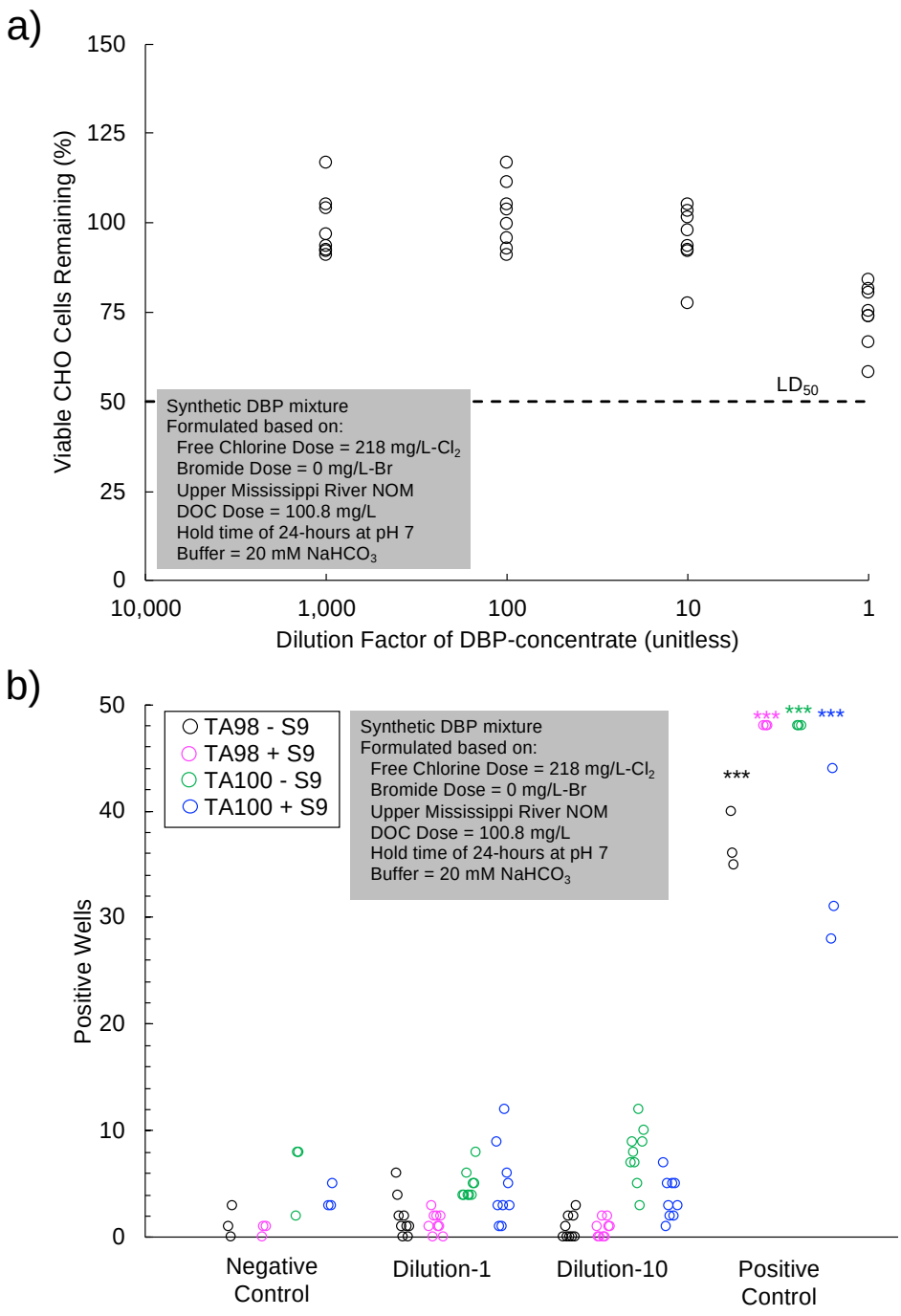

Figure 3-3. Bioassay results for a synthetic disinfection byproduct mixture-analog with the DBP profile shown in Table 3-2 corresponding to the mixture formulated by reacting 218 mg/L-Cl_2 free chlorine with 100.8 mg/L dissolved organic carbon for 24-hours at pH 7. (a) cytotoxicity bio-assay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

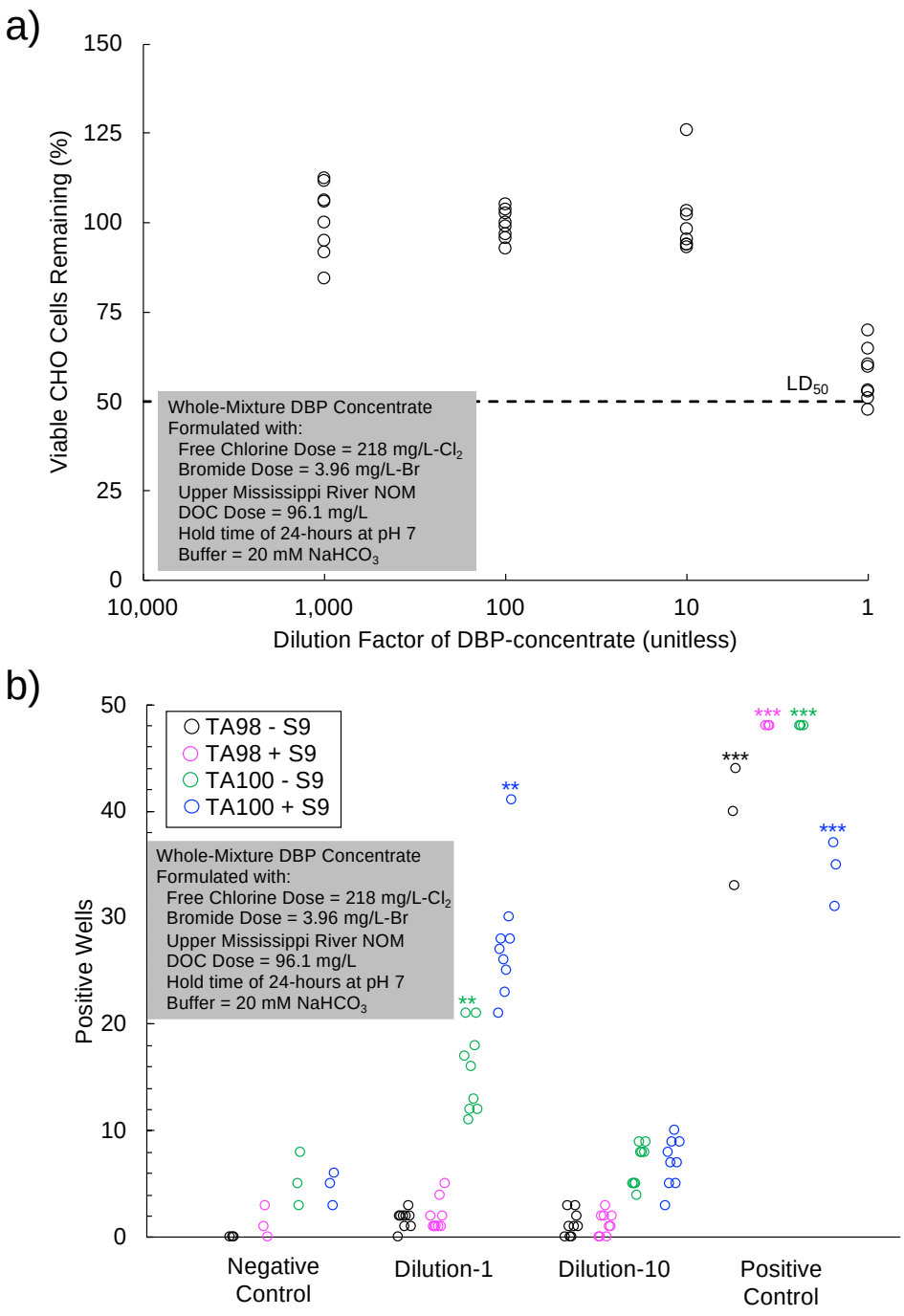

Figure 3-4. Bioassay results for a disinfection byproduct mixture-concentrate formulated by reacting 218 mg/L-Cl_2 free chlorine with 96.1 mg/L dissolved organic carbon and 3.96 mg/L bromide for 24-hours at pH 7. (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

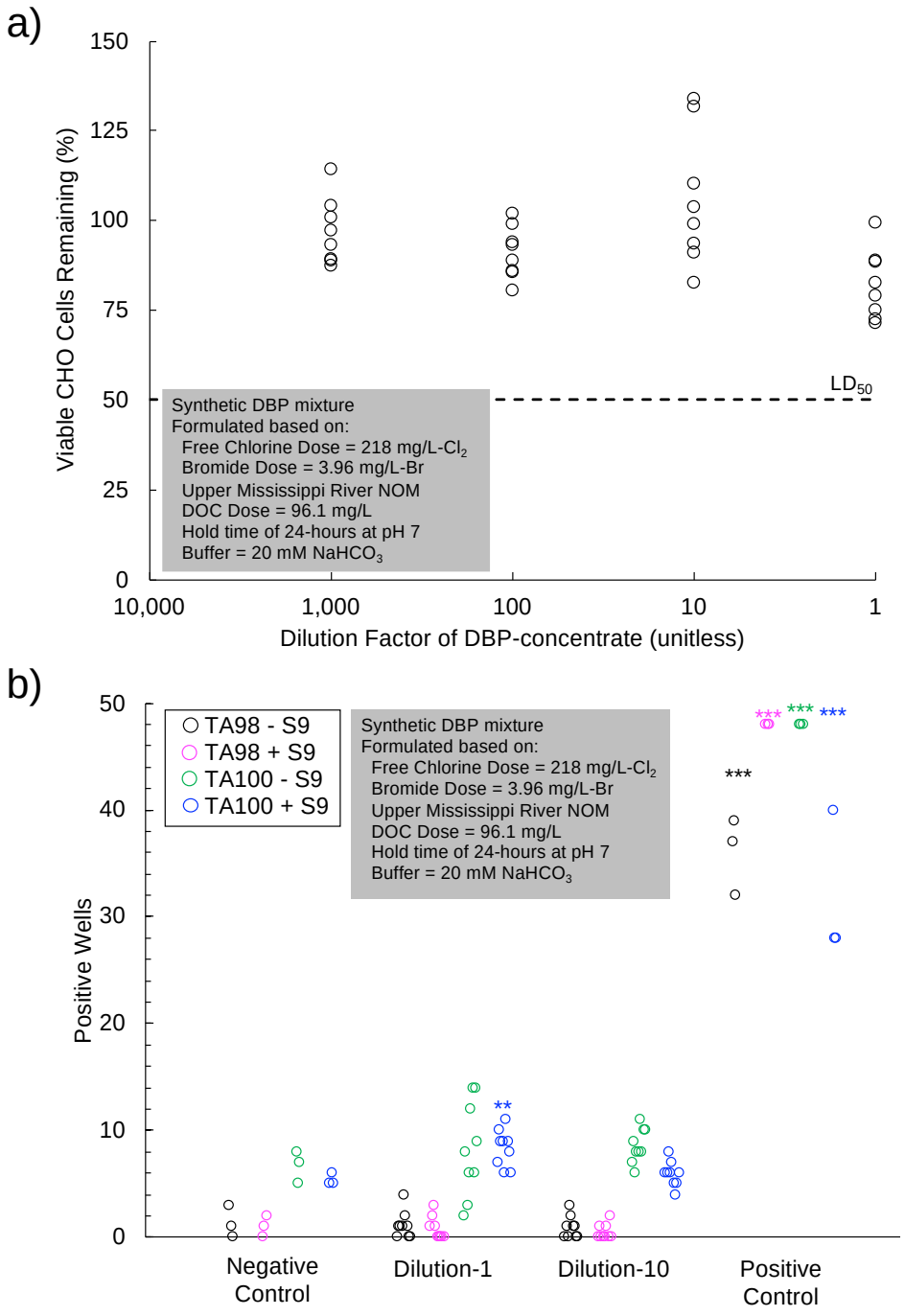

Figure 3-5. Bioassay results for a synthetic disinfection byproduct mixture-analog with the DBP profile shown in Table 3-2 corresponding to the mixture formulated by reacting 218 mg/L-Cl_2 free chlorine with 96.1 mg/L dissolved organic carbon and 3.96 mg/L bromide for 24-hours at pH 7. (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

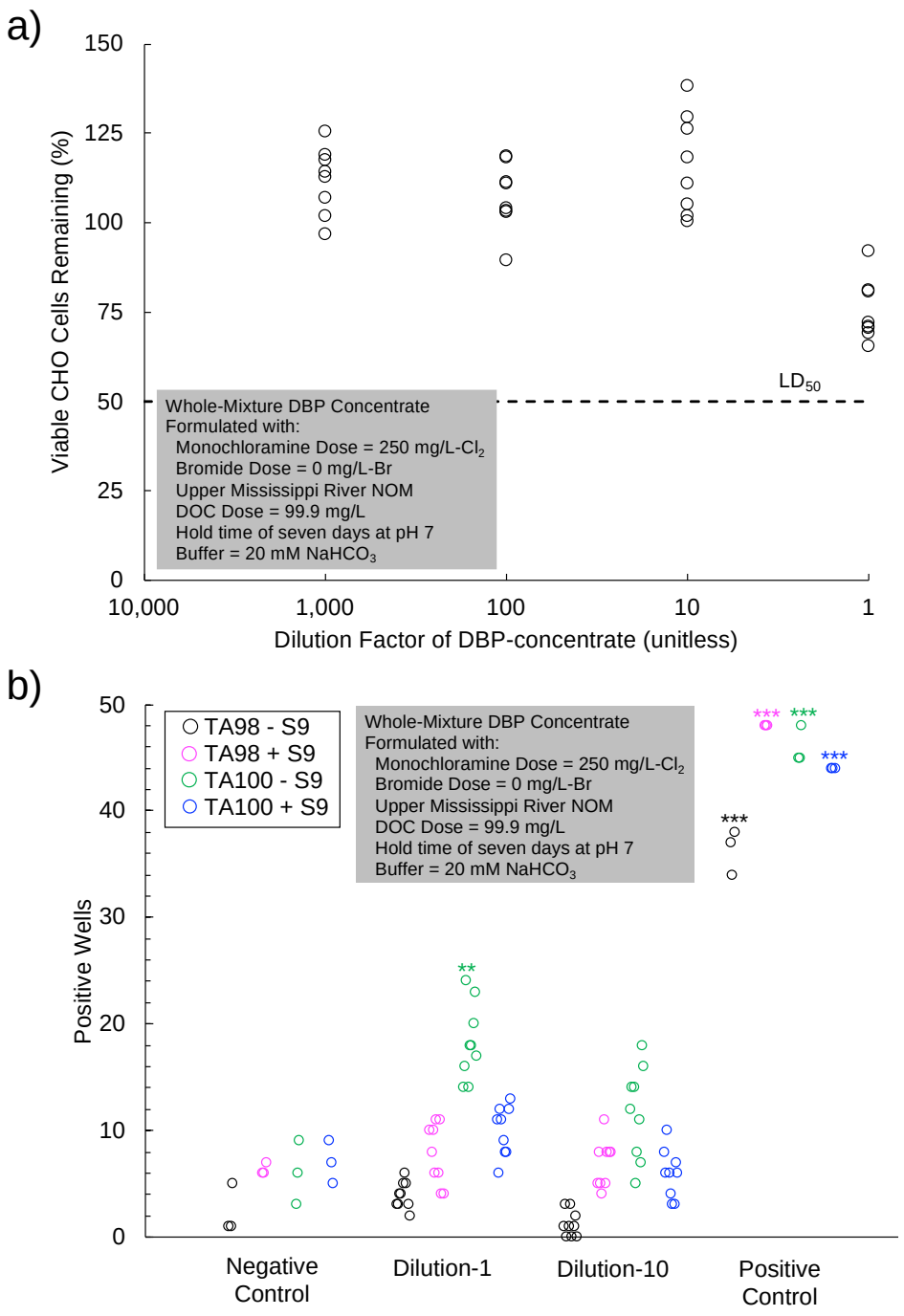

Figure 3-6. Bioassay results for a disinfection byproduct mixture-concentrate formulated by re-acting 250 mg/L-Cl_2 monochloramine with 99.9 mg/L dissolved organic carbon for 7-days at pH 7. (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

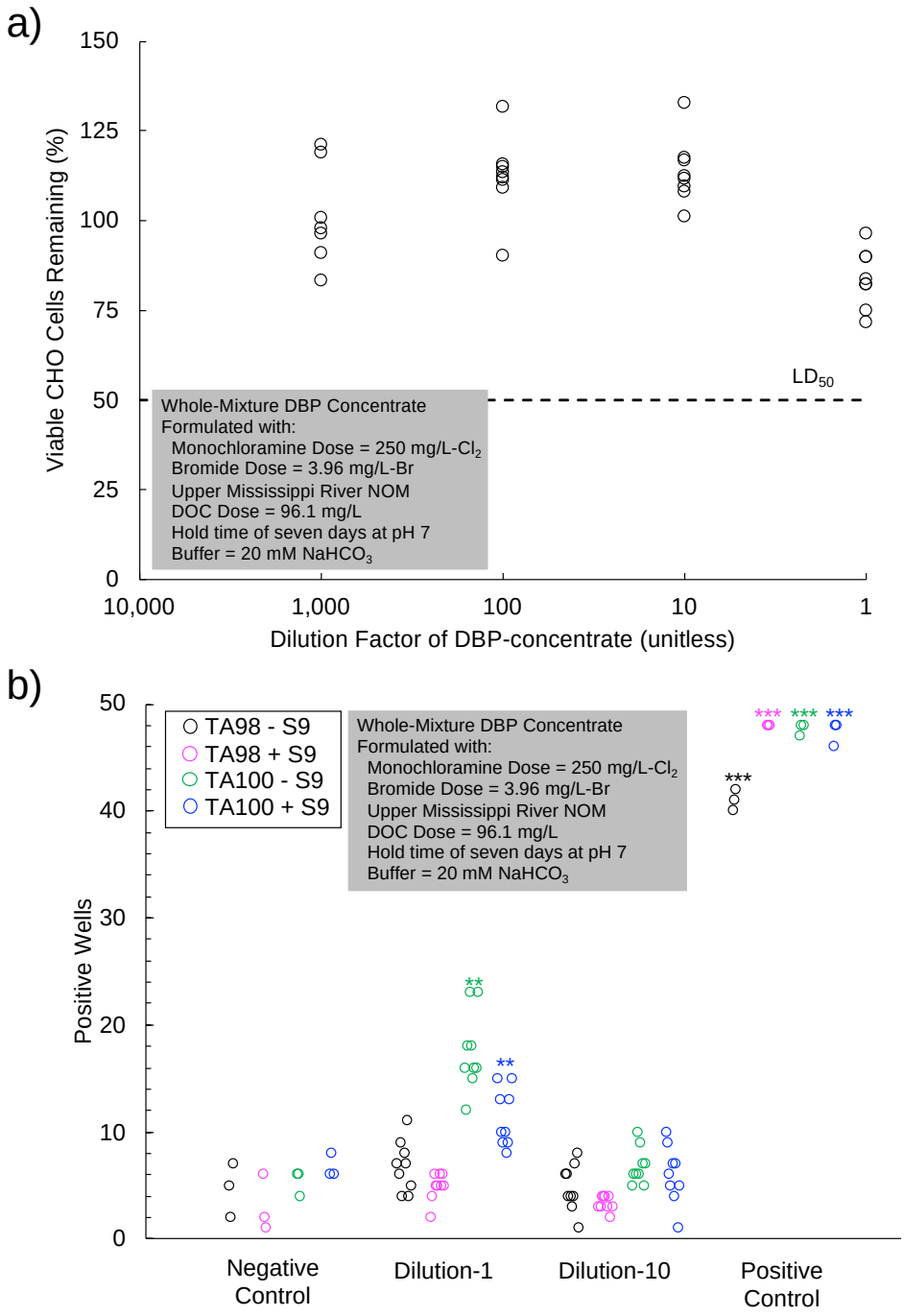

Figure 3-7. Bioassay results for a disinfection byproduct mixture-concentrate formulated by reacting 250 mg/L-Cl_2 monochloramine with 96.1 mg/L dissolved organic carbon and 3.96 mg/L bromide for 7-days at pH 7. (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

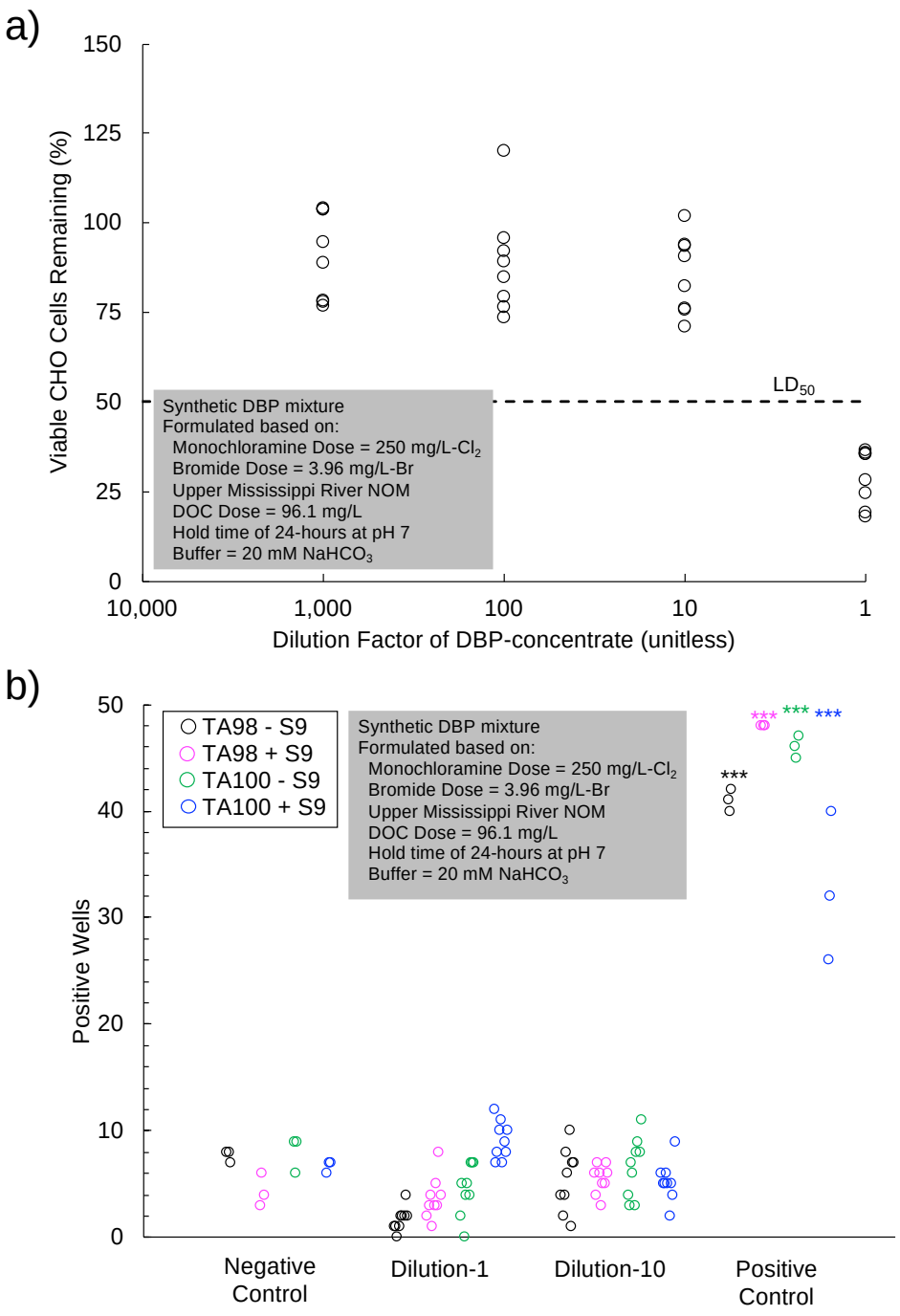

Figure 3-8. Bioassay results for a synthetic disinfection byproduct mixture-analog with the DBP profile shown in Table 3-2 corresponding to the mixture formulated by reacting 250 mg/L-Cl_2 monochloramine with 96.1 mg/L dissolved organic carbon and 3.96 mg/L bromide for 24-hours at pH 7. (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

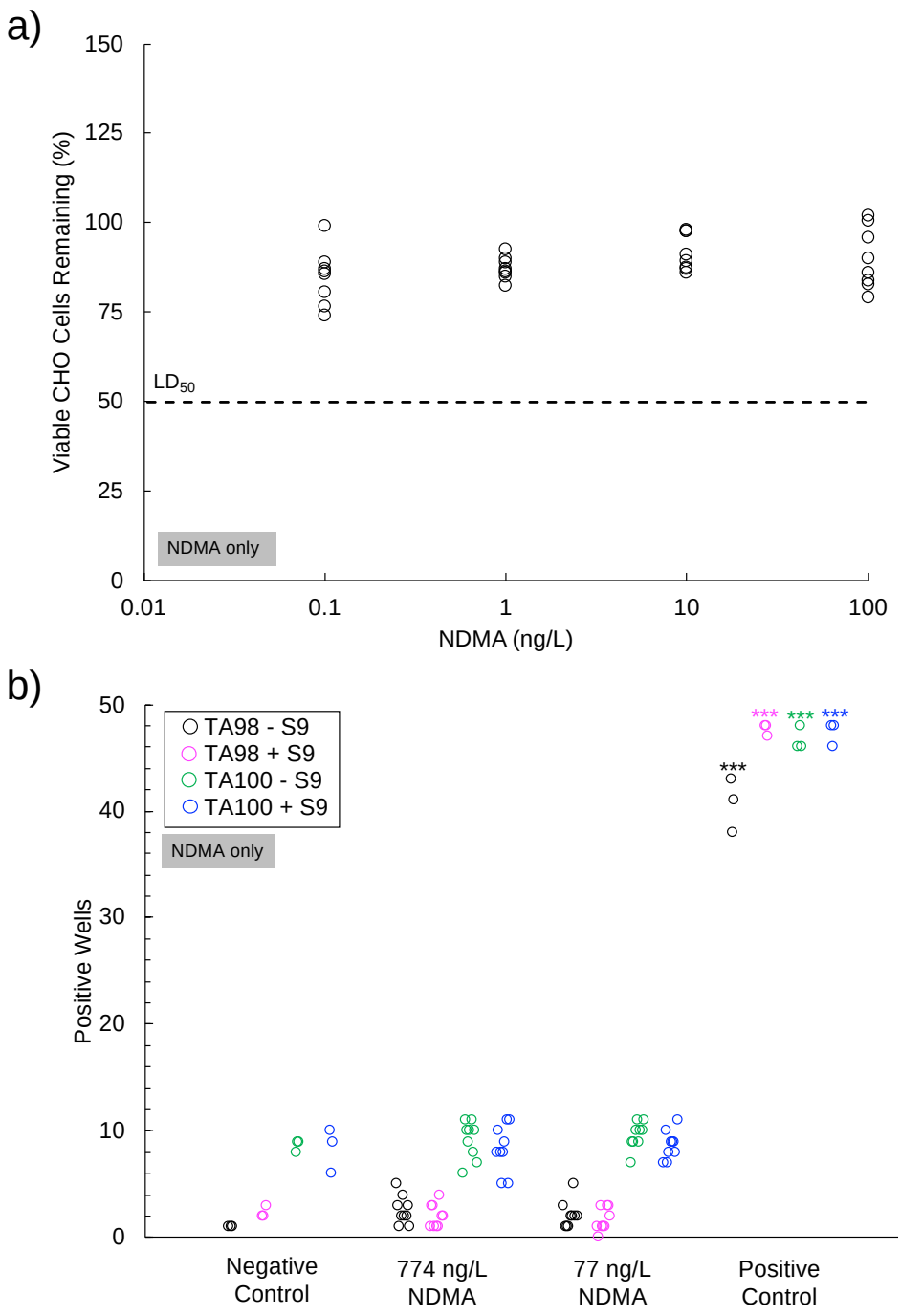

Figure 3-9. Bioassay results for *N*-Nitrosodimethylamine (NDMA) only. (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

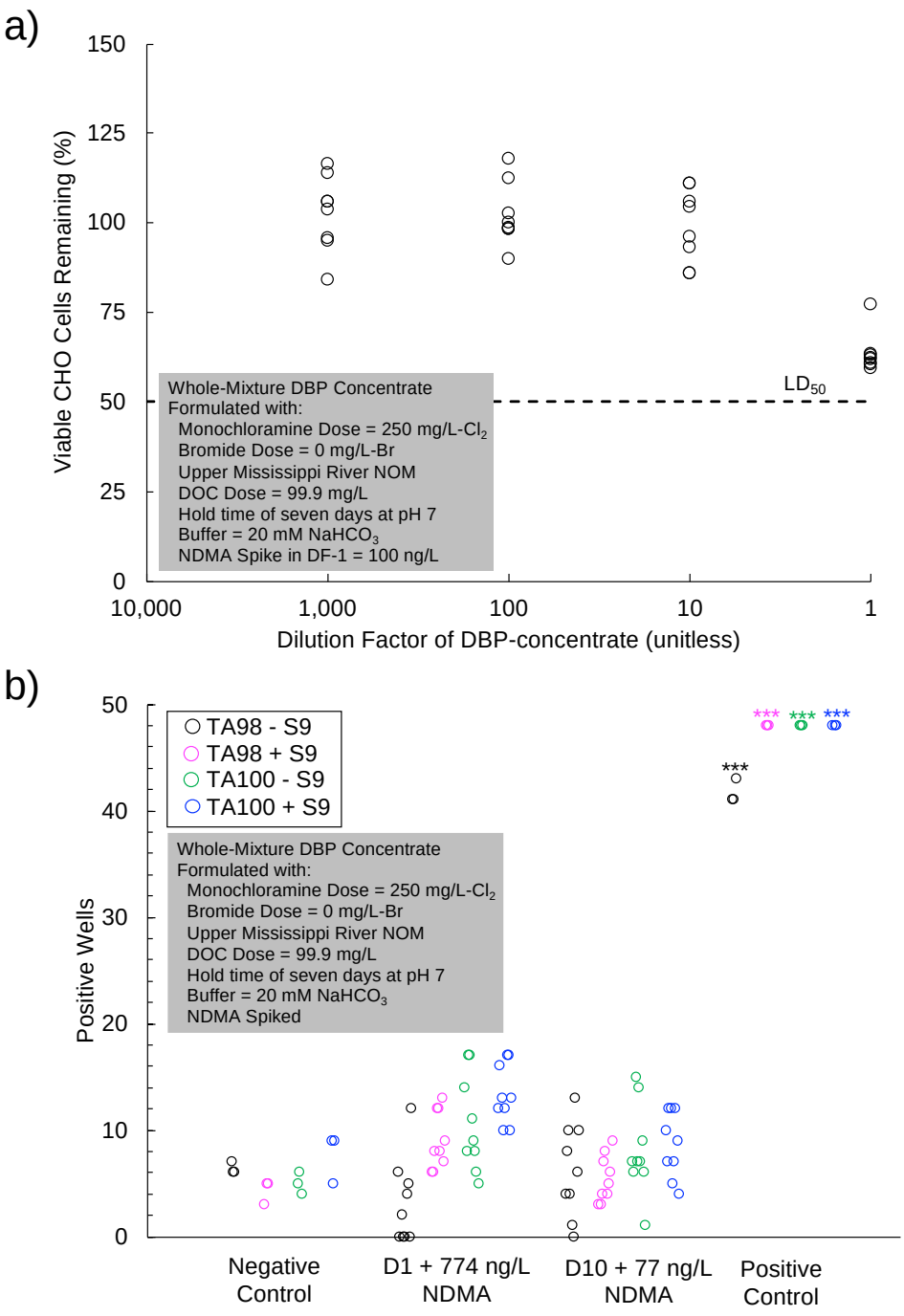

Figure 3-10. Bioassay results for a disinfection byproduct mixture-concentrate formulated by reacting 250 mg/L-Cl_2 monochloramine with 99.9 mg/L dissolved organic carbon for 7-days at pH 7 and amended with *N*-Nitrosodimethylamine (NDMA). (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

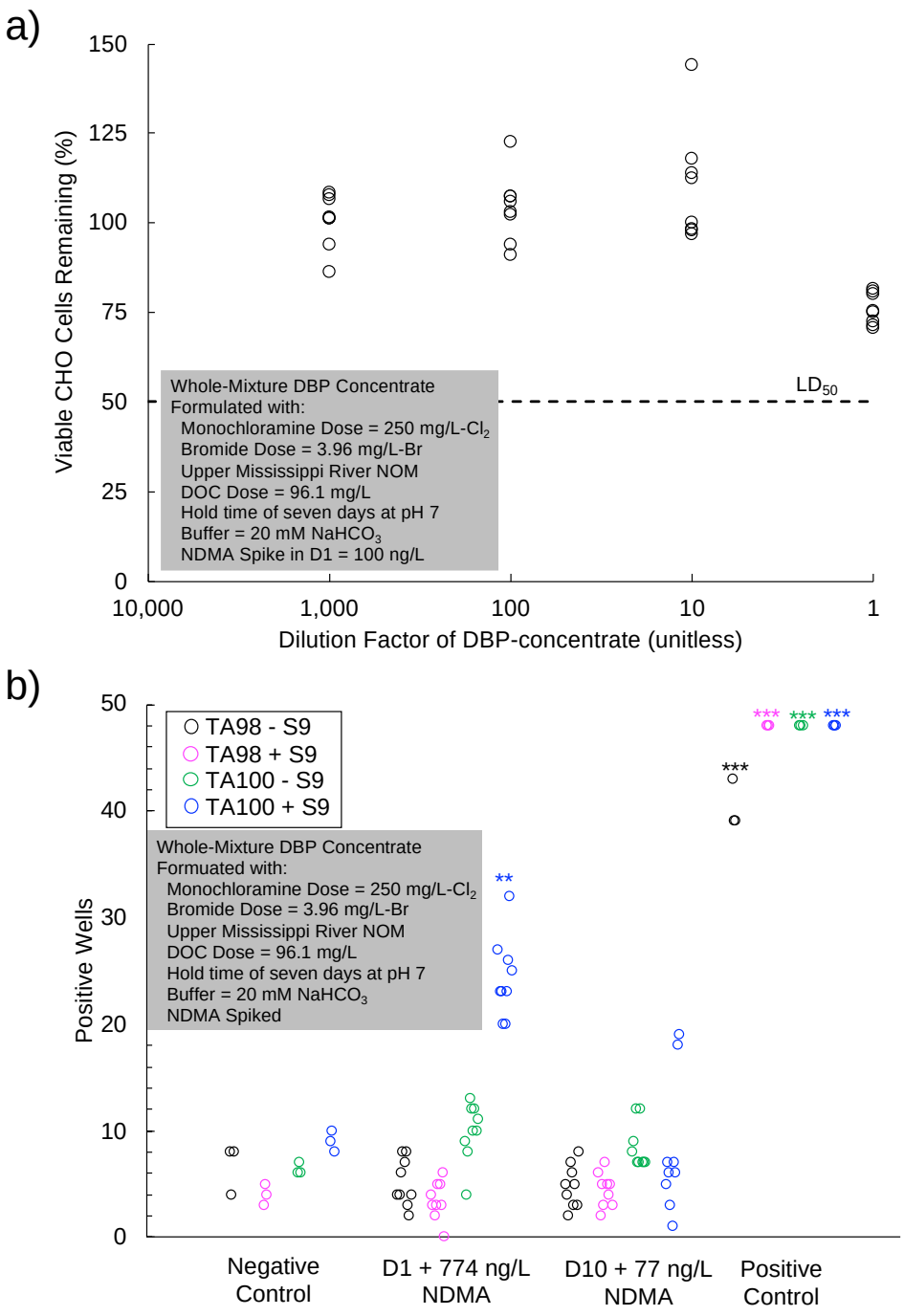

Figure 3-11. Bioassay results for a disinfection byproduct mixture-concentrate formulated by re-acting 250 mg/L-Cl_2 monochloramine with 96.1 mg/L dissolved organic carbon and 3.96 mg/L bromide for 7-days at pH 7 and amended with *N*-Nitrosodimethylamine (NDMA). (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

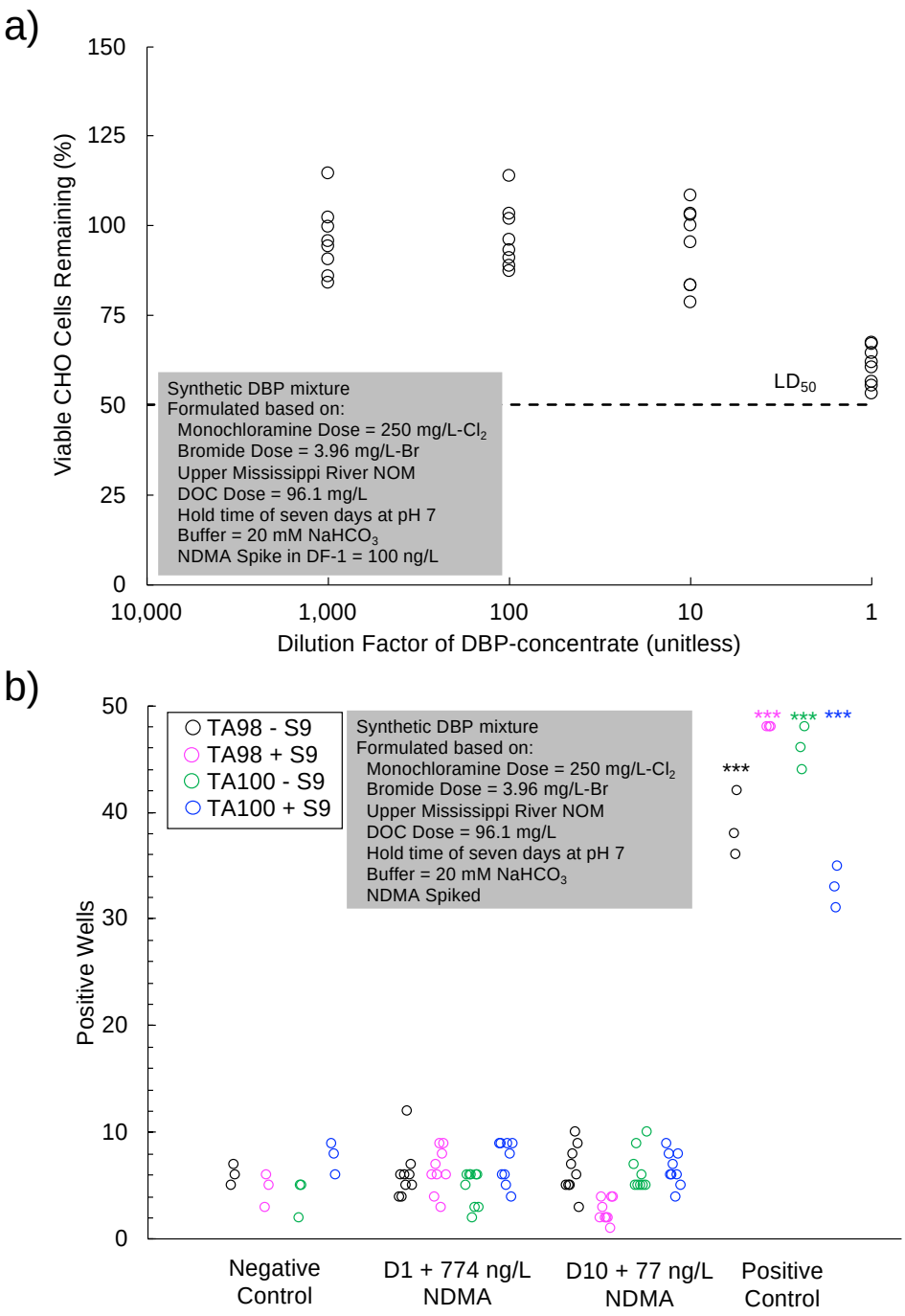

Figure 3-12. Bioassay results for a synthetic disinfection byproduct mixture-analog with the DBP profile shown in Table 3-2 corresponding to the mixture formulated by reacting 250 mg/L-Cl_2 monochloramine with 96.1 mg/L dissolved organic carbon and 3.96 mg/L bromide for 24-hours at pH 7 and amended with *N*-Nitrosodimethylamine (NDMA). (a) cytotoxicity bioassay with Chinese Hamster Ovary cells and (b) Ames test with TA-98 and TA-100 bacteria with and without S9 rat liver enzyme. Asterisk(s) indicate the number of positive wells is greater at the α = 0.01 significance-level than the *Negative Control only, **Dilution-10 and Negative Control, and ***Dilution-1, Dilution-10, and Negative Control.

4 Discussion

4.1 Cytotoxicity Tests with CHO Cells

At dilution factors of 10, 100, and 1,000, no cytotoxicity was exerted relative to *100% Viable CHO Cells Remaining* in any whole-mixture DBP-concentrates or their synthetic analogs (α = 0.01, Figures 3-1a to 3-12a). However, the following undiluted (e.g., dilution factor = 1) whole-mixture DBP-concentrates exerted some cytotoxicity, with the percentage of viable CHO cells remaining crossing the LD_{50} in at least one of the eight observations:

- 60 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose (Figure 3-1a)
- 218 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose (Figure 3-2a)
- 218 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose with 4 $mg \cdot L^{-1}$ bromide (Figure 3-4a)

Among the full-strength dilutions, the whole-mixture DBP-concentrates formulated using free chlorine were ***not*** more cytotoxic than those formulated with monochloramine (α = 0.01). The addition of 100 $ng \cdot L^{-1}$ NDMA did not increase the cytotoxicity (α = 0.01) of the whole-mixture DBP-concentrates formulated with monochloramine (Figures 3-6a vs. 3-10a) or monochloramine with bromide (Figures 3-7a vs. 3-11a). Comparison the full-strength whole-mixture DBP-concentrates to their synthetic analogs showed no difference in cytotoxicity (α = 0.01) in the following cases:

- 218 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose (Figure 3-2a vs. Figure 3-3a)
- 218 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose with 4 $mg \cdot L^{-1}$ bromide (Figure 3-4a vs. Figure 3-5a)
- 250 $mg \cdot L^{-1}$ as Cl_2 monochloramine dose with 4 $mg \cdot L^{-1}$ bromide and 100 $ng \cdot L^{-1}$ NDMA (Figure 3-11a vs. Figure 3-12a)

In contrast, for 250 $mg \cdot L^{-1}$ as Cl_2 monochloramine dose with 4 $mg \cdot L^{-1}$ bromide, the synthetic analog mixture was more cytotoxic (α = 0.01) than its whole mixture counterpart (Figure 3-7a vs.

Figure 3-8a). This result suggests mild antagonism in the whole mixture. On balance, however, these results indicate that the suites of commercially-available DBPs in the synthetic analog mixtures (see Table 3-2) *could* account for the observed cytotoxicity in the whole-mixture DBP-concentrates. The specific drivers of cytotoxicity cannot be determined from these data, whether it stems from specific trihalomethanes, haloacetonitriles, or haloacetic acids, and/or synergistic interactions amongst these DBPs. Further, these data support the conclusion that there were no significant differences in cytotoxicity in the DBP mixtures formulated with the two disinfectant types (free chlorine vs. monochloramine), bromide levels (0 vs. 4 $mg \cdot L^{-1}$), and for monochloramine only, NDMA levels (0 vs 100 $ng \cdot L^{-1}$).

4.2 Ames Mutagenicity Tests with *Salmonella typhimurium*

Ames tests were completed using TA-98 and TA-100 in the absence and presence of S9 rat liver enzyme, including seven tests on whole-mixture DBP concentrates and five tests on synthetic DBP analog mixtures (Figures 3-1b through 3-12b). All tests were valid, with ten or less revertant wells in the negative controls and thirty to forty-eight revertant wells in the positive controls. For TA-98, the number of revertant wells induced by the Dilution-1 and Dilution-10 mixtures were *not* significantly greater than their corresponding negative controls ($\alpha = 0.01$), indicating frameshift mutations in *his*D3052 were not produced by the DBP-concentrates or synthetic analogs. This result is similar to that of other researchers that have used TA-98 to assess the mutagenicity of DBPs (Gong et al., 2005; Manasfi et al., 2017), although different DBP species were assessed in these studies.

For TA-100, the Dilution-10 mixtures did not produce a significant number of revertant wells compared to their corresponding negative controls ($\alpha = 0.01$). In contrast, however, the Dilution-1 whole-mixture DBP-concentrates produced a significant number of revertant wells in TA-100 in the absence and presence of S9 in eleven out of fourteen cases (see Table 4-1). This

indicates a dose-response relationship and a base-substitution mutation in *his*G46 (Hengstler and Oesch, 2001) in the following whole-mixture DBP concentrates:

- 60 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose (Figure 3-1b)
    - *less* mutagenic with S9
- 218 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose (Figure 3-2b)
    - *less* mutagenic with S9
- 218 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose with 4 $mg \cdot L^{-1}$ bromide (Figure 3-4b)
    - *more* mutagenic with S9
- 250 $mg \cdot L^{-1}$ as Cl_2 monochloramine dose (Figure 3-6b)
    - *less* mutagenic with S9
- 250 $mg \cdot L^{-1}$ as Cl_2 monochloramine dose with 4 $mg \cdot L^{-1}$ bromide (Figure 3-7b)
    - *less* mutagenic with S9
- 250 $mg \cdot L^{-1}$ as Cl_2 monochloramine dose with 4 $mg \cdot L^{-1}$ bromide and 774 $ng \cdot L^{-1}$ NDMA (Figure 3-11b)
    - *more* mutagenic with S9

In four out of these six cases, the whole-mixture DBP concentrates were less mutagenic with S9 ($\alpha = 0.01$), in agreement with the findings of others (Bull, 1982). In the two aberrant cases in which the whole-mixture DBP concentrates were more mutagenic with S9 ($\alpha = 0.01$), bromide was added prior to oxidant addition to form bromine-substituted DBPs. As shown in Table 4-1, the median number of revertant wells was greater in the presence of S9 in the whole-mixture DBP concentrates formulated with a (1) 218 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose and 4 $mg \cdot L^{-1}$ bromide and (2) 250 $mg \cdot L^{-1}$ as Cl_2 monochloramine dose, 4 $mg \cdot L^{-1}$ bromide, and 774 $ng \cdot L^{-1}$ NDMA. The addition of S9 is intended to simulate mammalian metabolism, so this result implicates bromine-substituted DBPs in driving mutagenicity in whole-mixture DBP concentrates. Table 4-1 summarizes the median number of revertant wells in the synthetic DBP analog mixtures

formulated using commercially-acquired DBPs using the recipes shown in Table 3-2. In nine out of the ten cases, the synthetic DBP analog mixture did not induce mutagenicity in TA-100 (α = 0.01).

Table 4-1. Median number of revertant wells for Dilution-1 in TA-100 with and without S9

Disinfectant	Dose $mg \cdot L^{-1}$-Cl_2	Bromide $mg \cdot L^{-1}$	NDMA $ng \cdot L^{-1}$	S9	Revertant Wells[1] Whole Mixture	Revertant Wells[1] Synthetic Analog*
Free Chlorine	60	0	0	No	20 (3-1)	NM
				Yes	14 (3-1)	NM
	218	0	0	No	18 (3-2)	4 [ns] (3-3)
				Yes	12 (3-2)	3 [ns] (3-3)
	218	4	0	No	16 (3-4)	8 [ns] (3-5)
				Yes	27 (3-4)	9 (3-5)
Monochlora-mine	250	0	0	No	18 (3-6)	NM
				Yes	11 (3-6)	NM
	250	4	0	No	16 (3-7)	5 [ns] (3-8)
				Yes	10 (3-7)	9 [ns] (3-8)
	250	0	774	No	9 [ns]	NM
				Yes	13 [ns]	NM
	250	4	774	No	10 [ns] (3-11)	6 [ns] (3-12)
				Yes	23 (3-11)	8 [ns] (3-12)
None	NA	0	774	No	NA	10 [ns] (3-9)
				Yes	NA	8 [ns] (3-9)

[1]Values without superscript ns were greater than the negative control at the α = 0.01 significance level
*formulated with commercially-acquired DBPs and concentrations shown in Table 3-2
[ns] not significantly greater than the negative control at the α = 0.01 significance level
NA – not applicable
Number is parentheses (3-x) refer to the corresponding figure number

The lone exception was the synthetic DBP analog mixture formulated based on the 218 $mg \cdot L^{-1}$ as Cl_2 free chlorine dose and 4 $mg \cdot L^{-1}$ bromide with a median of nine revertant wells which was determined to be statistically greater than the corresponding negative control (Figure 3-5). However, there were 27 revertant wells in the corresponding whole-mixture DBP concentrate (Table 4-1 and Figure 3-4), which was the most mutagenic mixture evaluated in this research.

Therefore, the commercially-acquired DBPs – including four trihalomethanes, three dihaloacetonitriles, 1,1-dichloro-2-propanone, chloropicrin, nine haloacetic acids, and dalapon – at their respective concentrations similar to the whole-mixture DBP concentrates (Tables 3-1 and 3-2) and their associated synergistic interactions with and without exogenous NDMA ***do not*** account for the observed mutagenicity in TA-100. Rather, unidentified and/or unknown DBPs and their associated interactions with each other and/or the identified DBPs (Table 3-2) are likely driving the observed mutagenicity.

NDMA at 774 $ng \cdot L^{-1}$ impacts mutagenicity, as shown in Table 4-1 and by comparing Figures 3-7b, 3-9b, and 3-11b. NDMA by itself is not mutagenic (Figure 3-9b), but when NDMA is added to the whole-mixture DBP concentrate formulated with monochloramine and bromide (Figure 3-11b), TA-100 with S9 has a significant number of revertant wells. In the absence of NDMA addition (Figure 3-7b), both TA-100 with and without S9 have a significant number of revertant wells compared to the negative control ($\alpha = 0.01$). Direct comparisons of the Dilution-1 dose for TA-100 in Figure 3-7b (monochloramine + bromide) with that in Figure 3-11b (monochloramine + bromide + NDMA) indicate NDMA is an antagonistic mutagen without S9 and synergistic mutagen with S9. This observation of NDMA as an antagonistic mutagen is also true for the whole-mixture DBP concentrates formulated with monochloramine only (no bromide), as shown by comparing Figures 3-6b, 3-9b, and 3-10b. For TA-100 without S9, the whole-mixture DBP concentrate formulated with monochloramine only shows a statistically significant number of revertant wells ($\alpha = 0.01$, Figure 3-6b); however, when NDMA is added, the number of revertant well decreases and is no longer significantly greater than the negative control (Figures 3-10b).

Overall, these twelve Ames tests reveal whole-mixture DBP concentrates formulated with free chlorine and monochloramine induce mutagenicity in TA-100 only. The presence of bromide enhances mutagenicity in mammalian metabolisms in whole-mixture DBP concentrates

formulated with free chlorine, but, for monochloramine the presence of bromide enhances mutagenicity only when exogenous NDMA was also added. NDMA impacts mutagenicity, acting antagonistically in TA-100 without S9 and synergistically with monochloramine and bromide with S9. The characterized DBPs – including four trihalomethanes, three dihaloacetonitriles, 1,1-dichloro-2-propanone, chloropicrin, nine haloacetic acids, and dalapon – and their respective interactions do not account for the observed mutagenicity. Therefore, future studies should include more advanced characterizations of whole-mixture DBP concentrates in an attempt to characterize a greater number of DBPs, with particular attention paid to brominated species. This will facilitate assessment of synthetic analog mixtures with a greater number of DBP species and hence a better chance of identifying the drivers of mutagenicity in whole-mixture concentrates.

5 Conclusions and Recommendation for Future Work

The conclusions of this research based on the results and discussion of the DBP bioassays are:

- The undiluted whole-mixture DBP concentrates were cytotoxic to CHO cells and approached or reached the LD50 in most cases; no statistically-significant trends were observed with disinfectant type (free chlorine or monochloramine), bromide addition, or exogenous NDMA addition; similar cytotoxicity was observed in the corresponding synthetic DBP analog mixtures indicating the cytotoxicity could be attributed to the commercially-acquired DBPs in these mixtures, which included four trihalomethanes, three dihaloacetonitriles, 1,1-dichloro-2-propanone, chloropicrin, nine haloacetic acids, dalapon, and NDMA.
- For TA-98, the number of revertant wells in the Dilution-1 and Dilution-10 mixtures were *not* significantly greater than their corresponding negative controls ($\alpha = 0.01$), indicating frameshift mutations in *his*D3052 were not produced by the DBP-concentrates or synthetic analog mixtures.
- For TA-100, the number of revertant wells in the Dilution-1 mixtures were significantly greater than their corresponding negative controls in the DBP concentrates formulated with free chlorine and monochloramine, indicating a base-substitution mutation in *his*G46.
    - The presence of bromide enhances mutagenicity in mammalian metabolisms in whole-mixture DBP concentrates formulated with free chlorine, but, for monochloramine, the presence of bromide enhances mutagenicity only when exogenous NDMA was also added.
    - NDMA impacts mutagenicity, acting antagonistically in TA-100 without S9 and synergistically with monochloramine and bromide with S9.

    - The characterized DBPs – including four trihalomethanes, three dihaloacetonitriles, 1,1-dichloro-2-propanone, chloropicrin, nine haloacetic acids, and dalapon – and their respective interactions do not account for the observed mutagenicity.
- Future studies should include more advanced characterizations of whole-mixture DBP concentrates to reveal a greater number of DBPs, with particular attention paid to brominated species. Assuming these DBPs are commercially-available, this will facilitate assessment of synthetic analog mixtures with a greater number of DBP species and hence a better chance of identifying the drivers of mutagenicity in whole-mixture concentrates.

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