Evaluating the Toxicity and Formation of Halobenzoquinones in Point-of-Use Chlorinated Drinking Water

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EVALUATING THE TOXICITY AND FORMATION OF HALOBENZOQUINONES IN POINT-OF-USE CHLORINATED DRINKING WATER

A Thesis Presented

By

STEPHANIE HUNG

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

September 2018

Environmental Health Science
EVALUATING THE TOXICITY AND FORMATION OF HALOBENZOQUINONES IN POINT-OF-USE CHLORINATED DRINKING WATER

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To my dear friend

Jonathan Shaw
ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincerest gratitude to my advisor Dr. Krystal J. Godri Pollitt for challenging me to do laboratory research and providing endless support for my Master’s thesis research along the way. I would not have come this far if it were not for her patience, encouragement, ambition and wealth of knowledge. I would like to extend my gratitude to my committee members Dr. Emily Kumpel, for her continuous support and guidance in a field of research that was completely foreign to me, Dr. Alicia R. Timme-Laragy, for her expertise and willingness to teach and share her knowledge over the past few years, and Dr. Timothy E. Ford, for his invaluable advice and support. Thanks are also due to Dr. Niveen S. Ismail and Dr. David A. Reckhow for their generosity and research guidance.

I would like to thank Aarthi Mohan, Dr. Ahmed Khalil, Dr. Rassil Sayess, Michael J. Young, and Mimi Alkattan for their knowledge and assistance with experiments. Special thanks to Patrick Wittbold for his help with water sampling, especially during frigid temperatures.

I wish to express my deep appreciation to my husband, Benjamin, for being by my side. I am grateful for his love, support, humor, and incredible efforts to always make sure I was taken care of. Last, but certainly not least, I want to thank my mom, Anne, for her love, encouragement, good laughs, long phone conversations, and hugs.
ABSTRACT
EVALUATING THE TOXICITY AND FORMATION OF HALOBENZOQUINONES IN POINT-OF-USE CHLORINATED DRINKING WATER
SEPTEMBER 2018
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Chlorine has effectively reduced the prevalence of waterborne diseases, however there are secondary consequences to this public health advancement. Disinfection by-products (DBPs) are chemicals formed when chlorine reacts with natural organic matter (NOM) in water. A new class of DBPs, halobenzoquinones (HBQs), has recently been identified and data suggests it could be potentially carcinogenic and up to 1000 times more toxic than some regulated DBPs. So far, in vitro studies have assessed HBQ toxicity without taking into account its transformation in cell media into potentially less toxic compounds. This study evaluated the toxic effects of one HBQ, 2,6-DCBQ, and its transformed derivatives on colon epithelial and liver hepatoma cell lines by measuring intracellular reactive oxygen species production and cell viability post-DCBQ exposure. In addition, to better quantify the trade-off between exposure to waterborne pathogens and 2,6-DCBQ, the inactivation of a virus indicator (MS2), and formation of DCBQ were determined in chlorinated surface waters. Dose-dependent toxic effects were observed in both cell lines and transformed DCBQs were observed to be less toxic than their parent
compound. MS2 inactivation occurred immediately post-chlorination, but DCBQ was detected simultaneously. Such findings indicate that this compound is toxic to human cells, including colon epithelial cells, which may be pertinent due to the possible association between chlorinated waters and colon cancer. Findings also suggest this DBP may be relevant in developing countries because HBQs may form in point-of-use chlorinated drinking waters. Furthermore, observed reduction in toxicity of media-transformed DCBQs calls current literature on HBQ toxicity into question.
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CHAPTER 1
LITERATURE REVIEW

1.1 Background

Chlorine is the most common and widely-used water disinfectant in the U.S., used to control microbial activity and remove inorganic compounds and certain heavy metals from water (CDC, 2016). Chlorination in water treatment systems was a significant public health advancement (Gordon et al., 1987) and first used in the early 1900s. The technique was popularized by the Pan American Health Organization (PAHO) and the U.S. Centers for Disease Control and Prevention (CDC) as part of the Safe Water Systems (SWS) program in the 1990s in the midst of the cholera epidemic in Latin America. Chlorination has since effectively reduced the incidence and prevalence of waterborne diseases (CDC, 2016). However, during the disinfection process, chlorine reacts with water to produce hypochlorous and hydrochloric acids, which further react with compounds in water, including natural organic matter (NOM), to generate disinfection byproducts (DBPs). Only a fraction of the suspected number of DBPs have been identified (Li et al., 2015), which are mostly regulated by governmental and international agencies, including the WHO. Prominent classes of DBPs that are currently regulated in the U.S. are chlorite, bromate, total trihalomethanes (TTHMs) and some haloacetic acids (EPA, 2005). Several studies have linked exposure to regulated DBPs to adverse health outcomes (Li et al., 2015; Lyon et al., 2014; Villanueva et al., 2007).
Halobenzoquinones (HBQs) are a recently identified class of DBPs found in various chlorinated waters, including swimming pools, water treatment plants and water distribution systems in the U.S. and Canada (Zhao et al., 2010; Zhao et al., 2012; Wang et al. 2013; Du et al., 2017). HBQs have been estimated to be 300 times more toxic than chloroform and up to 1000 times more toxic than other regulated DBPs (Hrudey & Charrois, 2012; Li et al., 2015; Zuo et al., 2017). However, HBQs, which are halogen substituted cyclic diones, are unstable in water at ~pH 7 and readily undergo hydrolysis, forming hydroxyl-HBQs (OH-HBQs) (Zhao et al., 2012; Wang et al., 2014; Du et al., 2017)—a potentially less toxic compound. A variety of factors, including physiochemical characteristics of the water, including temperature, pH, chlorine levels, NOM levels, and amino acid composition (Qin et al., 2010; Mohan 2015; Du et al., 2017) may impact HBQ transformation. Four OH-HBQs (OH-DCBQ, OH-DCMBQ, OH-TriCBQ, and OH-DBBQ) have been detected in five water treatment plants (Wang et al., 2014). Their foundational structure, 1,4-benzoquinone, is a known carcinogen (Figure 1).

**Figure 1.** Structures of various HBQs detected in treated surface waters, water treatment plants and distribution systems in the U.S. and Canada.
Not all HBQs have been studied as DBPs; one HBQ, tetrachloro-1,4-benzoquinone (TCBQ), has been studied as a toxicant produced during industrial processes. TCBQ is a metabolite of pentachlorophenol (PCP), which was a widely used wood preservative and biocide by the lumber industry in the United States and internationally. Tight regulations on PCP were implemented in the United States in the mid-1980s due to well-documented adverse health effects associated with exposure to this toxicant. TCBQ has also been linked to adverse health effects and known to cause DNA damage.

A literature review on the mechanisms of HBQ toxicity was conducted. Cytotoxicity, oxidative stress, and DNA damage, as well as potential health outcomes from HBQ exposure have been explored. This review provides insight into informational gaps and unanswered questions on HBQ toxicity and health-relevant future directions of research.

1.2 Cytotoxicity

Cytotoxicity is a crude parameter of toxicological testing that measures the toxicity of a compound based on its potential to reduce cell viability and/or proliferation. The cytotoxicity of various HBQs has been evaluated in vitro using Chinese Hamster Ovary (CHO-K1), human colon carcinoma (Caco-2), and bladder carcinoma (T24) cell models. The human neural stem (hNSCs) and embryonic kidney 239 (HEK239) cell lines, as well as the Luminous bacterium Q67, have also been used to evaluate HBQ cytotoxicity (Fu et al., 2016; Du et al., 2017; Li et al., 2017). Several isomer- and halogen-specific HBQs have been researched to date: 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), 2,5-dichloro-1,4-benzoquinone (2,5-DCBQ), 2,6-dibromo-1,4-benzoquinone (2,6-DBBQ), 2,5-dibromo-1,4-benzoquinone (2,5-DBBQ), 2,6-tribromo-1,4-benzoquino-
none (TBBQ), 2,3,6-trichloro-1,4- benzoquinone (TriCBQ), tetrachloro-1,4- benzoquinone (TetraCBQ), 2- chloro-1,4-benzoquinone (2-CBQ), 2,3-diiodo-BQ (2,3-DIBQ), tetrafluoro-1,4-benzoquinone (TFBQ), tetrabromo-1,4-benzoquinone (TBBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), 3-hydroxy-2,6-dichloro-1,4-benzoquinone (OH-DCBQ), 5-hydroxy-2,3,6-trichloro-1,4-benzoquinone (OH-TriCBQ), 6-hydroxy-3,5-dichloro-2-methyl-1,4-benzoquinone (OH-DCMBQ), and 3-hydroxy-2,6-dibromo-1,4-benzoquinone (OH-DBBQ).

Exposure to increased HBQ concentrations has been positively correlated with decreased cell viability (Li et al., 2014, 2015, 2016; Prochazka et al., 2015; Fu et al., 2017). Real-time cell analysis (RTCA)/ real-time cell electronic sensing (RT-CES), as well as the methyltetrazolium salt (MTS) and lactate dehydrogenase (LDH) assays have been used to evaluate HBQ cytotoxicity following 12, 24 and 60-hour exposure periods. The MTS and LDH assays were also used to explore whether HBQ-induced cytotoxicity can be mitigated by treatment with an external source of antioxidants. Investigators selected to use T24 cells, given the suspected association between HBQs and bladder cancer and prior use of T24 cells in DBP toxicity testing (Du et al., 2013; McGuigan dissertation, 2014;). A concentration-dependent (HQB range: 1 to 500 µM) decrease in cell viability was reported following a 24-hour exposure. T24 cells treated with HBQ in the presence of an antioxidant (1mM N-acetyl-L-cysteine, NAC or 10mM glutathione, GSH) were demonstrated to exhibit decreased cytotoxicity. This suggests that at least one major toxic pathway of tested HBQs (DCBQ, TCBQ, DCMBQ, DBBQ) is related to oxidative stress. HBQs have also exhibited cytotoxic effects on undifferentiated human neural stem hNSCs cells. The cell cycle of undifferentiated hNSC cells that were treated with low
doses of 2,6-DCBQ and 2,6-DBBQ (0.1 and 1 µM) for 96 hours was severely disrupted; HBQs induced S-phase cell cycle arrest at low concentration (1 µM). A 12-day exposure to low doses of HBQs also curbed cell differentiation. Cell differentiation and cycles are sensitive indicators, and the effects on these endpoints demonstrate a potential to cause cytotoxic effects at higher doses and nervous system development toxicity at lower doses. The quantitative structural toxicity relationship (QSTR) has been determined for various HBQs to assist in predicting the metabolism of compounds and the potential interactions between xenobiotics and endogenous molecules. QSTR has been estimated using various properties of HBQs, including the log octanol-water partition coefficient (log $K_{ow}$), highest occupied molecular orbital ($E_{HOMO}$), lowest unoccupied molecular orbital ($E_{LUMO}$), dipole moment, molar refractivity and molar surface area (Bull et al., 2006).

HBQs have been reported to exhibit a wide range of cytotoxicity that are cell-, isomer- and halogen-specific. Iodinated HBQs have been found to be more toxic than their brominated and chlorinated counterparts, respectively, and the number of chlorine substituents also influenced the toxicity of the compounds (Table 1). Different cell types had varying sensitivities towards HBQs; CHO-K1 had a lower 24 h IC$_{50}$ compared to that of T24 and Caco-2 cells after being exposed to DCBQ and DBBQ. Other parameters, such as steric properties, were also positively associated with cytotoxicity. These findings could elucidate the ability of different HBQs to induce toxicity, including oxidative stress.
1.3 Oxidative Stress

1.3.1. ROS production & glutathione pathway

Oxidative stress is a mechanism of HBQ-induced toxicity. Excessive production of reactive oxygen species (ROS) can tip the redox balance within cells and induce a state of oxidative stress. The intracellular antioxidant defense system may be perturbed or compromised by HBQs and other \( \rho \)-benzoquinones via the production of semiquinones—free radicals that can directly react with oxygen to form ROS (Li et al., 2015; Li et al., 2018) (Figure 2). Studies have reported HBQs directly and indirectly form ROS (Zhu et al., 2007; Li et al., 2018). It has been postulated that HBQ-induced oxidative stress affects the glutathione pathway. GSH is an abundant non-enzymatic antioxidant that plays a major role in the detoxification process of xenobiotics by acting as a strong reducing agent (Tedeschi et al., 1990). GSH is catalyzed by glutathione S-transferase (GST) when forming conjugates for metabolism and is readily oxidized to glutathione disulfide (GSSG).
Figure 1.2. Semiquinone and oxygen radical formation from HBQ. One- and two-step electron reduction of benzoquinone to semiquinones.

The effect of HBQs on overall ROS production and intracellular GSH levels has been studied in T24 (Du et al., 2013; Li et al., 2014; Xu et al., 2018), CHO-K1 (Li et al., 2016), and HepG2 cells (Wang et al., 2018), as well as reaction mixtures (Zhu et al., 2011). Wang et al., (2018) deduced that HBQs (2,6-DCBQ, DCMBQ, TriCBQ, 2,6-DBBQ) activate the glutathione reaction pathway via formation of halosemiquinone free radicals, Michael addition of GSH, GSH substitution of the chlorine groups, and methyl-substitution (in DCMBQ). Decreases in cell proliferation were correlated with decreased GSH levels following treatment with individual HBQs (2,3-DIBQ, 2,5-DCBQ, 2,6-DCBQ, 2,5-DBBQ, 2,6-DBBQ, 2-CBQ, TriCBQ, TBBQ). Exposure to these eight HBQs was reported to increase ROS production and decrease GSH levels, suggestive of an oxidative stress response. T24 cells exposed to HQBs were also found to have decreased viability, similar to when they were treated with a GSH inhibitor, buthionine sulfoximine. However, an increase in cell viability occurred afterwards when cells were treated with exogenous sources of GSH. Significant increases in GST and glutathione peroxidase (GPx)—an enzyme involved with the detoxification of peroxides—activity were also observed, further supporting oxidative stress as a key mechanism of HBQ-induced cytotoxicity. Both CHO-K1 and T24 cells exhibited an increase in intracellular ROS levels, measured with fluorescent probe 2’7’-dichlorofluorescein (DCFH-DA). CHO-K1 experienced varying levels of ROS production: following a 12-hour exposure, the highest fluorescence intensity, indicating ROS production, was observed for all tested HBQs and then decreased across all test compounds when a 24-hour exposure period was evaluated (Li et
al., 2016). In another study, GSH conjugates with 2,6-DCBQ, TriCBQ and 2,6-DBBQ (36 – 85 µM) were identified in HepG2 cells post-exposure (Wang et al., 2018) using liquid chromatography mass spectroscopy (LC/MS). The analysis showed that GSH:HBQ ratios formed intracellularly were the same as those formed in reaction mixtures containing HBQs and GSH, without cells. These findings suggest that the mechanisms behind GSH-mediated detoxification of HBQs in reaction mixtures (halosemiquinone free radical formation, Michael addition of GSH and HBQ chlorine substitution) may be a mechanistic explanation for the toxic action of HBQs on glutathione pathway within cells.

Zhu et al. (2001) observed the potential of HBQs to form hydroxyl radicals when combined with H₂O₂— independent of their traditional metal ion reaction synthesis. Zhu et al. used electron spin resonance (ESR), a method extensively used to determine the presence of lone electrons characteristic of free radicals, and the spin-trapping agent 5,5-dimethyl-1-pyroline N-oxide (DMPO), to identify adducts formed as a result of the generation of the hydroxyl radical. After combining TCBQ and H₂O₂, the resulting ESR spectra revealed that the two chemicals form hydroxyl radicals and a variety of free radicals known to be downstream products or adducts of the highly unstable hydroxyl radicals. Further ESR analyses using other HBQs showed the formation of hydroxyl radicals with 2,5-dichloro and 2-chloro-1,4-benzoquinone being more efficient in generating hydroxyl radicals than TCBQ.

In addition to being time-dependent, ROS formation also appears to be a function of isomer type and halogen substitution groups: 2,5-HBQs demonstrated increased toxicity compared to their 2,6-HBQ counterparts and the highest levels of ROS were measured for iodo-HQBIs followed by bromo-HBQs and then chloro-HBQs (Li et al., 2016).
In summary, studies have shown that HBQs likely activate the oxidative stress pathway via nucleophilic attack against GSH or by reacting with other molecules to create free radicals and other ROS within the cell cytosol. An inverse association has been reported between HBQ exposure and intracellular level of GSH. This is compelling evidence in support of the GSH-mediated HBQ detoxification hypothesis.

1.3.1.1. Nrf2/ARE

The antioxidant response element (ARE)/Nrf2 signaling pathway is critical for adaptive stress response and cell damage prevention. Perturbations in the ARE/Nrf2 pathway have been associated with different types of cancers (Motohashi et al., 2004; Sporn et al., 2012). The Nrf2 transcription factor activates the ARE, which initiates Phase II metabolism. One way the ARE/Nrf2 pathway provides cellular defense to toxic insults is by upregulating antioxidant genes that govern enzymes, such as glutathione reductase (GSR) and NAD(P)H:quinone oxidoreductase 1 (NQO1). This determines the production, regeneration and utilization of endogenous GSH (Ha et al. 2006). Dysfunction of this adaptive stress response signaling pathway could lead to lower levels of intracellular GSH and induction of ARE/Nrf2-mediated oxidative stress. This pathway is important for overall cell viability and attenuation of potential HBQ-related carcinogenesis resulting from oxidative damage to DNA. Induction of the ARE/Nrf2 pathway by HBQs has been investigated using a human liver carcinoma (HepG2) cell line (Prochazka et al., 2015). Following a 15-hour exposure to 2,6-DCBQ and 2,6-DBBQ, a similar increase was found in ARE/Nrf2 pathway activity, suggesting that the ARE/Nrf2 pathway may be a target of HBQs and a mechanism of cytotoxicity.
The Nrf2/ARE and GSH pathways have both been shown to play a role in HBQ detoxification. However, results from supplementation with sulforaphane, a catalyst of the Nrf2 pathway, and catalase, an antioxidant defense enzyme, did not lessen the effects of benzoquinone-induced toxicity (Philbrook et al., 2015), contradicting previously mentioned findings that show antioxidant co-incubation curtailing the toxic responses of cells. Further exploration on Nrf-2/ARE- and GSH-mediated detoxification of HBQ is needed.

1.4 Genotoxicity

1.4.1. P53 Activation

The tumor suppressor gene, p53, responds to DNA damage and is an important tumor formation inhibitor. The p53 transcription factor triggers a series of intracellular events that protect DNA and preserves genome stability (Zilfou & Lowe, 2009). Activation of the p53 pathway may interfere with the normal functioning of DNA repair, cell cycle arrest, senescence, and apoptosis processes (Bates et al., 1998). Modification of the p53 pathway has been linked with various types of cancer. HBQs (2,5-DBBQ, 2,6-DBBQ, 2,5-DCBQ, 2,3-DIBQ) have been reported to increase p53 protein levels in CHO-K1 cells following exposure periods of 12 or 24 hours (Li et al., 2016). Prochazka et al. (2015) reported a larger increase in activation of p53 than the ARE/Nrf2 pathway, which suggests that oxidative stress may not be the only mechanism of cytotoxicity, consistent with findings from Li et al. (2016).

1.4.1.1. 8-hydro-2’-deoxyguanosine (8-OHdG) formation

8-hydro-2’-deoxyguanosine (8-OHdG) is commonly used biomarker of oxidative stress-related DNA damage and early signs of carcinogenesis (Musarrat et al., 1996; Pil-
Several studies have evaluated the association between oxidative DNA damage following quinone and benzoquinone exposure and 8-OhdG formation (Dalhaus et al., 1996; Lin et al. 2001(b); Dong et al., 2014; Xu et al., 2017, 2018). HBQs have been demonstrated to increase 8-OhdG levels in a concentration dependent manner in in vitro studies (Yin et al., 2013; Du et al., 2013; Li et al., 2016; Xu et al., 2018). CHO-K1 cells exhibited this effect after being treated with four HBQs individually (TriCBQ, 2-CBQ, DIBQ, TetraCBQ) for 12 and 24 hours (Li et al., 2016). Following a 24-hour exposure, DCMBQ and DCBQ were found to be the most potent oxidants in T24 cells compared to DBBQ, TCBQ, and 2,6-tribromo-1,4-benzoquinone (TBBQ) (25 – 125 µM) (Du et al., 2013; Xu et al., 2018). Complete toxicity ranking of HBQs are shown in Table 1.

HBQs, such as TFBQ, also may have the ability to modify other amino acids. A study found that the phenolic hydroxyl group of tyrosine residue readily forms conjugates with polyhalogenated quinones (Qu et al., 2016). Co-incubation yielded the formation of fluoroquinone-O-tyrosine conjugates along with their hydroxylated derivatives and the inhibition of tyrosine phosphorylation at low doses of TFBQ (50 nM). This could have significant implications for protein function and signaling but has yet to be tested in vitro.

1.4.1.1.1. DNA alkylation

Changes in DNA alkylation induced by halogenated DBPs and haloquinones have been reported in several studies (Tao et al. 2005; Coffin et al., 2000; Salas et al., 2015). Furthermore, DNA methylation changes have been observed in cells post-HBQ treatment. The cytosine of cytosine-phosphate-guanine (CpG) islands on DNA strands are
mostly methylated in mammalian cells. However, hypomethylation of CpG islands leading to the formation of 5hmC have been documented. This is significant when evaluating carcinogenicity because hypomethylation of cytosine and the 5hmC cancer biomarker have been detected in cancer cells and been shown to cause the activation of oncogenes (Hur et al., 2014). Human fetal lung fibroblast cells (MRC-5), human lung adenocarcinoma cells (A549) and HepG2 cells exhibited a two-fold increase in levels of 5hmC (from 5mC) post-HBQ exposure (CBQ, TBrBQ, DCBQ, DBBQ). A549 cells exhibited hypomethylation changes when exposed to HBQs.

In addition, MRC-5 cells had 5hmC-mediated significantly enriched genes that were important for key biological functions. Zhao et al. (2014) suggested that TCBQ-induced oxidation of 5mC was due to increased ten-eleven translocation (Tet) protein activity. This hypothesis was tested by exposing Tet1/Tet2 knockout and wild type mouse embryonic stem cells to TCBQ. Low levels of 5hmC were detected in the Tet double knockout cells, while wild type cells were found to have elevated 5hmC levels. Increased iron (II)—an important cofactor of the Tet protein—and iron storage protein levels correlated with formation of 5hmC. Based on these findings, TCBQ may have increased cellular iron (II) levels, upregulated Tet protein activity and thus, 5hmC. Most recently, this pathway was further confirmed by Li et al. (2017), who demonstrated TCBQ-induced 5hmC formation in mouse embryonic stem cells.

Although minimal evidence exists for HBQ-related DNA alkylation, several studies have documented methylation changes from other DBPs that also contain halogen substitution groups. Tao et al. (2005) determined the DNA-hypomethylation potential of several chlorinated DBPs, including trichloroacetic acid, in the kidneys of mice and rats.
Other studies reported that trihalomethanes, a tetrahedral molecule containing three halogen substitution groups, were also capable of inducing DNA methylation changes (Coffin et al., 2000; Salas et al., 2015). Although HBQs are structurally different than THMs and haloacetic acids, they share similarities in possible mechanisms of DNA damage, including formation of 8-OHdG and glutathione conjugation (Potter et al., 1996; Ross & Peagram, 2004).

1.4.1.1.1. DNA adducts

DNA adducts are considered important markers of carcinogenesis and have been researched extensively in carcinogen studies (La & Swenberg, 1996). Due to HBQs’ electrophilic nature and their potential to directly and indirectly form adducts at nucleophilic sites of DNA, HBQ-related DNA adduct formation has been investigated by a number of studies (Lin et al., 2001a; Anichina et al., 2010; Jia et al., 2010; Lai 2011; Wang et al., 2012; Huang et al., 2015). DCBQ and DBBQ have been demonstrated to have DNA adduct formation potential, which was detected between oligodeoxynucleotides (Anichina et al., 2010). A reaction mixture of DNA nucleotides was also found to accumulate over time for covalently bonded HBQs (TetraCBQ and tetrachloro-hydroquinone) (Lin et al., 2001a). TetraCBQ, TCBQ, tetrachloro-hydroquinone and tetrafluoro-1,4-benzoquinone (TFBQ) were also reported to cause DNA adduct formation (Jia, et al., 2010). The DNA adduct formation for TFBQ and TCBQ was investigated by another study using the photocurrent of a DNA intercalating agent pre- and post- HBQ exposure. Cells that were exposed to 300 μM TFBQ and TCBQ for 30 min had a drastic 40% decrease in photocurrent, indicating that DNA adduction led to degradation of DNA.
Interestingly, photoelectrochemical results showed that HBQ-induced DNA adduction was not exacerbated by presence of hydrogen peroxide. Also, semiquinones were surprisingly not found to form DNA adducts (Wangpradit et al. 2016). Further investigation into specific mechanism(s) and possible intermediary factors of HBQ-induced DNA adduction is needed.

1.4.1.1.1.1.1. DNA intercalation, strand breaks & AP sites

DNA strand breaks are considered a major indicator of cancer development risk (Khanna & Jackson, 2001). DNA single-strand breaks have been observed in several cell lines after exposure to HBQs (Lin et al., 2001(a); Lin et al., 2001(b); Jia et al., 2010; Tung et al., 2012; Dong et al., 2015). Other commonly found DNA mutations within the genome are Apurinic/apyrimidinic (AP) sites and DNA intercalation. DNA intercalation occurs when molecules are inserted between planar bases of DNA, which may lead to modulation of physiological functions. DNA intercalation, strand breaks and AP sites have pertinent implications for gene expression modification, biological pathways, and carcinogenesis.

Intercalation effects of HBQs were first reported by Yin et al. (2013) when investigating several polyhalogenated benzoquinones (range: 20 to 1320 nM) for DNA damage potential. Investigators used mass spectrometry (UPLC-ESI-MS/MS) to examine oxidation of nucleoside deoxyguanosine (dG) and DNA intercalation with HBQs in dsDNA. HBQs were found to promote the generation of hydroxyl radicals near nucleophilic sites of DNA, causing oxidation of dsDNA. The potential of HBQs to intercalate were ranked in descending order (Table 1). Increase in AP sites and DNA strand breaks have also been correlated with HBQ exposure.
Two other quinone metabolites of the PCP, tetrachlorohydroquinone (Cl₄HQ) and tetrabromohydroquinone (Br₄HQ), were also found to increase AP sites and induced DNA single-strand breaks (Lin et al., 2001a & b). An inference could be made from these findings on the genotoxic potential of HBQs. HeLa S3 tumor cells were treated with a range of HBQ concentrations (0 - 300 µM) over different exposure periods (0.5 to 2 hours) (Lin et al., 2001b). The maximum increase in AP sites was observed at 300 µM after a 0.5-hour exposure time. Calf thymus DNA was also isolated and exposed to Cl₄HQ (100 - 1000 µM) and Br₄HQ (range: 1 to 5000 µM) with and without Cu(II), an intermediate factor suspected to cause oxidation of HBQs. DNA single-strand breaks were dose-dependent with co-treatment of Cu(II).

<table>
<thead>
<tr>
<th>Rank</th>
<th>Endpoint</th>
<th>Cytotoxicity</th>
<th>Oxidative Stress</th>
<th>Nrf/ARE</th>
<th>DNA Intercalation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,3-DIBQ</td>
<td>2,3-DIBQ</td>
<td>DCMBQ</td>
<td>TBrBQ</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2-CBQ</td>
<td>2,5-DBBQ</td>
<td>2,5-DCBQ, 2,6-DCBQ</td>
<td>TCBQ</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2,5-DBBQ</td>
<td>TetraCBQ</td>
<td>TCBQ</td>
<td>TriCBQ</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>TetraCBQ, 2,5-DCBQ</td>
<td>2,5-DCBQ</td>
<td>TBBQ</td>
<td>2,5-DCBQ</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2,6-DBBQ</td>
<td>2-CBQ</td>
<td>2,3-DCBQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2,6-DCBQ</td>
<td>2,6-DBBQ</td>
<td>2,6-DCBQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>TriCBQ</td>
<td>TriCBQ</td>
<td>2-CBQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>2,6-DCBQ</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.5 Lipid and Protein Modification
HBQs have also been reported to alter protein expression. RAD51—a protein that promotes annealing of DNA strands at damaged sites (Haaf et al., 1995)—was downregulated by p53 following TCBQ exposure (Song et al., 2016). The human breast carcinoma (MDA-MB-231) cell line was exposed to 0-50 μM of TCBQ for 1, 3, 6, 12 and 24 h. Western blot analysis showed increased p53 and RAD51 interaction after immediate exposure to TCBQ, indicating DNA damage. However, this interaction decreased after 3 hours, as p53 downregulated RAD51. Perhaps this was part of its protective programmed cell cycle arrest mechanism. The p53 pathway and the role of RAD51 are crucial for maintenance of DNA integrity and abatement of key cancer precursors, thus the effects of HBQs on genome surveillance pathways should be further investigated and elucidated.

Protein carbonylation and adduction potential of HBQs was shown by Du et al. (2013). After T24 cells were exposed to either DCBQ, DBBQ, DCMBQ, and TCBQ (25 - 125 μM) for a 24-hour exposure period, protein samples were measured by the protein carbonylation and malondialdehyde (MDA) adduct assays. Protein carbonyls were produced with increasing HBQ concentrations, suggestive of significant oxidative stress. However, measurements of MDA protein adducts did not correspond with this trend; T24 cells did not experience significant changes in MDA adducts after being exposed to the four types of HBQs. Since MDA is a reactive product of high levels of lipid peroxidation, authors mentioned the possibilities of lipids not being a target of HBQ-related oxidative stress and/or MDA not being the primary product of lipid peroxidation caused by HBQs.

1.6 HBQ Transformation

Much of what has been reported on HBQ transformation has been its hydrolysis in water. HBQs are unstable at pH 7 and 8, and transform into OH-HBQs (Mohan, 2015;
Du et al, 2017). There does not appear to be documentation in the literature of the mechanism for the formation of OH-HBQs. Cell media is mostly comprised of water and also has a pH of approximately 7. Most toxicity testing of HBQs is performed *in vitro*, therefore it is important to understand HBQ transformation in cell media.

One study reported HBQ transformation within one hour of addition to cell medium (Prochazka et al., 2015): the IC$_{50}$ value of CHO-K1 cells treated with reacted HBQ media for $\geq$ 20 minutes increased ($\sim$40 µM) compared to the IC$_{50}$ value of cells that were treated with immediately spiked DCBQ in media ($\sim$10 µM). Authors hypothesized that some components within the cell medium may react directly with HBQs to form less toxic compounds. However, another study reported that the IC$_{50}$ value of Caco-2 cells remained unchanged after a one-hour delay. This finding suggests that there might be different degrees of sensitivity to transformed HBQs between cell line models. The characterization of changes in toxicity of transformed HBQs is critical for incubation time periods and IC$_{50}$ values. Du et al. (2017) investigated transformation of HBQs in the presence of amino acids. Histidine, an amino acid that was shown to be highly reactive with HBQs, is also a nonessential amino acid found in some cell media.
HBQs can also undergo biotransformation. Knowledge regarding the metabolism of HBQs is limited. Documented routes of exposure of DBPs have been primarily dermal absorption, ingestion, and inhalation (Richardson et al., 2007), however, specific routes of HBQs exposure have not yet been identified. No studies have investigated how the detoxification of HBQs is carried out in the human body, and what structural form(s) are reduced when it reaches organs responsible for excretion (e.g., bladder and colon). Overall kinetics of HBQs and their biotransformation should continue to be determined and evaluated for future toxicological studies.

1.7 HBQ-related Human Health Effects

Several epidemiological studies have demonstrated DBP exposure to be associated with adverse health outcomes. Well characterized DBPs such as trihalomethanes (THMs), and other major classes of DBPs, such as haloacetic acid (HAAs) have been
linked to negative reproductive health outcomes (Nieuwenhuijsen et al., 2000), bladder cancer (Villanueva et al., 2006; Costet et al., 2011; Villaneuva et al., 2014), brain cancer (Cantor et al., 1999) and various respiratory conditions (Richardson & Postigo, 2011). Evidence of DBP-related colorectal cancer risk remains scant and inconsistent and has primarily focused on THMs. Several studies have reported lifetime exposure to THMs was positively associated with increased risk of colon cancer (Hildesheim et al., 1998; King et al., 2010). However, Villanueva et al. (2017) conducted a case-control study in Italy and Spain from 2008 to 2013 on colorectal cancer risk and long-term exposure to THMs and reported no association between cases and THM exposure. It is possible the DBPs that have been investigated for their association to colon cancer, such as THMs, are not the primary cancer-inducing agent. Exposure to emerging classes of DBPs should also be considered, including HBQs.

1.8 HBQ Exposure in Developing Countries

Water disinfection is essential for reducing the primary risk of exposure to waterborne pathogens and preventing waterborne diseases such as schistosomiasis, typhoid, cholera, and diarrheal diseases (UNICEF, 2012). DBPs are a major health concern in developed countries because water treatment infrastructures that use chlorine are continuously releasing the chemical disinfectant into treatment and distribution systems. Exposure to chlorine DBPs, many of which are known carcinogens, from point-of-use (POU) treated drinking waters in developing countries is a secondary health risk that is poorly understood (Lantagne et al., 2008). According to toxicological data mentioned in previous sections, HBQs are suspected carcinogenic DBPs, yet no study has been conducted on HBQ exposure in developing countries. Furthermore, quantification of the trade-off between exposure
to HBQs and waterborne pathogens in POU drinking water has also not yet been done. Several DBPs fall under the U.S. Environmental Protection Agency's disinfection byproduct rules, which include detection limits for each regulated DBP in public drinking water, but no regulations exist for POU-disinfected waters. POU chlorination with sodium hypochlorite has been a popular household water treatment and safe storage (HWTS) method around the world in areas where water disinfection is often inadequate or missing (Lantagne et al., 2008; Preston et al., 2010).

1.8.1. HBQ precursors in surface waters of developing countries
Various surface waters (e.g. rivers, ponds, streams, and lakes) are sources of drinking water around the world. Natural organic matter (NOM) in water has been accepted as a major precursor for DBP formation (Singer & Reckhow, 1990; Singer, 1999; Gerecke & Sedlack 2003). Most NOM is comprised of humic substances—including fulvic acids, amino acids, aromatic acids, aliphatic hydrocarbons, carbohydrates, and phenols—and biopolymers. Factors such as vegetation, geography, physiochemical water characteristics (e.g., pH, turbidity), water distribution system characteristics, and climate also influence the NOM present in water, and thus the kinds of DBPs formed. For example, NOM derived from water distribution systems due to microbial activity (e.g. proteins and amino acids) may be important for trihalomethane formation but contribute insignificantly to formation of chloral phenols (Leenheer & Croue 2003). A study identified 21 aromatic compounds that were precursors of 2,6-DCBQ and concluded that phenols and chlorinated phenols, as well as para-substituted phenolic species and aromatic amines derived from NOM were found to contribute highly to DCBQ formation (Kosaka et al., 2017). Waters in vastly different regions, such as the global south and global north, may have drastically different
concentrations and classes of DBPs. That being said, DBP levels in the same geographical region may also vary significantly due to local activity.

![Figure 1.4. 2,6-DCBQ formation from hypochlorite reaction with NOM in water.](image)

Documented total organic carbon (TOC) and dissolved organic carbon (DOC) concentrations, commonly used surrogate measurements for NOM concentrations, from several natural tropical waters in Kenya and Tanzania range from 0-270 ppm, (Lantagne et al., 2007; Aschermann et al., 2016), whereas DOC concentrations from uncontaminated rivers in the U.S. generally range from 0-10 ppm (Malcom & Durum 1976). Organic carbon levels differ between tropical and non-tropical waters, in part due to the differences in geology, contamination sources, climate and local economies (Wantzen et al., 2008). Tropical waters have higher temperatures year-round, which increases DBP precursors such as DOC released from soils into water sources and hydrophobic acids. (Wang et al., 2009). Furthermore, anthropogenic climate change has exacerbated and extended dry and wet seasons in
tropical countries, drastically altering global precipitation patterns, which highly influence quantity and quality of NOM (humic versus biopolymer) in surface waters.

In 2017, over 150 million people in the world still collected water from for drinking directly from surface water sources, more than half of who live in sub-Saharan Africa (WHO/UNICEF 2017). Even sources such as groundwater or piped water are frequently contaminated: in 2010, it was estimated that 1.8 billion people in the world, or one-quarter of the world’s population, drank water that was unsafe (Lee & Schwab 2005; Pickering et al., 2010; Kumpel et al., 2015). Lack of adequate and reliable drinking water supplies has lead to the continual use of surface water in many developing countries. The often-contaminated state of surface water is a primary reason point-of-use chlorination is a popular drinking water disinfection method employed in household settings. Many surface waters have low flow velocity or are stagnant, which encourages algal blooms. Low flow rate of water, high temperatures, and anthropogenic sources of nutrients promote algal growth, which can contribute to HBQ and other DBP formation (Yang et al., 2011; Wang et al., 2014; Ge et al., 2017). Anthropogenic sources of nutrients include pollution from agricultural run-off, open defecation, broken pit latrines, improper waste disposal etc. and may also increase intracellular organic matter (IOM) and algal organic matter (AOM), which are major precursors of DCBQ. Eutrophication of waters and waters with biodegradable substances may be significant for HBQs because biopolymer NOM has been reported as an important predictor of HBQ formation (Wang et al., 2014).

Liquid sodium hypochlorite is a common method of household water treatment (WHO 2016). The doses of chlorine and source waters used vary with context: accessibil-
ity, degree of contamination, individuals' knowledge about water disinfection, water storage method, and aesthetic factors, such as taste and odor (Crider et al., 2017). However, the consequences of exposure to pathogens and DBPs due to over- or under-chlorination of different raw source waters that contain varying level of NOM remain largely unexplored. Moreover, the relationship between exposure to pathogens and HBQs in drinking water in the context of POU chlorination has not yet been quantified.

1.9 Conclusions

POU chlorination of surface water that contains NOM is a potential source of HBQs. NOM is a major precursor of HBQs, namely phenols, chlorinated phenols and para-substituted aromatic amines. The cytotoxicity of HBQs has been hypothesized based on QSTR modeling and reported in several in vitro studies. Recent studies have shed light on potential molecular pathways impacted by HBQ exposure, namely the glutathione pathway, DNA methylation, lipid and protein modification, DNA adduction, DNA strand breaks, and DNA intercalation. However, gaps still remain about the toxic action of HBQs regarding specific mechanisms of toxicity and effects of HBQ exposure on normal non-cancerous cell lines. Furthermore, toxicological data in the current literature may not accurately reflect the true potency of HBQs based on the lack of data on the toxicity of transformed HBQ compounds in cell media. More toxicological research is needed in order to better understand the toxic effects of HBQs on human health.

Furthermore, HBQs in water have been shown to undergo hydrolysis, transforming into OH-HBQs. Only two studies have evaluated the toxicity of OH-HBQs in vitro, and the exposure lengths raise similar concerns to that of HBQs. The toxicity of transformed HBQs needs to be further investigated.
1.10 Thesis Overview

There has been growing interest in understanding the toxicity of HBQs since this class of DBPs was first identified in drinking water in 2010 (Zhao et al., 2010). Increasing evidence of genotoxic and mutagenic effects of HBQs are emerging within the HBQ literature. While published toxicological studies on HBQs provide insight into possible mechanisms of toxicity, the most significant unanswered question is whether these findings can be extrapolated to predict HBQ toxicity toward humans. To date, the human cell lines that have been primarily used to evaluate the toxic action of HBQs have been cancer cell lines. Cancer cell lines are commonly used in general toxicity testing when evaluating parameters, including cell growth and vital staining (Ekwall et al., 1990). However, cancer cell lines may not be the most optimal choice for measuring precise endpoints such as GSH levels or damage in DNA. One study reported that certain cancer cell lines have different levels of endogenous GSH compared to analogous cells from healthy tissues (Gamcsik et al., 2012). Breast and liver cancer cells were reported to have elevated levels of GSH compared to corresponding disease-free cells and other cancer cell lines. This is important to consider because results from cancer cell lines may not reflect how disease-free cells respond to HBQ-induced oxidative stress, due to variations in glutathione levels, thus making it difficult to determine human health relevance. There are still many informational gaps regarding specific endpoint comparisons between cancerous and healthy cells.

*In vitro* testing using non-human cell lines has been done to determine specific cell function and metabolic signaling tests in toxicology. One caveat to using non-human
cell lines is when insufficient data can yield inaccurate comparison between the cell models. For example, the genome of the Chinese hamster contains many similar homologs as that of humans (19,711 out of 24,383 genes), however, according to GO class assignments, <1% of genes related to important biological events, such as “anatomical structure morphogenesis”, are relevant to humans (Xu et al., 2011). In addition, despite the same genes being present in CHO-K1 cells, some genes were not expressed, especially after exponential growth. Studies should select an exposure model based on whether results are likely to be relevant to human health.

The research scope and health implications of HBQs have been primarily in water treatment plants, distribution systems, and swimming pools in North America. Much of the global population may not have similar exposures to HBQs, based on the vastly different types of source waters treated for drinking and the various disinfection methods applied around the world. Determining the occurrence of potent DBPs, like HBQs, in POU-treated waters is critical for identifying the settings in which HBQ exposure is relevant. Studies to date are concerned with potential chronic effects of HBQs, such as cancer. The life expectancy in many parts of the world that employ the POU chlorine water treatment method, such as rural areas in Latin America, exceeds the age range of the highest cancer incidence rates, making HBQs a potential exposure of concern. In order to quantify the health trade-off between HBQ exposure and exposure to waterborne pathogens an assessment of HBQ formation is needed.

1.11 Thesis Aims

In order to better understand the health relevance of HBQs, particularly in the context of the developing world, this thesis will report preliminary findings on:
1) Evaluation of the toxic effects of 2,6-DCBQ on viability and oxidative stress in a healthy human colon cell model (Chapter 2)

2) Conduct a comparative assessment of the toxicity of 2,6-DCBQ and its transformed derivatives in water and cell medium (Chapter 2)

3) Evaluation of the log removal of a surrogate measurement for waterborne viruses—the bacteriophage MS2 post-chlorination and formation of HBQs in chlorinated raw river waters (Chapter 3).
CHAPTER 2
COMPARATIVE TOXICITY ASSESSMENT OF 2,6-DCBQ AND ITS TRANSFORMED DERIVATIVES

2.1 Background

HBQ cytotoxicity data and IC$_{50}$ values reported in previous studies have been based on exposure periods ranging from 6 to 24 hours. The extended length of these exposure periods disregarded the stability of HBQs and the potential implications on the measured toxicity induced by the transformed HBQ products. HQBs in water have been demonstrated to have relatively short half-lives (~7 hours) (Wang et al., 2014; Mohan 2015), rapidly reacting to form OH-HBQs. The half-life of HBQs in cell cultures was found to be shorter than in water (< 1 hour) (Figure 2.1). Recently, studies found HBQ degradation and OH-HBQ formation from reaction with amino acids present in water (Du et al., 2017; Ge et al., 2018). The amino acids that were found to react with HBQs (Phe, Cys, Thr, His), forming AA-HBQs, OH-HBQs-AA, and OH-HBQs, are also contained in many different types of cell media. It is possible that HBQs reacted with amino acids or some other ingredient(s) in the cell medium, which transformed HBQs into OH-HBQs or some unknown derivative(s). Studies have suggested that OH-HBQs are less toxic than their parent compounds (Wang et al., 2014; Prochazka et al., 2015; Du et al., 2017). This raises questions regarding the accuracy of HBQ toxicity results previously reported in studies investigating exposure periods longer than one hour.
Figure 2.1. Transformation of DCBQ. Transformation of DCBQ in water. LC/MS analysis of DCBQ and OH-DCBQ concentrations (µM) in water at pH 7.2. The OH-DCBQ formation (orange) is directly correlated with DCBQ (blue) degradation in water over time at ambient temperature (25°C). DCBQ in cell media with and without phenol red rapidly transforms in under 60 minutes at ambient temperature (25°C).

Previous studies along with preliminary kinetics tests have demonstrated that HBQs may react with components of commercially available cell media. Studies have suggested that transformed HBQ, whether from hydrolysis or interaction with amino acids (e.g., His-HBQ, OH-HBQ, OH-HBQ-His) may induce decreased toxicity (Du et al., 2017; Wang et al., 2014; Prochazka et al., 2015). To test this hypothesis, a comparative assessment of toxicity was conducted evaluating DCBQ and transformed derivatives. Toxicity was investigated using the PrestoBlue and DCFH-DA assays on both the immortalized (non-neoplastic) normal colon cell line (CDD 841 CoN) and liver hepatoma cell lines (HepG2).

The study presented in this chapter investigated the dose-response effect of one HBQ, 2,6-DCBQ, on the induced cytotoxicity and oxidative stress in HepG2 and CDD 841...
We hypothesized a DCBQ concentration-dependent effect on cells and a reduced toxic response from transformed DCBQ exposure.

2.2 Methods

2.2.1. Chemicals and Reagents
Eagles’ Minimum Essential Medium (EMEM), Dulbecco’s Phosphate Buffered Saline (D-PBS), penicillin (100 U)/ streptomycin (1 mg/mL) antibiotic, trypsin-EDTA (1X), fetal bovine serum (FBS), and dimethylsulfoxide (DMSO) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Triton X-100 (Molecular Biology grade) was purchased from Promega. Minimum Essential Medium (MEM containing no phenol red) (Fisher Scientific), 2’7’-dichlorofluorescein-diacetate ≥ 97% (Sigma Aldrich), and Hank’s Balanced Salt Solution with magnesium and calcium (HBSS with Mg++, Ca++; Fisher Scientific) were used in the DCFH-DA assay. 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ) stock was made by dissolving 2,6-DCBQ (Sigma Aldrich) in DMSO.

2.2.1.1. Cell Culture
HepG2 and CDD 841 CoN were obtained from ATCC. Cells were inspected for macromolecular signs of contamination upon receipt and were cryopreserved in -80 °C until culturing.

2.2.1.1.1. Aseptic working environment
An aseptic working environment was prepared and maintained by washing, autoclaving, and spraying down parts of the biological safety cabinet (BSC) and incubators with 70% 200-proof laboratory grade ethanol. The BSC sash was drawn at the proper position at all times to maintain laminar flow and the incubator was regularly inspected for contamination. All reagents and materials used were packaged sterile or autoclaved.
2.2.1.1.1. Thawing

Cells stored in cryopreservation (~1.0 x 10^6 cells per 1 mL freezing media) were rapidly thawed in a 37°C water bath with gentle agitation. Freezing media was comprised of EMEM with 20% FBS, 10% DMSO, 1% penicillin/streptomycin. Cells were then transferred to a sterile 15 mL conical tube and 10 mL of EMEM-C (EMEM with 10% FBS and 1% penicillin/ streptomycin) were added to the cells. Cells were pelleted by centrifugation at 3000 rpm for 5 minutes. The supernatant was removed with a sterilized Pasteur pipette attached to a vacuum pump and 2 mL of new medium were added to cells.

2.2.1.1.1.1. Counting

Cells were resuspended in 2 mL of medium by flicking the conical tube and pipetting the medium up and down with a 1000 µL pipette. After cells had been uniformly suspended in EMEM-C, a 1:1 ratio of cell suspension and Trypan Blue (0.4%) (Fisher Scientific) was added to an Eppendorf tube. The Trypan Blue dye was gently mixed with cells in EMEM-C and 20 µL of cell suspension was loaded onto a clean and dry hemocytometer with a cover slip (Fisher Scientific) for counting. The hemocytometer was placed under an inverted microscope (Fisher Scientific) and viewed at 100x magnification. Cells within the four quadrants were counted and the mean of the cells was calculated and applied to the equation below:

\[
\text{Average \# of cells} \times \text{dilution factor} \times 10^4 = \text{Total \# of cells / mL}
\]

Since both HepG2 and CDD 841 CoN cell lines are anchorage-dependent, they were transferred to tissue culture-treated vessels, T- 25 cm^2, T- 75 cm^2 flasks, or 100mm
x 15 mm petri dishes, based on the total number of cells, in the appropriate medium volume for the vessel and kept in the incubator at 37°C with 5% CO₂. A water tray was kept in the incubator to maintain proper pH and humidity levels.

2.2.1.1.1.1.1.1.1. Sub-culturing

Cells were observed under the microscope daily for morphological changes and inspected for indicators of contamination. Cells were kept at log phase of growth and split 1:2 to two vessels after having grown in monolayer to 70-80% confluence. First, all reagents were pre-warmed and then cells were washed with about 2 mL of D-PBS and incubated with about 3 mL of trypsin-EDTA for three minutes. After all cells were resuspended, about 5 mL of EMEM-C were added to the flask. Cells were transferred to a sterile 15 mL conical tube and pelleted by centrifugation at 3000 rpm for 5 minutes. After pelleting, the supernatant was removed by using a Pasteur pipette attached to a vacuum pump and cells were counted using the method described in the previous section. Cells were sub-cultured with a 1:2 split ratio and transferred to the appropriate vessels for growth or seeded onto 96-well plates (~0.1x10⁵ cells per well with 100 µL EMEM-C) to be used for experimental assays. Passage number of adapting cells were noted after each sub-culture.

2.2.2 PrestoBlue Cell Viability Assay

CDD 841 CoN were obtained from ATCC at passage ~7 and cells used for experiments were between passages 7-12. HepG2 cells used for experiments were between passages ~25-30. The PrestoBlue Cell Viability assay (Fisher Scientific) was used to evaluate the cytotoxicity of 2,6-DCBQ on both cell lines.
DCBQ was directly spiked into EMEM-C immediately prior to the start of the experiment. OH-DCBQ was prepared by spiking 75 mg/mL DCBQ into sterile DNase-free water (Fisher Scientific). The half-life of DCBQ in aqueous solution at pH 7 was reported to be roughly 6-7 h (Qin et al., 2010). The DCBQ in sterile water was left on the bench at room temperature for at least 42 h (7 half-lives) to ensure that the majority of DCBQ was transformed to OH-DCBQ. OH-HBQs are stable in water at pH 7 (Wang et al., 2014).

2.2.2.1. HBQ Exposure

Cells were seeded in a clear, tissue culture-treated, sterile, 96-well plate with EMEM-C and cultured as previously described. Once cells had reached 70-80% confluence, EMEM-C was removed from each well and cells were washed once with 100 µL of pre-warmed D-PBS. Pre-thawed 2,6-DCBQ in DMSO was spiked into several sterile conical tubes containing EMEM-C and then immediately decanted onto separate pipette basins, and pipetted into the wells. The plates were placed in a humidified incubator for one hour at 37°C with 5% CO₂. Following the exposure period, supernatant was aspirated off and each well was washed once with 100 µL D-PBS. PrestoBlue in pre-warmed EMEM-C (1:10 ratio) was prepared in the dark and transferred into all wells containing cells followed by a one hour incubation at 37°C with 5% CO₂. The dye was transferred into every other column on standard, clear 96-well plates and fluorescence was measured using Spectramax i3 microplate reader (Molecular Devices, San Jose, CA) at excitation/emission wavelengths of 560 nm/ 590 nm. A four-point bottom read was done with three flashes. Exposures were performed at minimum in triplicate on separate days, yielding 12-24 technical replicates per concentration. Cell viability was evaluated based on the
mean viability values (expressed in relative fluorescence units, RFU) from all experiments and the associated standard error for each value. Percent viability was calculated as follows:

\[
\text{\% Viability} = \frac{RFU_{\text{sample}}}{RFU_{\text{control}}}
\]

One-hour IC\(_{50}\) values were estimated using the equation of the trend lines.

### 2.2.3 DCFH-DA assay

#### 2.2.3.1. Quantifying ROS production

Total ROS production was measured using the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay. The fluorogenic probe DCFH-DA penetrates the cell membrane and the diacetate group is cleaved by cellular esterases to non-fluorescent compound 2′7′-dichloro-dihydrofluorescein (DCF). DCF is oxidized by ROS within the cellular cytosol, such as by superoxide anion, hydrogen peroxide and hydroxyl radicals, to the fluorescent compound 2′7′-dichlorofluorescein (DCF). DCF fluorescence intensity is correlated with overall cytosolic ROS levels and can be quantified by flow cytometry or fluorescence microscopy. In the current study, DCF intensity was measured by fluorescence microscopy using the SpectraMax i3 microplate reader (Molecular Devices, San Jose, CA) at excitation and emission wavelengths of 485 and 535 nm.

After HepG2 cells reached 70-80\% confluence within the black, clear-bottom 96-well plates (Corning) in 37°C with 5\% CO\(_2\), they were washed once with HBSS supplemented with magnesium and calcium and treated with 5 \(\mu\)M DCFH-DA in MEM without phenol red for 30 minutes in the dark. After DCFH-DA had permeated the cell membrane and been retained within the cells, they were washed once with HBSS (Mg\(^{++}\), Ca\(^{++}\)) and
incubated in 2,6-DCBQ (75 - 750 µM) for one hour at 37°C with 5% CO₂ in darkness. A stock of 75 mg/mL 2,6-DCBQ was spiked in phenol-red free medium immediately after the second wash step and loaded onto cells as quickly as possible to ensure that the cells were being treated with untransformed DCBQ within that time. The range of concentrations was guided by viability tests described in the Results section. DCBQ concentrations used in DCFH-DA experiments were above the one-hour viability IC₅₀ concentration (~420 µM). The percent DMSO for all control and experimental groups was kept at ≤ 1%. A solvent control group (MEM medium with ≤ 1% DMSO) was included in every biological replicate, as well as a high concentration of DCBQ (750 µM) as a positive control. After DCBQ treatment, cells were rinsed once and imaged with HBSS (Mg⁺⁺, Ca⁺⁺).

The microplate reader conducted a 12-point bottom-read of the 96-well plate. RFU was measured for each well by taking the arithmetic mean of the fluorescence of 12 points. This experiment was done in triplicate and each DCBQ concentration had at least five technical replicates (maximum eight replicates). At least four biological replicates and between 12 and 24 experimental replicates were used to determine percent ROS increase of each DCBQ concentration. Percent increase in ROS was determined by the following equation:

\[
\% \text{ increase ROS} = \frac{(RFU_{\text{sample}} - RFU_{\text{control}})}{RFU_{\text{control}}}
\]

One-hour IC₅₀ values were estimated using the equation from the trend lines.

2.2.3.1.1. DCBQ-DCF calibration curve

During optimization of the DCFH-DA assay, DCBQ was found to quench DCF fluorescence. DCF, the fluorescent product formed when DCFH reacts with ROS, was
mixed with a range of DCBQ concentrations. DCF was initially dissolved in 1 mL of DMSO, then 1 mM DCF aliquots diluted in 1 mL phenol red-free medium were made and transferred to 1.5 mL Eppendorf tubes and stored at -20°C. DCF stock (1 mM) was thawed and serially diluted in phenol red-free medium to 1 µM DCF to achieve a less than 1% DMSO concentration. DCF solution (100 µL) was pipetted onto a standard, clear 96-well plate (Santa Cruz). DCBQ (100 µL) at concentrations of 250, 350, 450 or 750 µM) were added to the DCF. One column also contained DCF with phenol red-free medium as a control. The plate was placed on the shaker within the microplate reader for 10 seconds and medium speed and an endpoint bottom-read was taken at the same excitation/emission wavelengths (485/535 nm).

A unique DCF standard curve was generated for four DCBQ concentrations (150, 350, 550, 750 µM). DCF stock (1 mM) was thawed and serially diluted to 1, 0.1, 0.01, 0.001 µM in 10 mL phenol red-free medium. Each DCF concentration (10 mL) was decanted onto a pipette basin and pipetted onto a 96-well plate, with one DCF concentration per every other column (8 wells) in order to avoid fluorescence interference from adjacent wells. Plates were covered with aluminium foil while DCBQ dilutions were being prepared to prevent stray light and air interactions. DCBQ stock (75 mg/mL) was spiked in phenol red-free media and mixed by inversion before decanting onto the pipette basin. Each DCBQ concentration (100 µL) was pipetted into wells of separate 96-well plates containing 100 µL of DCF. Plates were shaken linearly at medium speed for 10 seconds before an endpoint bottom-read. The entire procedure was conducted in darkness.

To ensure that this observation was indeed a result of DCBQ interference with DCF fluorescence, rather than an occurrence caused by an additional compound, this
quench test was repeated using iodoacetic acids (IAA) (5 - 1000 µM), another class of DBPs.

2.2.4 Determination of Transformed DCBQ Toxicity

2.2.4.1. PrestoBlue Cell Viability Assay

OH-DCBQ in water was vortexed and spiked into EMEM-C to achieve <0.01% DMSO. Then, the same concentrations of all experimental compounds—DCBQ directly spiked into medium, OH-DCBQ, and media-transformed DCBQ—were pipetted onto the cells for a one-hour exposure period (at least four experimental replicates). Solvent controls were included in all experiments. The percent viability for OH-DCBQ replicates was calculated using the negative control containing media, DMSO, and water as the baseline. The CCD 841 CoN cells experienced total decline in viability in wells containing OH-DCBQ dissolved in water and the solvent control, thus OH-DCBQ experiments were no longer conducted with this cell line. Instead, only DCBQ and media-transformed DCBQ were used on CDD 841 CoN cells. DCBQ, OH-DCBQ, and media-transformed DCBQ used on HepG2 cells.

2.2.4.1.1. DCFH-DA Assay

The same concentration of DCBQ, OH-DCBQ and media-transformed DCBQ were diluted in MEM without phenol red. Compounds were pipetted onto HepG2 cells (at least four experimental replicates) for a one-hour exposure period. Percent increase in ROS production was determined for each compound.
2.2.5 Statistical Analysis

Standard error for each concentration was calculated by dividing standard deviation by the number of technical replicates. The standard error for each concentration was propagated within individual biological replicates using the following equation:

$$\frac{\partial Q}{|Q|} = \sqrt{\left(\frac{\partial a}{a}\right)^2 + \left(\frac{\partial b}{b}\right)^2 + \cdots + \left(\frac{\partial n}{n}\right)^2}$$

where,

- $Q$ = mean RFU for a single DCBQ concentration,
- $\partial Q$ = standard error of the mean RFU,
- $a, b, \ldots, n$ = mean RFU value for each concentration per replicate

The coefficient of variation (CV) was determined by dividing standard deviation by the mean RFU of each concentration.

Viability and ROS production results were analysed using a one-way analysis of variance (ANOVA) on each dose for all biological replicates together to determine a statistically significant effect of DCBQ exposure ($p \leq 0.05$) and a simple linear regression was performed. A one-way ANOVA was done as a regression for each biological replicate separately ($p \leq 0.05$). The difference in response from DCBQ and transformed compounds (OH-DCBQ and media-transformed DCBQ) was determined with a two-sample t-test ($p \leq 0.05$). Another t-test was conducted to assess the groups OH-DCBQ and media-transformed DCBQ, although there was not a significant difference ($p \geq 0.05$). All statistical analysis was performed in R Studio.
2.3 Results

There was a linear concentration-response observed in both cell lines. HepG2 cells were marginally less susceptible to 2,6-DCBQ than colon epithelial cells. The one-hour IC$_{50}$ values were ~455 µM for colon epithelial cells and ~540 µM for liver hepatoma cells (Figures 2.2 and 2.3).

**Figure 2.2.** CCD841 CoN viability. DCBQ dose-dependent decrease in CDD 841 CoN cells viability (p=0.03) after a 1-hour exposure.

**Figure 2.3.** HepG2 viability. DCBQ dose-dependent decrease in HepG2 cell viability (p=0.001) post 1-hour exposure (150-750 µM).
2.3.1. DCFH-DA Method Optimization

The DCFH-DA assay has been widely used across disciplines and within fields related to molecular biology (Lebel 1992). Despite its popularity within redox biology, there lacks a standardized method for this assay. Length of incubation, concentration of DCFH-DA, and solvent type varied between studies (LeBel et al., 1992; Osseni et al., 2000; Cheung et al., 2004; Sohn et al., 2005; Fu et al., 2008; Wang et al., 2008; Ericksen et al., 2010; Chen et al., 2012; Zhang et al., 2011; Aranda et al., 2013; Mostafavi-Pour et al., 2013; Wang & Roper, 2014; Janda et al., 2015; Lu et al., 2016; Azimzadeh et al., 2017; Krause et al., 2017; Scarpa et al., 2017). For example, Cheung et al. (2004), Sohn et al. (2005) and Wang et al. (2008), along with several other studies, assessed increase in ROS production in the HepG2 cell line, but treated cells with different concentrations of DCFH-DA (5 - 100 μM) for different lengths of time, ranging from 30 to 240 minutes. A general literature search of the DCFH-DA assay used on the HepG2 cell line was conducted; the most common concentrations used were between 5 and 25 μM, incubation time ranged from 15 to 45 minutes, and experimental buffers used were PBS, HBSS (with and without Mg²⁺ and Ca²⁺), or medium (without phenol red). In order to create the optimal protocol for the DCFH-DA assay for the HepG2 cell line, different concentrations, incubation times, and experimental and imaging buffers were tested. DCFH-DA optimization experiments indicated that incubation of 30 minutes with 5 μM DCFH-DA in media without phenol red was optimal for HepG2 cells (Figure 4); few cells were left adhered to the wells at concentrations above 5 μM and a incubation times longer than 45 minutes (data not shown). Each experimental group was incubated with the same concen-
tation of DCFH-DA for the same length of time and each group had four biological replicates. This experiment was performed once with solvent controls (experimental buffer containing less than 0.1% DMSO).

Fluorescence settings were also optimized on the Spectramax i3 (Figures 2.4 and 2.5). The SpectraMax i3 microplate reader has several flash interval settings for measuring re-radiated light of cells and quantifying fluorescence intensity. There are many benefits to increasing the number of flashes per read (increasing the read time per well), such as potentially improving delta F values and reducing background noise of each well, however, photo-bleaching is a possibility in the event of too much absorbed light. To find the optimal flash interval or read time per well for this particular assay, one (minimum number of flashes), three, and six (default) flashes per read were tested on cells treated with 30% laboratory grade hydrogen peroxide solution (Sigma Aldrich, St. Louis, MO), a potent oxidizing agent that causes intracellular ROS. Based on preliminary tests, bottom-read with 6 flashes and PMT Gain set to “High” was the most suitable setting for DCF fluorescence quantification. All experimental groups were treated with DCFH-DA diluted in PBS for the same amount of time and were exposed to 2.5 and 20 µM of H₂O₂ diluted in PBS. Solvent control groups were included (PBS containing ≤ 0.1% DMSO). There were four biological replicates per experimental group and this experiment was performed once.
Figure 2.4. DCFH-DA experimental buffer optimization. HepG2 cells in different experimental buffers were treated with the same concentration of DCFH-DA and incubated with 5 and 50 µM DCBQ. Their response, expressed in relative fluorescence units (RFU), was measured using the Spectramax i3 microplate reader at excitation and emission 485 nm and 535 nm. Experimental buffer media without phenol red (blue) had a linear response to increasing DCBQ concentration, whereas PBS (orange) and HBSS supplemented with magnesium and calcium (grey) had trends that did not correspond to DCBQ concentration. The negative control groups were cells treated with experimental buffers without HBQs.

Figure 2.5. DCFH-DA number of flashes optimization. HepG2 cells exposed to 2.5 and 20 µM H₂O₂ were treated with the same concentration of DCFH-DA for the same length of time and DCF fluorescence was measured at excitation and emission wavelengths 485 and 535 nm. A dose-dependent fluorescence was observed with the six flashes (grey), as opposed to one (blue) or three (orange) flashes.
2.3.1.1. DCBQ-DCF calibration curve

DCF calibration curves were generated to quantify the DCF fluorescence quenched by DCBQ. RFU was found to decrease with increasing DCBQ concentrations, which confirmed the fluorescence quenching phenomenon (Figure 2.6A). The presence of IAAs did not interfere with DCF fluorescence (Figure 2.6B). The RFU values of all DCFH-DA replicates were adjusted based on DCBQ-DCF calibration curves to account for fluorescence quenching.

![Graph A](image)

(A)

![Graph B](image)

(B)

**Figure 2.6.** Fluorescence quenching. DCBQs may interfere with DCF fluorescence. (A) DCBQ (concentration 0 - 750 µM) incubated with DCF was found quench DCF fluorescence in a dose-dependent manner. (B) IAA (0 - 1000 µM) incubated with DCF demonstrated no quenching effect of DCF fluorescence.
**Figure 2.7.** DCF calibration curves. (A) Calibration curves for four DCBQ concentrations (75, 150, 350, 750 µM), demonstrating an inverse relationship between concentration and fluorescence (RFU). (B) Quenching phenomenon at 1µM DCF, with RFU values decreasing with increasing DCBQ concentrations.
2.3.1.1.1. Concentration-dependent DCBQ induction of ROS formation

HepG2 cells exposed to 2,6-DCBQ for one hour experienced a dose-dependent increase in ROS production (Figure 2.8).

![Figure 2.8](image)

**Figure 2.8.** HepG2 12- and 24-hour ROS production. A comparison of percent increase in ROS after exposure lengths of one (blue) and 24 hours (orange) with DCBQ. The 24-hour DCBQ exposure was repeated in duplicate and the one hour exposure were repeated in quadruplicate.

In HepG2 cells, a range of DCBQ concentrations were used for measuring ROS production following a one-hour exposure period, results presented in Figure 2.8. Each point on the curve represents the mean percent increase in ROS for each DCBQ concentration across four biological replicates with their propagated standard error. Percent ROS increase was adjusted based on the DCBQ-DCF calibration curves. A positive correlation was found between DCBQ exposure concentration and ROS production \((p=0.000028)\). Traditionally, exposure periods of 12 and 24 hours were conducted, thus 24-hour exposures to DCBQ were also performed to compare ROS production levels with extant literature. The percent increase in ROS following a 24-hour exposure to a range of DCBQ concentrations (250, 450, 550, 650, 750 µM) was lower than the one-hour exposure. IC\textsubscript{50}
values for HepG2 cells exposed to DCBQ for one hour was ~ 420 µM. The 24 IC_{50} value was roughly ~ 487 µM. These results demonstrate that the shorter exposure time increased oxidative stress more than a longer incubation with DCBQ. These findings may indicate a reduced toxicity of transformed DCBQ compounds present in the cell culture medium over the extended exposure time.

Several concentrations of DCBQ, OH-DCBQ and media-transformed DCBQ (50, 150, 300 µM) were used to evaluate relative toxicity. For each concentration, the transformed DCBQs induced similar responses relative to the parent compound (data not shown). In both cell lines, cells exposed to transformed DCBQ compounds (media-transformed DCBQ and OH-DCBQ) exhibited decreased cytotoxicity responses compared to exposure to DCBQ (Figures 2.9A and B). Similarly, both cell lines also experienced decreased ROS production (Figure 2.10) and viability in HepG2 and CCD cells. DCBQ was also found to be more toxic than OH-DCBQ ($p=0.019$), however there was no difference in viability between OH-DCBQ and media-transformed DCBQ ($p= 0.489$). There was no difference between the biological replicates.
Figure 2.9. DCBQ- and transformed DCBQ-induced cell viability. Viability results of CCD and HepG2 cells treated with the same concentration of DCBQ and transformed DCBQ. (A) Percent viability of CCD cells relative to the control was significantly higher in media-transformed DCBQ compared to that of DCBQ (p=0.03), suggesting that transformed DCBQ is marginally less toxic than its parent compound. Percent viability of DCBQ- and media-transformed DCBQ-treated cells were adjusted relative to the media and DMSO control. (B) There was a significant difference between HepG2 viability post-treatment of DCBQ and OH-DCBQ (p=0.019). There was no significant difference in cell viability between DCBQ- and media-transformed DCBQ-treated groups (p=0.061), and OH-DCBQ and media-transformed-DCBQ-treated groups (p=0.763). Although there was no statistical difference in responses between the parent compound and media-transformed DCBQ groups, analysis results were approaching significance, indicating that the parent compound is more toxic than both transformed products.
Figure 2.10. DCBQ- and transformed DCBQ-induced ROS production. Percent increase in ROS production relative to the control in HepG2 cells after being treated with the same concentration of DCBQ, OH-DCBQ and media-transformed DCBQ for one hour. OH-DCBQ treatment groups were adjusted relative to the media, DMSO and water control. Other treatment groups were adjusted relative to the control containing media and DMSO. Percent increase in ROS was statistically significantly higher in DCBQ compared to that of OH-DCBQ (p=0.05) and transformed DCBQ (p=0.01). ROS production also differed between cells treated with OH-DCBQ and media-transformed DCBQ (p=0.05). This assay was performed in triplicate with at least five technical replicates per experimental group per experiment.

2.4 Discussion

DCBQ induction of ROS production was observed in a concentration-dependent manner in both cell lines. There are two possible pathways for this to occur: 1) DCBQ permeated the cell membrane and directly induced oxidative stress by increasing ROS levels or 2) no permeation of DCBQ through the cell membrane occurred and ROS production was induced indirectly. After the diacetate group of DCFH-DA is cleaved by cellular esterases, DCFH is stabilized and retained within the cell to react with ROS. Thus, if DCBQ permeated the cell membrane, it is highly probable that it was directly quenching DCF fluorescence. However, if DCBQ does not permeate the cell membrane, the compound is removed with the supernatant and washed away during the third wash step and
does not interfere with DCF fluorescence. Both pathways of toxicity are plausible, but there are certain toxicokinetic factors that influence selective permeability of the plasma membrane. Lipinski's "rule of 5" postulates that physical properties of a compound, such as octanol-water coefficient ($\log K_{ow}$), molecular weight, size, and charge, govern the permeability of a compound across the phospholipid bilayer. According to the PubChem profile of 1,4-BQ—the foundational structure of HBQ—the compound is only slightly soluble in water with a $\log K_{ow}$ of 0.2. The addition of two chlorine groups to $p$-benzoquinone increases both the polarity and lipophilicity of the compound, which increases its absorption and permeability of the plasma membrane. DCBQ fulfills each of Lipinski's "rule of 5" criterion and it is possible that DCBQ may enter the cell by diffusion and directly induce ROS production intracellularly, thus interfering with DCF fluorescence. Furthermore, a study by Yasukawa et al. (1997) found that redox species, such as $p$-hydroquinone and $p$-benzoquinone, that had large permeability coefficients were able to permeate the membranes of protoplasts by passive diffusion. Based on this information, it is assumed that DCBQ was able to directly cause oxidative stress intracellularly and quench the fluorescence of DCF.

DCFH-DA results demonstrated a dose-dependent increase in ROS production following exposure to 2,6-DCBQ. This finding is consistent with the current literature (Du et al., 2013; Li et al., 2016; Xu et al., 2018). The HepG2 cells displayed an enhanced oxidative stress response following exposure to DCBQ for the one-hour period compared to their response after exposure for 24 hours. Reactions between HBQs and substances within cell media may have transformed the HBQs, as preliminary kinetics tests have shown, and resulted in less toxic derivatives of HBQs (e.g., OH-HBQs, AA-HBQs, OH-
HBQ-AAs etc.). The 24 IC\textsubscript{50} value for was estimated to be \(~487\, \mu\text{M}\). This was a notable finding because 24-hour IC\textsubscript{50} values for DCBQ in other mammalian cell lines have been reported to be lower, ranging from \(~1\) to \(30\, \mu\text{M}\) (Li et al., 2015). Limited by the kinetics of 2,6-DCBQ in cell medium, the short incubation time of just one hour could have been the reason DCBQ concentrations could span a wide range (up to 750 \(\mu\text{M}\)) without achieving a total cytotoxic effect on the cells. The HepG2 cell line has naturally abundant endogenous GSH levels compared to other cell lines used to evaluate HBQ-induced ROS production, such as CHO-K1, T24 and Caco-2, which could account for the higher IC\textsubscript{50} values observed in this study. The DCFH-DA results confirmed the dose-response effects of the parent DCBQ compound and pointed to the possibility of a difference in toxicity between parent HBQ compound and transformed compound in medium. One DCFH-DA experiment was performed on colon epithelial cells using the same method as HepG2 experiments, however, a concentration-dependent effect was not observed. Assay optimization may have been needed for this cell line, as it has many different properties and overall biological profile than HepG2 cells. Human epithelial cell lines also have low endogenous levels of GSH, which is an antioxidant pathway that HBQs are suspected to inhibit (Song et al., 2009; Bull et al., 2011; Li et al., 2014). One study found that maspin, a serine protease-inhibitor, is naturally expressed in epithelial cells and that it interacts with isoforms of GST found in human cells (Yin et al., 2005). Investigators reported a positive association between GST/maspin interaction and oxidative stress, which resulted in curbed ROS production. This may offer mechanistic insight into why human epithelial cells may be poor cell models for the study of oxidative stress. The CCD 841 CoN cell
line has not been commonly used in toxicological studies. Some studies that have assessed toxicological endpoints comparing CCD 841 CoN cells to their cancerous counterparts (i.e., Caco-2, HCT-116, HT-29) have found CCD 841 CoN to be less affected by toxic agents (Leong et al., 2016; Hajiaghaalipour et al., 2017) and had relatively lower antioxidant responses during stress (Li & Shah, 2017). Ultimately, this assay was not performed on the CCD 841 CoN cell line, although the DCFH-DA probe was successfully used with CCD 841 CoN cells in other studies (Regmi et al., 2014; Packiriswamy et al., 2017).

The toxicological data of DCBQ and its corresponding transformed products suggest the parent compound is more toxic. It was postulated that due to the detoxification effect of DCBQ transformation, longer exposure to DCBQ (24 hours), counterintuitively, elicited a lower toxic response in treated cells. Cell viability improved in both cell lines and oxidative stress response was curtailed in liver cells. Our findings support our previous hypothesis that transformed 2,6-DCBQ is less toxic than its respective parent compound. Because there was no significant difference detected between media-transformed DCBQ and OH-DCBQ, the composition of unknown transformed DCBQ compounds in media may include OH-DCBQ. Alternatively, uncharacterized transformed DCBQ compounds in media (e.g., AA-HBQ) may activate the same pathways in these cell lines, leading to the similarities in cell response.

Such findings shed light on the importance of investigating compound interactions with substances in cell media when conducting in vitro studies. Furthermore, this study demonstrates that there is a clear need to investigate biotransformation and metabolism of compounds in order to conclude on the effect of a compound on human health. A
recent study found that phenolic compounds alter the structure of molecules in cell media, which may have potential confounding effects on endpoints of interest and overall cell response (Aragones et al., 2017). Thus, newly formed metabolites should be characterized in future studies, especially given the difference in toxicity. One possible next step is to explore how to differentiate the cell responses from all metabolites present in cell medium and the responses to OH-DCBQ. Another possibility is to identify the toxicity of metabolites using novel methods, such as using lab-on-a-chip devices. This method uniquely allows human hepatocytes and other cells from tissues that comprise of the GI tract to metabolize a compound prior to reaching the target cells or cell line of interest, mimicking the metabolic processes it would likely undergo inside the human body.
CHAPTER 3
EVALUATION OF DCBQ FORMATION AND INACTIVATION OF A SURROGATE MEASUREMENT FOR WATERBORNE VIRUSES

3.1 Background

Untreated surface waters are still frequently used for drinking around the world. According to the 2017 WHO Joint Monitoring Report, 159 million people continue to collect drinking water directly from surface water sources (WHO, 2017). NOM in raw waters and water distribution systems are precursors of many classes of DBPs after disinfection, such as chlorination and chloramination (Qian et al., 2013). HBQs (DCBQ, DBBQ, DCMBQ and TriCBQ) have been identified in chlorinated waters in the U.S. and Canada (Wang et al., 2014; Mohan, 2015), but there remains a large data gap for these potentially highly toxic compounds in household chlorination of water. However, household water treatment systems, including in-home chlorination, is commonly used in low and middle-income countries (WHO, 2012). Since many source waters being chlorinated in at-home storage containers are contaminated with both organic matter and/or pathogenic viruses, bacteria, or protozoa (WHO, 2016), there are two causes of concern: 1) free chlorine levels may reduce quickly after chlorine is added due to high NOM levels, reducing its efficacy in inactivating pathogenic microorganisms and 2) high NOM levels may contribute to DBP exposure, including likely toxic compounds such as HBQs. It is hypothesized that MS2 inactivation and HBQ formation will be dependent on chlorine dose and that HBQ concentration would increase with highly contaminated source waters. It is recognized that chlorine is essential for preventing waterborne diseases and the health consequences of not disinfecting drinking water are definitive and immediate compared
to the potential health consequences from DBP exposure. This chapter merely aims to quantify the relationship between HBQ formation and inactivation of MS2 in chlorinated surface water in order to better understand human exposure to HBQs via POU chlorinated drinking water.

3.2 Methods

The laboratory was set up to reflect the setting in which household chlorination might take place in order to accurately study the implications and exposures of POU chlorination. Raw river water and primary wastewater were used to mimic source waters in developing countries. Bench-scale water disinfection studies often use "challenge waters"--waters that are prepared with added content in order to simulate sources of drinking water --to determine the efficacy of water treatment methods. For this experiment, untreated water from the Mill River in Amherst, Massachusetts, and untreated Mill River water spiked with primary wastewater effluent were used as challenge waters. These were collected on three separate days in December 2017 and February and March 2018. Surrogate measurements for determining NOM concentrations in source waters - total organic carbon (TOC) and ultraviolet absorbance at 254 nm (UV$_{254}$) - were measured in real-time using THM 100 (AMC) and Real UV254 (Real Tech Inc.) and are summarized in Table 3.1.

3.2.1. Chemicals

Sodium hypochlorite was purchased from Fisher Scientific. Ferrous ammonium sulfate (FAS), N, N-diethyl-p-phenylenediamine (DPD) indicator, 88% formic acid and sodium thiosulfate were purchased from Sigma Aldrich.
All glassware and equipment used during chlorination experiments were cleaned by soaking overnight in soapy water then in a 5% nitric acid bath, and rinsed with MilliQ water multiple times prior to drying.

3.2.1.1. Chlorine Residual
A series of chlorine residual measurements were taken over an 8-hour period (Table 3.2) to determine when the chlorine residual fell below 0.2 mg/L, which is the chlorine residual standard set by the CDC (CDC, 2014). Since the measured chlorine demand (Cl\textsubscript{dose} – Cl\textsubscript{residual}) of undiluted, raw river water was relatively low (~0.7), indicating possibly low levels of NOM and DBP precursors, the challenge water (river water) was spiked with several dilutions of primary wastewater effluent to better reflect the characteristics of the surface waters being used in POU and household disinfection. The chlorine residual was measured using the Hach Pocket Colorimeter Total and Free Chlorine Kit (Hach, Loveland, CO) or the DPD Titration Method. Stock sodium hypochlorite was calibrated by DPD titration with MilliQ water prior to use. When using the Hach chlorine kit, the colorimeter was blanked with 10 mL of sample water, then DPD Reagent Powder Pillow packets were added to the sample and a free chlorine measurement was taken using absorbance. The DPD titration method was carried out by adding 5 mL of phosphate buffer and 5 mL of DPD indicator together in an Erlenmeyer flask followed by adding 100 mL of the chlorinated sample. The DPD amine is oxidized by small amounts of chlorine to a Würster dye, which is magenta appearance. The red/magenta solution was rapidly titrated with 2.28 mM FAS from a burette or serological pipette, which reduced the magenta free radical species to the original colorless amine. The volume of titrant was converted to concentration (mg/L) of chlorine:
NaOCl $\rightarrow$ Na$^+$ + ClO$^-$ + (NH$_4$)$_2$Fe(SO$_4$)$_2$•6H$_2$O

ClO$^-$ + 2Fe$^{2+}$ + 2H$^+$ $\rightarrow$ 2Fe$^{3+}$ + H$_2$O + Cl$^-$

2 mmol FAS = mmol NaOCl

2 (2.82 mmol/L FAS * L FAS) = 2 * mmol FAS = mmol NaOCl

$\frac{\text{mmol NaOCl} \times \text{mg/mmol NaOCl}}{\text{L NaOCl}} = \text{mg/L NaOCl}$

3.2.1.1.1. NOM surrogate measurements
SUVA$_{254}$, UV$_{254}$ and DOC concentration of three the challenge waters were measured in order to quantify HBQ precursors. SUVA$_{254}$ is a common measure to determine the aromaticity of the water. Samples were filtered with GF/F filters and acidified with hypochlorous acid. UV$_{254}$ and DOC concentration were measured using an Agilent 8453 UV-Vis spectrophotometer (Agilent, Santa Clara, CA) and TOC-VCPH total organic carbon analyzer (Shimadzu Corp., Kyoto Japan). SUVA$_{254}$ was calculated using the following equation:

$$SUVA_{254} = \frac{\text{UV absorbance (UV}_{254}, m^{-1})}{\text{DOC concentration (mg/L)}} \times 100$$

3.2.1.1.1. HBQ formation
Wastewater and river water were adjusted to a pH of 7. Wastewater was then diluted with river water in ratios of 1:10, 1:100 and 1:250. Each of the water mixtures were then dosed with NaOCl (4 mg/L) and stored in 500 mL sample bottles. At each time point, samples were quenched with 0.25% formic acid and stored at 4°C. For LC/MS analysis, all samples were taken out of the cold room (4°C) and brought to room temperature for the solid-phase extraction (SPE) procedure. SPE was conducted following an optimized protocol by the Reckhow lab group. All solvents used during the extraction process were acidified with 0.25% formic acid. DCBQ standards were prepared in acidified
methanol/water (v/v 60/40). 6 mL Water Oasis HLB cartridges with a 200 mg adsorbent were conditioned with methanol and then water prior to mounting onto the SPE vacuum manifold (VAC Elute SPE 24). Due to the high turbidity of the primary treated waste water effluent, samples spiked with waste water effluent were first filtered using a GF/F filter (Fisher Scientific) to prevent the over saturation of the adsorbent. After filtration, 500 mL of sample were loaded onto the cartridges while maintaining steady flow rates. After the cartridges were washed with 6 mL of acidified water and then 6 mL methanol/water (v/v 50/50) solvents. The analyte was eluted with 6 mL of acidified methanol and 0.4 mL of acidified water was added to the collected eluate. The eluate was dried down to a volume of 0.5 mL under nitrogen at 13-20 psi in an evaporator (Zymark TurboVap LV).

Samples were transferred into 250 µL glass inserts (JM, Denver, CO) and 1 µL was injected into the LC for analysis.

An HPLC separation module (Alliance Waters) and a triple quadrupole tandem mass spectrometer (Quattro Micro API-QAA) were used to analyze the samples. A Luna C18 (2) column was used with mobile phase, comprising of 0.25% formic acid in water and 0.25% formic acid in methanol. At a constant flow rate of 0.15 mL/min, 100 µL of extracts was injected with a solvent gradient of 20% of methanol/formic acid solution. After 20 minutes it was increased to 90% and held for 5 minutes, then lowered to 20% at 25 minutes and held for 15 minutes. The instrument was operated under negative electrospray ionization (ESI) conditions with multiple reaction monitoring (MRM). Direct injection and extraction calibration curves were generated with 0, 25, 50, 100, 200 ng/L DCBQ standards and used to calculate DCBQ recoveries (> 70%).
3.2.1.1.1.1. MS2 Enumeration

MS2 is a group I male-specific coliphage that infects *Escherichia coli* (*E. coli*) and other *Enterobacteriaceae* members by attachment to the F-pili. MS2 was used as a model organism of enteric viruses and its log removal was measured after chlorinating raw surface water. The MS2 model is a widely used surrogate measurement for water-borne pathogens, especially viruses, due to similarities in physical structure. The single-stranded RNA bacteriophage MS2 has been used as a surrogate measurement for eukaryotic viruses, such as the norovirus and poliovirus, and has been documented to be more resistant to chemical disinfectants, such as chlorine, which qualifies it as a sensitive indicator organism for assessing the efficacy of water disinfectants on pathogen removal (Tree et al., 2005; Fisher et al., 2010; Hornstra et al., 2012). The aim of this experimental set up was to simulate household chlorine disinfection of contaminated surface water. All MS2 experiments were performed in the laboratory of Dr. Niveen Ismail at Smith College (Northampton, MA).

Raw river water collected from the UMass Mill River Monitoring Facility or directly from the river waterbody was stored in a cold room at 4°C. The river water was brought to room temperature and 100 mL of water was decanted into separate beakers for chlorine residual measurement using the DPD titration method.

Prior to conducting chlorination experiments, the stock concentration of MS2 was enumerated. Bottom-agar plates were prepared with ~10-12 mL autoclaved Tryptic Soy Agar (TSA) containing 1X Amp-Strep antibiotics and *E. coli* *F*~amp~ host. TSA was evenly poured onto 100 x 15 mm petri dishes. After the bottom agar had solidified, the plates
were stored in a 4°C room. Tryptic Soy Broth (TSB) was prepared in DI water and autoclaved at 121°C. 100 µL of “Generation 2” E. coli F<sub>amp</sub> host bacteria was thawed from -80°C and spiked in a conical tube with 25 mL TSB containing 1X Amp-Strep antibiotics (Fisher Scientific). Sterile technique was carefully followed to prevent contamination. The host was placed on a shaker at 37°C and propagated for ~12 h. After overnight incubation, the TSB should have a cloudy appearance, indicating that the host propagated successfully. 50 µL of host was inoculated into fresh 25 mL of TSB with 1X Amp-Strep and returned to shaker in the warm room for ~4 h. The F-pilus is better expressed in E. coli F<sub>amp</sub> during this phase of growth (exponential) rather than the stationary phase. At this time, TSA (Fisher Scientific) was prepared in DI water and boiled, then autoclaved at 121°C. After the E. coli F<sub>amp</sub> finished incubating, it was rested on the bench at room temperature until further use.

The autoclaved TSA was placed in a water bath. After cooling down to 50°C, 1X Amp-Strep antibiotics was added and stirred briefly. Then, ~7 mL TSA was pipetted into 13 mm glass tubes inside the water bath. MS2 was serially diluted in PBS. 50 µL of E. coli F<sub>amp</sub> host was aliquoted into each agar-filled tube and vortexed. Then, 100 µL of 10<sup>-6</sup>, 10<sup>-5</sup>, 10<sup>-4</sup>, and 10<sup>-3</sup> diluted samples were spiked into each tube and vortexed and immediately poured onto bottom-agar plates. A host control and MS2 control (without host) were also included. When the top agar solidified, plates were inverted and incubated at 37°C for 18-24 hours. The next day, plates with 30-300 MS2 plaques were counted. MS2 concentration was determined by the volume-weighted average, adhering to the upper counting limit. The stock concentration was 149 x 10<sup>5</sup> plaque forming units (PFU)/mL.
MS2 inactivation was measured using two concentrations of NaOCl (2 and 4 mg/L) over a 2-8-hour period, depending on the chlorine residual. 1 mL of the MS2 stock was spiked into 100 mL of raw river water, which diluted the MS2 concentration to $149 \times 10^3$ PFU. 200 µL of the spiked river water along with $10^{-1}$ and $10^{-2}$ dilutions in PBS were aliquoted into 1 mL Eppendorf tubes and quenched with sodium thiosulfate (10 mg/L) at the designated time point following the same protocol described above. As a control, enumeration of MS2 in an unchlorinated sample of raw river water was also performed in parallel.

### 3.3 Results

DCBQ formation experiments were designed according to chlorine residual levels over time (Table 3.2). The DCBQ was detected in two POU chlorinated waters: raw river water and a 1:250 dilution of wastewater to river water. DBCQ was not detected in 1:10 and 1:100 wastewater:river water dilutions. In other words, samples with less wastewater had higher concentrations of DCBQ. Specific ultraviolet absorbance ($SUVA_{254}$) was also higher in raw river water, then dropped as the concentration of wastewater increased (and the concentration of river water increased) (Table 3.4). $SUVA_{254}$ values were < 3, indicating the NOM in waters were likely to be non-humic and hydrophilic substances. However, the $SUVA_{254}$ value for the raw river water was relatively close to 3, so it is difficult to characterize the NOM. In addition, high nitrate levels in lower DOC waters may interfere with this measurement, which may explain the inconsistent $SUVA_{254}$ value observed for the 1:100 wastewater: river water dilution.

Previous studies have indicated that biopolymer NOM was a major precursor of DCBQs, however wastewater—likely containing higher levels of biopolymer NOM (Ma
et al., 2001)—did not seem to be a precursor of DCBQ formation. However, due to the high chlorine demand and low chlorine residuals of samples containing wastewater (Table 3.3), it is possible that not enough free chlorine was available to react with NOM and form DCBQ. There is insufficient data to speculate which factors and mechanism(s) contributed to DCBQ formation.

MS2 inactivation in raw river water was observed immediately post-chlorination (2 and 4 mg/L NaOCl), but some plaques were still detected at 2 hours, although below the detection limit. There was a 1.49 log reduction in MS2 immediately after the addition of 4 mg/L of NaOCl and 1.71 log reduction in MS2 after 2 hours. There was a 1.31 log reduction in MS2 immediately after the addition of 2 mg/L NaOCl and 2.37 log reduction after 2 hours. No MS2 plaques were detected at 4 hours (Figure 3.1).

**Figure 3.1.** Log reduction of MS2 with 2 mg/L and 4 mg/L NaOCl in raw river water. Time -1 hours refers to measuring MS2 concentration prior to spiking with chlorine.
UV$_{254}$ absorbance, pH levels, TOC levels, THM and chloroform concentrations of raw river waters were collected on the day of sampling which took place on three separate days during the winter (Table 3.1). These parameters indicated that the raw river water was at a relatively neutral pH level, which is optimal for modelling potable water. Waters that are too acidic or alkaline can affect HBQ formation; HBQs are more stable at low pH levels. UV$_{254}$ and TOC measurements were relatively high, which indicated that the raw river water likely contained HBQ precursors. Similarly, THM and chloroform levels were also detected in the microgram per liter levels, which also indicate the presence of HBQ precursors.

Table 3.1 NOM surrogate measurements. Live measurements of UV absorbance at 254 nm (UV$_{254}$), pH level, TOC, THM, and chloroform concentrations were recorded. The pH values of the Mill River water were frequently measured by a pH meter by personnel at the water monitoring facility, which was reported to be consistently between 6.0 and 8.0. Day 1, Day 2, and Day 3 of sampling took place in December 2017, February 2018 and March 2018, respectively.

<table>
<thead>
<tr>
<th></th>
<th>UV$_{254}$</th>
<th>pH</th>
<th>TOC (ppm)</th>
<th>THM (µg/L)</th>
<th>Chloroform (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.113</td>
<td>6.0 - 8.0</td>
<td>4.29</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.076</td>
<td>6.0 - 8.0</td>
<td>5.31</td>
<td>236</td>
<td>233.1</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.077</td>
<td>6.0 - 8.0</td>
<td>5.30</td>
<td>322.1</td>
<td>314.0</td>
</tr>
</tbody>
</table>

Table 3.2 8-hour 4 mg/L chlorine residual measurements of various challenge waters: undiluted river water, treated primary wastewater effluent diluted with river water in ratios of 1:10, 1:100, and 1:250.
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>River water (no wastewater)</th>
<th>1:10 wastewater</th>
<th>1:100 wastewater</th>
<th>1:250 wastewater</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>0.12</td>
<td>0.19</td>
<td>2.7</td>
</tr>
<tr>
<td>0.5</td>
<td>4.8</td>
<td>0.12</td>
<td>0.13</td>
<td>1.86</td>
</tr>
<tr>
<td>1</td>
<td>3.7</td>
<td>0.06</td>
<td>0.05</td>
<td>0.76</td>
</tr>
<tr>
<td>2</td>
<td>2.7</td>
<td>0.04</td>
<td>0.04</td>
<td>0.55</td>
</tr>
<tr>
<td>5</td>
<td>1.01</td>
<td>0.04</td>
<td>0.05</td>
<td>0.31</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>0.03</td>
<td>0.04</td>
<td>0.21</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>0</td>
<td>0.01</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Table 3.3 MS2 PFU counts. MS2 plaque forming units (PFUs) counted post enumeration various dilutions of MS2 after addition of 4 mg/L NaOCl at different time points. $T_{0(a)} =$ before addition of chlorine, $T_{0(b)} =$ immediately after addition of chlorine, $T_1 =$ 2 hours after addition of chlorine, $T_2 =$ 4 hours after addition of chlorine. “N/A” refers to defective plates that were excluded from enumeration. PFU of various dilutions of MS2 after addition of 2 mg/L NaOCl at different time points are shown.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>4 mg/L NaOCL</th>
<th>2 mg/L NaOCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted (PFU/200 µL)</td>
<td>$10^{-1}$ (PFU/200 µL)</td>
</tr>
<tr>
<td>$T_{0(a)}$</td>
<td>N/A</td>
<td>98</td>
</tr>
<tr>
<td>$T_{0(b)}$</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>$T_1$</td>
<td>19</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 3.2. DCBQ formation. DCBQ concentrations in raw river water (blue) and 1:250 primary wastewater: raw river water (orange) after chlorination (4 mg/L NaOCl). DCBQ was not detected in 1:10 and 1:100 wastewater: river water dilutions (data not shown).

Table 3.4 DOC, UV\textsubscript{254} and SUVA\textsubscript{254} measurements. SUVA was lower in samples that contained wastewater. It also seemed to be decreasing as the concentration of wastewater increased. DCBQ concentration directly correlated with SUVA\textsubscript{254} values, with the exception of 1:100 wastewater: river water dilution.

<table>
<thead>
<tr>
<th></th>
<th>DOC</th>
<th>UV\textsubscript{254}</th>
<th>SUVA\textsubscript{254}</th>
</tr>
</thead>
<tbody>
<tr>
<td>River water</td>
<td>4.36</td>
<td>0.12</td>
<td>2.67</td>
</tr>
<tr>
<td>1:250</td>
<td>4.18</td>
<td>0.08</td>
<td>1.83</td>
</tr>
<tr>
<td>1:100</td>
<td>3.29</td>
<td>0.08</td>
<td>N/A</td>
</tr>
<tr>
<td>1:10</td>
<td>6.03</td>
<td>0.10</td>
<td>1.69</td>
</tr>
</tbody>
</table>

3.4 Discussion
This study should act as methodological guide for the design of future studies aiming to explore POU chlorination and HBQ formation further. However, the results from these preliminary experiments suggest implications for how to design studies to fully explore this issue.

The 2,6-DCBQ concentrations found in this study are in concordance with that of analogous studies in the literature. In other studies, 2,6-DCBQ was detected in water treatment plants that used NaOCl (~135-275 ng/L) (Zhao et al. 2012), chlorinated tap water (~71 ng/L) and chlorinated swimming pool waters (~27-299 ng/L) (Wang et al., 2013). The chlorine residual of the swimming pool waters was between 3 and 5 mg/L and the chlorine doses used at the water treatment plants were not mentioned in the study. Chlorinated NOM waters in this study yielded 2,6-DCBQ concentrations within the range of 56 – 145 ng/L. These preliminary results suggest that POU-treated drinking water using raw river water as source waters may have comparable 2,6-DCBQ concentrations as drinking waters found in water treatment plants and distribution systems in the U.S. and Canada. However, due to the limited number of experimental replicates and large variability associated with the numerous factors that influence DCBQ precursors, no conclusions on human exposure to POU-treated drinking water should be drawn from this data.

DCBQ production was associated with unadulterated raw river water and not found in treated primary wastewater, except at the most dilute ratio of 1:250 wastewater:raw river water. This was contrary to our original assumption that DCBQ formation would be higher in primary wastewater due to the likelihood of larger biopolymer fractions. Our hypothesis was based off of findings from Wang et al. (2014), which reported biopolymer NOM to be the strongest predictor of 2,6-DCBQ formation in raw waters.
However, their original study also mentioned the key role that large humic NOM fractions played in DCBQ formation and that $R^2$ values were much higher for humic substances as precursors of DCBQ when assessing the raw surface waters separately. SUVA$_{254}$ values calculated in this study correspond with observed DCBQ formation trends; unadulterated raw river water had the highest SUVA$_{254}$ value and the lowest value was detected in the least diluted wastewater. This shows that unadulterated raw river water likely consisted of higher levels of aromatic substances, which were able to react with hypochlorous acid to form DCBQ. Another important factor to note is the lower levels of free chlorine in samples containing wastewater (due to higher chlorine demand of wastewater). It is possible that chlorine attack on other organic substances present occurred at a faster rate than with HBQ precursors upon spiking, which out competed the formation of DCBQ. In contrast, the lower chlorine demand of undiluted raw river and 1:250 wastewater:river waters yielded more free chlorine to react with HBQ precursors to form DCBQ.

The removal of MS2 was almost immediate after chlorination with 2 and 4 mg/L of NaOCl and HBQ formation was observed after a couple of minutes of addition of 4 mg/L. Based on this preliminary finding, there does not seem to be a period in which one would not be exposed to either waterborne pathogens or HBQs at the set chlorine concentration and reaction time of the experiments. $\log_{10}$-reduction of MS2 has been observed at much lower levels of different chlorine disinfectants ($\leq0.5$ mg/L) (Zyara et al., 2016), thus conducting this study using lower doses of chlorine would be important for determining the dose-response effect of chlorine on MS2 and the dose threshold for HBQ formation. Technical replicates have not yet been done for MS2 removal in raw river water,
and no experiments have been done to determine MS2 removal in wastewater-diluted water, thus there is little that can be inferred from this preliminary data.

To continue answering this research question requires expanding the parameters of the experimental design to include lower concentrations of chlorine and other sources of raw waters. Source waters from other geographical locations could be used for the purposes of varying NOM and other factors that influence HBQ formation. For example, higher temperatures were found to be significantly associated with higher levels of HBQ occurrence in waters (Wang, 2016). Raw water was collected from the Mill River during the winter time, from a generally cold region of the U.S., which may affect DCBQ production. The cold climate that the raw water was collected from may not model HBQ occurrence in tropical surface waters. Using waters from other milder or warmer locations may more accurately reflect POU chlorination conditions in developing countries, many of which are located in warmer climates.

The scope of this study only examined the relationship between MS2 inactivation and DCBQ formation. Formation of other DBPs and HBQs were not determined. Occurrence of other DBPs and HBQs may be possible in source waters and may not overlap with DCBQ. In other words, there may be other DBPs forming simultaneously with or after DCBQ formation. If other DBPs are formed at the same time or overlap with DCBQ, there could be an exposure to a mixture of DBPs. If there are DBPs that have a slower rate of formation than that of DCBQ, there could also be a subsequent exposure to DBPs even after DCBQs concentrations have declined. The environmental conditions, as well as the kinetics and mechanisms of DBP formation will determine cumulative human exposure to DBPs.
Although this study detected DCBQ in POU chlorinated waters, disinfection is imperative for preventing immediate illnesses and symptoms that may lead to other long-term adverse health outcomes. For example, enteric diseases incurred from inadequate drinking water disinfection via pathogens may cause short-term effects, such as diarrhea, but chronic diarrhea may lead to stunted growth and improper development, possibly leading to increased susceptibility to diseases throughout an individual’s life. Chlorine is a low-cost intervention that is crucial for public health. The findings of this study are not meant to deter individuals from employing POU chlorination, but to guide future research on DBPs in POU chlorination settings.
CHAPTER 4
DISCUSSION AND CONCLUSIONS

4.1 Toxicity of HBQs

In the current study, 2,6-DCBQ demonstrated its ability to cause cytotoxicity in liver hepatoma cells (HepG2) and normal colon epithelial (CCD 841 CoN) cell lines. A linear decline in cell viability was associated with increased DCBQ concentration following a one hour exposure period. A concentration-dependent increase in ROS production was also observed in HepG2 cells following DCBQ exposure. Toxic effects of DCBQ, hydrolyzed DCBQ (OH-DCBQ) and media-transformed DCBQ were compared. Results demonstrated that in both cell lines, OH-DCBQ and media-transformed DCBQ exposures yielded decreased ROS production and enhanced cell viability compared to DCBQ exposure. The toxicological data from this study was in agreement with our hypotheses of 1) concentration-dependent responses to HBQ exposure and 2) reduced toxicity of transformed products.

Experiments from this study used higher concentrations of DCBQ compared to those reported in the literature, however, cell viability was maintained. Cells were examined under the microscope after each experiment to ensure that a majority of cells were still adhered to the bottom of the well. We postulate that the short exposure time was insufficient to elicit significant cytotoxic response, thus higher concentrations of DCBQ were required in order to observe a dose-dependent effect on cell viability.

It was expected that normal colon epithelial cells would be more sensitive to DCBQ exposure compared to HepG2 cells, however, calculated IC$_{50}$ values for the colon
cells (~450 µM) were only marginally lower than the HepG2 cell line (~550 µM). Another unexpected finding from this study was that cells exhibited ≤ 2-fold decrease in viability after exposure to transformed compounds, contradicting previously reported findings (Wang et al., 2014).

Formation experiments showed that 2,6-DCBQ was detected in POU-chlorinated waters: preliminary findings showed that addition of NaOCl (4 mg/L) to various raw waters with different levels of NOM led to formation of DCBQ over a 6-hour period. The highest formation of DCBQ occurred in unadulterated raw river water, which may have had higher levels of HBQ precursors compared to wastewater diluted with river water. Approximately 2-log removal of the model organism, MS2, was achieved upon addition of chlorine (2 and 4 mg/L). The selected doses of NaOCl in these experiments were determined by preliminary chlorine residual tests. The two source waters in which DCBQ was detected had 6-hour chlorine residuals (0.2 and 1 mg/L) that are in line with WHO drinking water guidelines. The CDC has deemed drinking water with up to 4 mg/L as safe—which is the dose chosen for these experiments. The chlorination experiments conducted in this study were modeled realistic conditions of point-of-use disinfection of drinking water.

In vitro studies that have examined 2,6-DCBQ toxicity have primarily used human cancer cell lines (e.g., T24, HepG2 and Caco-2), as well as animal cell lines (e.g. CHO-K1). As noted in Chapter 1, there are certain caveats to using non-healthy human cell lines, especially when measuring a specific endpoint, such as glutathione levels. The current study explored the toxic effects of 2,6-DCBQ on a colon epithelial cell line that has not been previously used to evaluate HBQ toxicity, which may be pertinent to human
health given the possible association between colon cancer and DBP exposure (Hildesheim et al., 1998; Lyon et al., 2010). Toxicity data from this study can expand the toxicological profile of HBQs and further complement current HBQ literature that has used HepG2 cells (Prochazka et al., 2015; Wang et al., 2018).

One-hour IC$_{50}$ values for cytotoxicity and ROS formation were determined for 2,6-DCBQ. This study is the first to report these values; IC$_{50}$ values have only been previously reported for longer exposure periods (≥12 hours). The rapid transformation of DCBQ in media occurs in < 1 hour, thus an incubation of ≥12 hours will have transformed DCBQ products. Since the effects of DCBQ and its transformed derivatives cannot be differentiated, the toxicity of the parent compound cannot be fully deduced. We assumed that by incubating for an hour, there was a greater ratio of parent DCBQ compound to transformed DCBQ compound(s). It was important to evaluate the toxicity of the parent DCBQ compound because of its occurrence in drinking water (Wang et al., 2014; Mohan, 2015). This study also reports novel findings on transformed HBQ compounds. Evidence of media-transformed HBQs is scant and one of the only endpoints that have been used to assess the toxicity of transformed HBQ compounds, including OH-DCBQ, is cytotoxicity. The current study demonstrated 1) ROS production ability of media-transformed DCBQ and OH-DCBQ and 2) relative difference in toxicity between media-transformed DCBQ and OH-DCBQ is negligible.

4.2 Tipping Point: Chlorination vs. DBPs

MS2 inactivation and DCBQ formation results were not in line with our original hypotheses. Due to the physical structure of MS2, resembling that of viruses, resistance
to chlorine was expected. However, in our study, we observed immediate inactivation of MS2 following chlorination. Log$_{10}$-reduction of MS2 was observed at lower chlorine disinfectant levels ($\leq 0.5$ mg/L) in other studies (Zyara et al., 2016). Therefore, repeating this study using lower doses of chlorine would be important for determining the dose-response effect of chlorine on MS2, as well as the dose threshold for HBQ formation. The raw river water had low chlorine demand, thus we expected low levels of NOM as HBQ precursors. However, the opposite was observed (Figure 3.2) and no DCBQ was detected in samples with higher wastewater: river water content (>10%).

4.3 Future direction

There are two major gaps in the current literature on human health effects from HBQ exposure. The first is the information gap of how HBQs are metabolized in the human body, thus much remains unknown about the whole picture of human exposure to HBQs. While determining the toxicity and formation of HBQs is important, a full understanding of exposure is also needed to understand human health relevance. The metabolism of xenobiotics is a key aspect of exposure that is missing from the current literature; we do not have sufficient evidence to show how this compound is biotransformed and which organs are being targeted. To gain a deeper understanding of the toxicity of HBQs, as well as their relationship to disease, the biotransformation of these compounds should be further researched. Numerous novel in vitro and in silico assays have been used to study the metabolism of compounds, such as lab-on-chip experiments that simulate different phases of metabolism (Neuzil et al., 2006). Perhaps future in vitro studies can employ these methods to provide answers to this question.
In \textit{in vitro} toxicity testing, compound-media interactions have not been widely explored. This study demonstrated that there is a clear need to consider DBP kinetics when conducting toxicological analysis. A recent paper that reviewed the metabolic fate of phenolic compounds in cell media identified several newly derived metabolites, including methyl, glucuronide and sulfate conjugate, post-incubation of test compounds in cell medium with and without cells (Aragones et al., 2016). These metabolites had both enhancing and limiting effects on the bioactivity of several cell lines in numerous assays. Thus, effects of compounds could be over- or under-extrapolated or estimated if the transformed products impact cells differently. Since many HBQs are structurally less stable than many phenols, given that they are not aromatic, the kinetics in cell medium and metabolized derivatives should be analyzed prior to \textit{in vitro} toxicity testing.

HBQ precursors may vary depending on the water characteristics of source waters. The water parameters, such as temperature and DOC and NOM levels, should be expanded in future HBQ formation experiments. Raw waters from other geographical locations could be used to more realistically model the source waters being used in POU disinfection in tropical developing countries. The raw water used in this study was collected from New England, during the winter. This water may not be an accurate model for surface water in tropical, developing countries as higher temperatures have been found to be significantly associated with higher levels of HBQ occurrence in waters (Wang dissertation, 2016). HBQ determination using lower doses of chlorine should also be performed.

It has been established that HBQs are likely toxicants that may occur in POU chlorinated waters. These attributes make them a relevant class of DBPs in developing countries. Exposure assessment of vulnerable populations, including infants and children,
should be prioritized. According to the U.S. EPA, infants and children have higher consumption of water per body weight, potentially increasing their exposure of water toxicants (EPA, 1999). The immune systems of children are also less efficient at detoxification. According to the WHO, significant health disparities exist between children in developing countries compared to children in developed countries, which indicates that their immune systems may already be immunocompromised from other environmental insults and diseases (WHO, 2011). These health factors make children in developing countries a potentially vulnerable group to exposure to toxic DBPs like HBQs. Future studies should conduct risk and exposure assessments of HBQs in household- or POU-chlorinated waters by prioritizing potential exposure of children.
4.4 Acknowledgements

LC/MS analysis, NOM surrogate measurements of source waters, and DCBQ kinetics experiments were performed by Aarthi Mohan.
REFERENCES


