Investigating Relationships Between Artificial Sweeteners and Water Quality: Waste Impact Indicators and Disinfection Byproduct Precursors

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Analytical and Environmental Toxicology

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Abstract

"It always seems impossible until it's done"
– Nelson Mandela (1918-2013)

Artificial sweeteners (ASs) are highly stable, man-made sugar alternatives used in many low and no-calorie foods and beverages. The massive consumption of ASs by the general population has resulted in their wide occurrence in waste and environmental waters. Wastewater treatment is not designed to remove ASs and therefore detection of ASs can be used as indicators of human impact on environmental waters. **Chapter 1** includes a critical review of literature on ASs and re-assessment of the specificity and stability assumptions that led to their use as waste-water indicators. I drew attention to accurate characterizing of waste sources before interpreting AS impact on receiving water bodies to account for specificity and natural variation in occurrence. Further, I emphasized the importance of sensitive and reliable analytical methods to facilitate proper utilization of ASs as WW impact indicators.

In **Chapter 2**, a high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method was developed for sensitive (ng/L) detection of two ASs, acesulfame (ACE) and sucralose (SUC) without sample preconcentration. The method was used to investigate the occurrence of ACE and SUC in 10 publicly accessible surface water bodies in the Thompson Region. SUC was only present at two sites while ACE was detected in every sample. Therefore, ACE is a better indicator for future water quality studies on human waste-impact in the Thompson Region surface waters.

In **Chapter 3**, I examined the application of ACE as a urine indicator in recreational waters. Water disinfection is essential to prevent the transmission of waterborne pathogens but unintentionally results in the formation of disinfection byproducts (DBPs). Nitrogenous compounds in urine can react with chlorine disinfectants to form irritating *N*-DBPs in swimming pools. Rapid HPLC-MS/MS analysis identified 100% occurrence of ACE in pool and hot tub samples from two Canadian cities. The recreational water samples contained significantly greater concentrations of ACE compared to the input tap water controls. Approximate estimates of 30 and 75 L of urine were calculated to be required to account for the average ACE in two pools.

In **Chapter 4**, I moved to examine ASs as potential DBP precursors. Previous studies have identified halobenzoquinones (HBQs) as more cyto- and genotoxic *in vitro* than commonly regulated DBPs. I therefore examined a commonly used AS, aspartame, an aromatic dipeptide composed of phenylalanine, aspartic acid, and methanol, as a precursor to 2,6-dichloro-1,4-benzoquinone (DCBQ). Under controlled chloramination conditions, HPLC-MS/MS analysis confirmed aspartame and phenylalanine as precursors of DCBQ. Further, dissolving one AS package (Equal® Original) in a cup of authentic tap water containing residual monochloramine can increase DCBQ concentration. Additionally, the presence of aspartame in water was found to reduce the transformation of DCBQ to HO-DCBQ. Boiling pre-treatment of tap water reduced residual chloramine, increased pH and significantly decreased the concentration of DCBQ.

In **Chapter 5**, I further examined formation of HBQs from aromatic amino acids (AAAs). Determining HBQ precursors is essential to reduce their formation in drinking water. AAAs share structural similarities and are readily found in natural water bodies.

DCBQ and 2,3,6-trichloro-1,4-benzoquinone (TriCBQ) formation was quantified with HPLC-MS/MS after chlorination of each AAA (phenylalanine, tyrosine, and tryptophan). Additionally, water sources high in bromide ions have been found to produce higher proportions of Br-DBP analogues after disinfection. Br-DBP species are more toxic than the corresponding chlorinated compounds. In the presence of bromide, chlorination of all AAAs yielded 2,6-dibromo-1,4-benzoquinone (DBBQ) in addition to DCBQ.

This thesis investigates ASs and their relation to water quality. Ubiquitous occurrence of ACE in B.C. surface waters and Canadian swimming pools and hot tubs provides a baseline for future waste impact studies on these environmental waterbodies. The confirmation of aspartame and phenylalanine as DCBQ precursors under chloramination and AAAs as HBQ precursors under chlorination conditions warrants further investigation into human exposure to DBPs via consumption of beverages prepared with disinfected tap water.

Preface

Parts of **Chapter 1** have been published as Jmaiff Blackstock, L.K.; Wawryk, N.J.P.; Jiang, P.; Hrudey, S.E.; Li, X.-F. Recent applications and critical evaluation of using artificial sweeteners to assess wastewater impact. Current Opinion in Environmental Health & Science. 2019, 7(2): 26-33. Copyright 2018 Elsevier. Available from: https://doi.org/10.1016/j.coesh.2018.09.002 . As the first author of this review I identified the topic concept, reviewed and annotated the recent literature, and composed the manuscript draft. Nicholas Wawryk and Dr. Ping Jiang contributed to the organization of the review outline, preparation of figures and tables, as well as editing responsibilities. Dr. Steve Hrudey and Dr. Xing-Fang Li, the supervisory author, participated in editing, and oversaw the manuscript composition and concept execution.

Parts of **Chapter 2** have been published as Jmaiff Blackstock, L.K.; Wawryk, N.J.P.; Jiang, P.; Hrudey, S.E.; Li, X.-F. Recent applications and critical evaluation of using artificial sweeteners to assess wastewater impact. Current Opinion in Environmental Health & Science. 2019, 7(2): 26-33. Reprinted with permission. Copyright 2018 Elsevier.

Chapter 3 of this thesis has been published as Jmaiff Blackstock, L.K.; Wang, W.; Vemula, S.; Jaeger, B.T.; Li, X.-F. Sweetened swimming pools and hot tubs. Environmental Science & Technology Letters. 2017, 4(4): 149-153. Reprinted with permission. Copyright 2017 American Chemical Society. Available from: https://pubs.acs.org/doi/abs/10.1021/acs/estlett/7b00043 . Further permission related to the material excerpted should be directed to the American Chemical Society (ACS). As the first author of this original research article, I participated in the concept development and method optimization and was responsible for the experimental design, collection of all British Columbia samples, all sample analysis, data interpretation, manuscript composition and editing. Wei Wang trained me to use the analytical instrumentation and interpret the experimental data, as well as guided me in the development of the analytical method and in subsequent troubleshooting throughout sample analysis. Sai Vemula was responsible for collection of Alberta samples and participated in sample preparation before analysis. Ben Jaeger was responsible for sample collection for the Alberta case study samples. As the

supervisory author, Xing-Fang Li participated in manuscript editing as well as concept development and execution. Parts of **Chapter 3** have been published as Zheng, Q.; Jmaiff Blackstock, L.K.; Deng, W.; Wang, H.; Le, X.C.; Li, X.-F. Keep swimming but stop peeing in the pools. Journal of Environmental Sciences. 2017, 53: 322-325. Copyright 2017 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Available from: https://doi.org/10.1016/j.jes.2017.03.006 . The co-authors of this commentary were jointly responsible for the manuscript outline, flow of ideas, written content and revisions.

"Whether you think you can, or think you can't, you're right"

– Henry Ford (1863-1947)

Acknowledgements

"Alone we can do so little; together we can do so much"
– Helen Keller (1880-1968)

To my supervisor and mentor, Dr. Xing-Fang Li, I truly appreciate all of the opportunities you have provided to me throughout my graduate education. I admire the fierce passion you have for the advancement of research in your field. The self-confidence and motivation I have gained to set and achieve goals in my future career were inspired by your leadership and guidance. Dr. Steve Hrudey and Dr. Hongquan Zhang, thank you for your sincere support, aspirational advice, and thoughtful direction while serving as my committee members. I am grateful to Dr. X. Chris Le for providing a platform for graduate students to succeed in the Division of Analytical and Environmental Toxicology (AET). I am very appreciative to all the supportive administrators and faculty in the AET division and the LABMP department, specifically Katerina Carastathis, Dianne Sergey, Cheryl Titus, and Dr. Monika Keelan, for ongoing guidance that kept me on track to achieve important milestones and deadlines each year. Thank you to the countless, supportive professors, and mentors during my undergraduate degree at Thompson Rivers University. Dr. Kingsley Donkor, Dr. Sharon Brewer, and Mr. Kent Watson, your foundational encouragement to engage in research motivated me to invest in this academic journey.

To all current and alumni members of the Li Research Group, thank you sincerely for your continuous constructive encouragement, training and re-training, writing and research collaboration, moral support, and overall friendship. Special thanks to Drs. Wei Wang, Yichao Qian, Jinhua Li, Birget Moe, Yanming Liu, Ping Jiang, Guang Huang, and future Drs. Caley Craven, and Nicholas Wawryk. Thank you to Ms. Xiufen Lu for ongoing laboratory training and support, and assistance in research execution. The ever-evolving group of AET division members continue to forge a welcoming and collegial community on the 10th floor of the Clinical Sciences Building. A round of applause to my not-yet-mentioned extraordinary friends, made along the way at the UofA, for the much-needed ongoing exchange of motivational talks, daydreaming, cheerleading, laughter, and shoulders to cry

on: Evelyn, Ashley, Angela, Ian, Aleks, Rebecca, Michael, and Teresa. I want to recognize the wide-ranging teammates and colleagues, near and far, who together worked tirelessly on research collaboration and publications, or on organizing and executing memorable community projects and events, especially those devoted members 'Women in Science Engineering and Research' Edmonton and the LABMP Graduate Student Group. Cheers to Harvest Pointe Starbucks, where I spent many, many months writing my thesis, building friendships with the baristas, and developing a moderate to severe caffeine addiction. I wish everyone successful and happy futures. I am tremendously honoured and extremely grateful to have received funding support throughout my graduate education. Thank you to the University of Alberta, Alberta Innovates, NSERC, PEO International, and the Dorothy J. and Izaak Walton Killam Memorials.

I would like to express my sincere gratitude to everyone in my community who has supported me throughout this academic journey. Above all, thank you to my parents who always encouraged excellence in any scholarly and extracurricular endeavors. They balanced a strong work ethic and high expectations with unconditional love and support throughout my life. Mom, you are amazing, I love you, thank you forever. Big hugs to the Blackstock family and McFarlane family for ongoing motivation. Thank you to my friends from home (xox - Alison, Karissa, Angelina, and Ashley) for being never too far for a phone call. Finally, and most importantly, thank you to my husband Colten for your selfless dedication and persevering commitment to our future during these 6 years away from our friends, family, and home in B.C. Thank you to the best boys: our son Alec, who has brought overwhelming joy and perspective into our lives since March 14, 2019; and our frug Donny, for all of the woofs, walks, entertainment, and emotional support snuggles since 2016. The endless stream of love, understanding, and encouragement from family and friends helped me focus on the finish line and will not be forgotten.

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a plan, and not quite enough time."
– Leonard Bernstein (1918-1990)

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%RSD	percent relative standard deviation
5HmdC	5-hydroxymethyl-2'-deoxycytidine
8-OhDG	8-hydroxy-deoxyguanosine
AA	ascorbic acid
AAA	aromatic amino acid
ACE	acesulfame
ANOVA	analysis of variance
API	atmospheric pressure ionization
ARE	antioxidant response element
AS	artificial sweetener
BC	British Columbia
BP	2,4-dihydroxybenzophenone
BSO	buthionine sulfoximine
C&EN	Chemistry and Environmental News
$Ca(Ocl)_2$	calcium hypochlorite
Caco-2	epithelial colorectal adenocarcinoma cells
CCD 841 CoN	normal human colon cells
CCL4	contaminant candidate list
CDC	Centre of Disease Control
CE	collision energy
CHO	Chinese hamster ovary cell line
CXP	collision cell exit potential
CYC	cyclamate
DBA	dibromoacetic acid
DBBQ	dibromobenzoquinone
2,6-DBBQ	2,6-dibromo-1,4-benzoquinone
2,5-DBBQ	2,5-dibromo-1,4-benzoquinone
DBDMBQ	dibromodimethylbenzoquinone
2,6-DBDMBQ	2,6-dibromo-3,5-dimethyl-1,4-benzoquinone
DBP	disinfection byproduct
DCA	dichloracetic acid
DCBQ	dichlorobenzoquinone
2,6-DCBQ	2,6-dichloro-1,4-benzoquinone
2,5-DCBQ	2,5-dichloro-1,4-benzoquinone
DCMBQ	2,6-dichloro-3-methyl-1,4-benzoquinone
DIBQ	diiodobenzoquinone

2,3-DIBQ	2,3-diiodo-1,4-benzoquinone
DOC	dissolved organic carbon
DOM	dissolved organic matter
DON	dissolved organic nitrogen
DP	declusterizing potential
DWTP	drinking water treatment plant
EC	(-)-epicatechin
ECG	(-)-epicatechin gallate
EGCG	(-)-epigallocatechin gallate
EPA	Environmental Protection Agency
EPR	electron paramagnetic resonance
ESI	electrospray ionization
FA	formic acid
FAC	free available chlorine
FP	formation potential
GA	gallic acid
GAC	granular activated carbon
GC-MS	gas chromatography mass spectrometry
GSH	glutathione
HAA5	haloacetic acids
HBQ	halobenzoquinone
Br-HBQs	bromohalobenzoquinones
Cl-HBQs	chlorohalobenzoquinones
I-HBQs	iodohalobenzoquinones
HepG2	human liver carcinoma cell line
HLB	Hydrophilic-Lipophilic Balanced co-polymer (Oasis®)
hNSCs	human neural stem cells
HOBr	hypobromous acid
HOCl	hypochlorous acid
HOI	hypoiodous acid
HPLC	high performance liquid chromatography
I-DBPs	iodo disinfection byproducts
IAA	iodoacetic acid
IC	ion chromatography
LOD	limit of detection
LOQ	limit of quantitation
MAC	maximum acceptable concentration

MBA	monobromoacetic acid
MeOH	methanol
MOF	metal organic framework
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
N-DBP	nitrogenous disinfection byproduct
N_2	nitrogen
NAC	*N*-acetylcysteine
NaOCl	sodium hypochlorite
ND	not detected
NDMA	*N*-nitrosomethylamine
NH2Cl	monochloramine
NOM	natural organic matter
Nrf2	nuclear factor (erythroid derived 2)-like 2
OH-HBQs	hydroxy-halo-benzoquinones
OH-DBBQ	3-hydroxy-2,6-dibromo-1,4-benzoquinone
OH-DCBQ	3-hydroxy-2,6-dichloro-1,4-benzoquinone
OH-DCMBQ	3-hydroxy-2,6-dichloro-5-methyl-1,4-benzoquinone
OH-TriCBQ	3-hydroxy-2,5,6-trichloro-1,4-benzoquinone
PAC	powdered activated carbon
PCP	personal care product
PHE	phenylalanine
PPCPs	pharmaceuticals and personal care products
ppt	parts per trillion
PVDF	polyvinylidene fluoride
QSTR	Quantitative Structure Toxicity Relationship
QTRAP	Quadrupole Ion Trap
ROS	reactive oxygen species
Rt	retention time
SAC	saccharin
SC	stem cell
SG	sulfur-glutathionyl
SG-BQ	sulfur-glutathionyl-benzoquinone
SPE	solid phase extraction
SD	standard deviation (also known as ‘St. Dev.’)
SUC	sucralose

SUVA	specific ultraviolet absorbance
SV-HUC-1	human uroepithelial cells
T24	human urinary bladder carcinoma cells
TBBQ	tetrabromobenzoquinone
TetraB-1,2-BQ	3,4,5,6-tetrabromo-1,2-benzoquinone
TetraB-1,4-BQ	2,3,4,5-tetrabromo-1,4-benzoquinone
TCA	trichloroacetic acid
TCBQ	tetrachlorobenzoquinone
TetraC-1,2-BQ	3,4,5,6-tetrachloro-1,2-benzoquinone
o-TCBQ	ortho-tetrachlorobenzoquinone
p-TCBQ	para-tetrachlorobenzoquinone
THM4	trihalomethanes
TIN	total inorganic nitrogen
TOP	Thompson Okanogan Plateau
TOX	total organic halogen species
TriCBQ	2,3,6-trichloro-1,4-benzoquinone
TRP	tryptophan
TRW	Thompson River watershed
TYR	tyrosine
UFLC	ultra-fast liquid chromatography
UN	United Nations
U.S.	United States
UV	ultraviolet
UV_{254}	ultraviolet signal at 254 nm
WHO	World Health Organization
WW	wastewater
WWTP	wastewater treatment plant

Chapter 1

Introduction*

"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less"
– Marie Curie (1867-1934)

1.1 Importance of Safe and Clean Water Sources

Water quality is of utmost importance due to its pervasive use in daily life. Humans rely on water for hydration, food preparation, bathing, and recreational purposes. The United Nations (UN) "Recognizes the right to safe and clean drinking water and sanitation as a human right that is essential for the full enjoyment of life and all human rights".[1] Safe drinking water is defined by the World Health Organization (WHO) as "free from micro-organisms, chemical substances and radiological hazards that constitute a threat to a person's health".[2] Although it is a basic human right, globally 884 million people do not have access to safe and clean drinking water.[3] A 2009 WHO Report on Global Health Risks[4] reported that the incidence of diarrhoeal diseases increased with inadequate access to sanitation, hygiene or water. Globally, unsafe water, sanitation or hygiene is the cause of most (88%) diarrhoeal deaths. Of which, 99% of deaths occur in developing countries and 84% are children.[5]

Both surface and groundwater are used as drinking water sources. In 2010, surface and groundwater was used by approximately 213 and 104 million U.S. citizens, respectively.[6] In Canada, 90.2% of municipal water distribution systems used surface water while 9.8% came from a ground water source. In 2009, 10.5% of the Canadian population obtained their water from private wells.[7] Surface water is found in lakes, rivers and wetlands as well as water bound up in snow, ice and glaciers.[8] Alternatively, ground water not in direct contact

* Parts of **Chapter 1** (i.e., **Sections** of **1.2.2** Critical Evaluation of Indicator Characteristics) have been published as Jmaiff Blackstock, L.K.; Wawryk, N.J.P.; Jiang, P.; Hrudey, S.E.; Li, X.-F. Recent applications and critical evaluation of using artificial sweeteners to assess wastewater impact. Current Opinion in Environmental Health & Science. 2019, 7: 26-33. Reprinted with permission. Copyright 2018 Elsevier.

with the atmosphere.[9] Beneath Earth's surface, water can be contained in pores,[10] in the form of soil moisture, or as water stored in aquifers.[8] Aquifers are geological formations of sand, gravel or permeable rock that can store and transmit water[8] and are protected from surges of natural organic matter (NOM) present in run off.[11] NOM is ubiquitous in natural waters[12,13] as a complex mixture, varying both regionally and temporally, that can be impacted by the surrounding environment.[14] For example, spring run-off causes large volumes of agriculturally impacted organics to be mobilized into the surrounding watershed or after increasingly common climate change events like wildfire followed by heavy rainfall and flooding over eroded soils.[15] By adsorbing to sediment, pathogens can travel in water sources.[16]

Exposure to pathogens present in untreated water can result in gastrointestinal distress and even in some cases death.[17,18] *Cryptosporidium*, *Giardia*, and *Escherichia coli* (E. coli) O157:H7 are a few examples of pathogens[19] commonly found in lakes and rivers especially those contaminated with animal and/or human fecal waste.[17,18,20] Although the majority of deaths from preventable diarrheal diseases occur in developing nations, [4,16,21] fatal waterborne disease outbreaks continue to occur in affluent nations when major contamination incidents and subsequent treatment failures take place at waste and/or drinking water untilities.[21–23] For example in 1993, sewage contaminated a drinking water source intake in Lake Michigan, which led to a *Cryptosporidium* outbreak in Milwaukee affecting an estimated 400 000 residents with mild to severe cases of gastrointestinal illness and contributed to over 50 deaths among immune compromised individuals in the following 2 years.[21,22,24,25] In 2000, heavy rainfall in Walkerton Ontario, Canada, led to minor *Campylobacter* and major *E. coli* O157:H7 contamination of the drinking water source by agricultural manure, resulting in 2300 cases and 7 deaths.[21,26] Since 2000, 24 outbreaks have been reported in affluent nations, including Canada, the U.S., Sweden, Spain, Norway, Iceland, Finland, Ireland, Switzerland, Montenegro, England, Denmark, and Greece.[27] Most recently, *Campylobacter* in sheep manure contaminated ground water in Havelock North, New Zealand causing 4 fatalities and an estimated 5500 cases of illness in 2016.[27] These outbreaks underline the acute importance of drinking water treatment through appropriate disinfection. To ensure safe water requires comprehensive understanding of the potential

contamination sources and subsequent treatment strategies for individually unique raw water sources. Therefore, it is necessary to develop detection methods for monitoring water contaminants and investigating treatment strategies.

1.2 Waste Indicators

As urbanization continues, ground and surface water bodies are increasingly impacted by wastewater (WW),[28] which has been contaminated with human, industrial, agricultural and/or animal waste,[29] affecting environmental and drinking water quality. Waterborne pathogens, shed in untreated fecal waste, can cause gastrointestinal distress and even death.[30] Nitrogen and phosphorus in nutrient rich WW effluent can result in eutrophication of aquatic environments[31] leading to potentially toxic cyanobacterial blooms and taste and odor issues.[32] Additionally, industrial and municipal wastes can contain environmental pollutants ranging from salts[33,34] and organic chemicals[35] to artificial sweeteners (ASs) as well as pharmaceuticals and personal care products (PPCPs).[36] Pristine fresh water sources continue to decrease, demanding better understanding and management of WW and its impact on the environment and our drinking water.[37] Municipal, industrial and some agricultural wastes are managed by WW treatment plants (WWTPs) to reduce the risks of discharge in surrounding water bodies. However, some anthropogenic contaminants, such as PPCPs and ASs, do not occur naturally in the environment and are incompletely removed by current WWTPs.[38] Using specific indicators of WW to trace impact in surface and ground waters can inform future treatment strategies designed to mitigate negative WW impacts in aquatic environments and to ensure safe, high quality drinking water.

1.2.1 Artificial Sweeteners as Wastewater Indicators

An ideal indicator for tracing WW impact should: 1) be specific to its source; 2) resist removal by WWTPs; 3) be quantifiable with sensitive methods capable of detecting change in receiving waters; 4) occur in WW at concentrations higher than background environmental levels; and 5) undergo negligible attenuation in the receiving water body.[39,40] Various contaminants previously used as indicators of WW contamination (e.g., PCPPs) have met a variety of challenges.[41] For example, pharmaceuticals often occur at trace levels with varying occurrence in municipal WW[42] because of differences in prescriptions and usage; caffeine

can degrade in the environment, and chloride ions are non-specific with seasonally confounding sources (e.g., road salts, fertilizer). Different anthropogenic contaminants, such as drug metabolites or X-ray contrast media, are being proposed[43,44] for their prospective improvement on inherent limitations with known WW tracers.

ASs have received increasing attention for both their potential and effective use as WW indicators[45–47] in ground and surface waters. In general, ASs originate from a specific point source, have high occurrence in WW, generally resist removal by WWTPs and environmental transformation processes, and have established, sensitive detection methods.[46,48] There are continuous advancements in understanding ASs from source to sample. Here, I highlight unique applications using ASs to assess WW impact on different water bodies. Additionally, I identify key AS characteristics evaluated in recent literature to be considered in future investigations.

1.2.2 Critical Evaluation of Indicator Characteristics

1.2.2.1 Specificity of ASs to WW

ASs are widely consumed[49] because of their pervasive use in processed foods, beverages, and pharmaceuticals. In the United States, 25% of children and 41% of adults reported consuming low-/no-calorie sweeteners.[50] ASs can be classified as either nutritive (e.g., fructose, isomalt, malitol, xylitol) or non-nutritive (i.e., acesulfame (ACE), sucralose (SUC), cyclamate (CYC), and saccharin (SAC); **Figure 1.1**) based on their ability to be metabolized and consequential caloric value.[51] Nutritive, low-calorie sweeteners, including sugar alcohols (e.g., steviol, malitol, or xylitol) or peptide-based compounds (e.g., aspartame or thaumatin) are broken down during digestion[52,53] and therefore are not introduced into WW. Due to the metabolism of these low-calorie nutritive sweeteners, they do not meet the criteria of effective waste indicators. Non-nutritive sweeteners are not metabolized, excreted unchanged in urine and/or feces (ACE: urine >99%; CYC: mixed urine and feces, SAC: urine 95% and feces 5%; SUC: 15% urine, 85% feces),[51,52,54] and inevitably transported to municipal WWTPs. Since ASs are not naturally occurring in the environment, their anthropogenic specificity is ideal to trace domestic WW. A major change in AS consumption, leading to the cessation of use in a population may impact its ability to trace WWTP effluents, but could still be used to monitor movement of legacy waste sources (i.e., septic tanks or

landfill leachate). However, a recent review of the global intake of low- and no-calories sweeteners found no evidence of a shift in artificial sweetener intake over time.[49]

Municipal WW is not the exclusive source of ASs in the environment. Landfill leachate can contain non-nutritive ASs that may contaminate ground and surface water.[55] Furthermore, in addition to human consumption, ASs are also used in agricultural operations. SAC, CYC, and ACE have been reported as components of livestock (i.e., piglet) feed in Europe[56] and China.[57] Ma *et al*. reported high concentrations of ASs in soils fertilized with AS-rich pig manure. Yet, this study found that AS concentrations decreased with soil depth, indicating a lower potential for ASs to contaminate groundwater.[57] While domestic WW is the major source of AS contamination in environmental waters, taking note of nearby landfills and agricultural livestock operations can eliminate potential confounders.

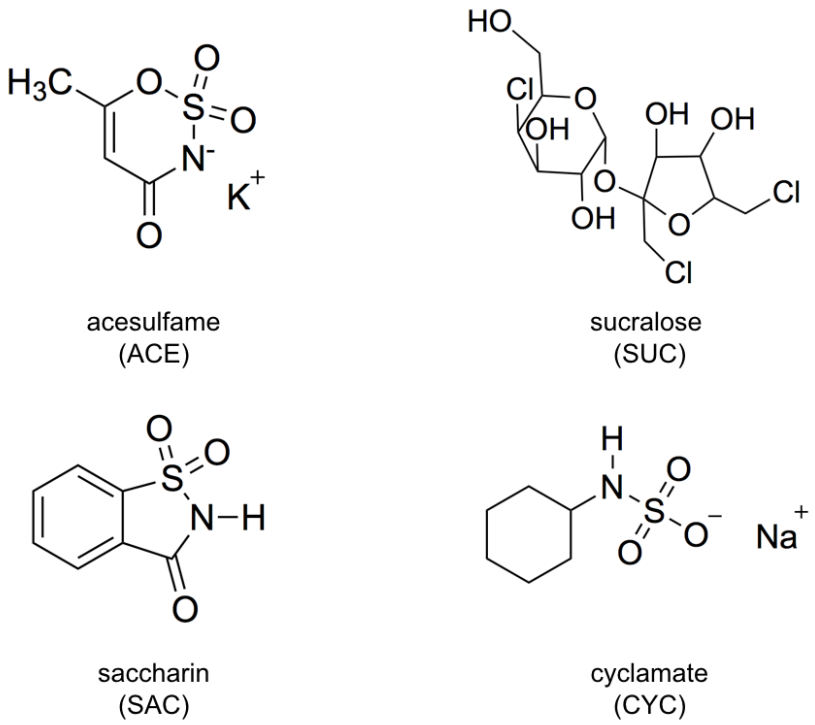

Figure 1.1 Structures of non-nutritive artificial sweeteners: ACE, SUC, CYC, and SAC; commonly investigated as human waste indicators in environmental waters

1.2.2.2 Removal of ASs by WWTPs

When a contaminant is eliminated or significantly removed by a WWTP, it is likely not detectable in water samples, thereby restricting WW impact assessment viability. While SAC and CYC are consistently observed to be almost completely removed,[45,58] previous studies found SUC and ACE to be stable throughout WW treatment processes.[59] However, recent research development has provided evidence that ACE may be susceptible to biologically mediated degradation (**Table 1.1**).[58,60–64] Interestingly, Kahl *et al*. suggested that the increased removal of ACE through WWTPs could be due to an evolution in the capability of microbial degradation processes[63] and indicated the microbial families: *Phyllobacteriaceae, Methylophilaceae,* and *Bradyrhizobiaceae*, as being involved in ACE metabolization or degradation.[64] Recent studies have also investigated the effects of ultra-violet (UV) irradiation on the fate of ASs.[65–69] Perkola *et al*. found that UV irradiation degraded ACE, SUC, SAC, and CYC, with ACE degrading three orders of magnitude faster than the other ASs.[69]

Overall, AS concentrations can be altered by different WWTP processes. Therefore, negligible removal of ACE or SUC from municipal WW influent should not be assumed, and concentrations of ASs in effluent must be used to accurately assess WW impacts downstream. Although removal of ASs by water treatment processes is evident, as long as the concentration of ASs in WW effluent is enough to be detectable, ASs could be utilized as WW impact markers in drinking water sources.

Table 1.1 Selected examples reporting evidence of ACE removal by WWTPs through biologically mediated degradation in recent literature

Treatment process	Experimental Scale	Percent Removal	Notes	Reference
biodegradation	5 WW treatment plants	13-28%	- denitrification under anoxic conditions yielded the highest removal efficiency for ACE	Li *et al.* 2018[58]
biodegradation	9 WW treatment plants	>85%	-indicated three microbial families commonly found in soils as being involved in ACE metabolization or degradation - increased removal of ACE could be due to an evolution in the efficiency of microbial degradation processes	Kahl *et al.* 2018[64]
biodegradation	13 WW treatment plants	59%-97%	-degradation of ACE occurred in both activated sludge and sand filters under oxic and denitrifying conditions but was not removed under anaerobic conditions in the absence of both dissolved oxygen and nitrate	Castronovo *et al*. 2017[62]
3-step treatment including: physical removal of solids, biological processing, and chlorine-based disinfection	1 WW treatment plant	>90%	-biological process includes four oxidation ditches with an aerobic and anaerobic zone followed by clarifiers	Cardenas *et al.* 2016[60]
activated sludge with oxic or anaerobic post treatment	1 WW treatment plant and laboratory scale	>60%	-removal of micropollutants in real municipal wastewater samples was investigated in 12-L sequencing batch reactors	Falas *et al.* 2016[61]

1.2.2.3 Methods for Determination of ASs

An indicator of WW contamination should be detectable by analytical methods capable of quantifying minor changes in concentration at realistic environmental concentrations. An in-depth overview of physiochemical properties and analytical methods for ASs has been compiled by Lange *et al.*[59] High performance liquid chromatography (HPLC) or ion chromatography (IC) separation coupled with electrospray ionization mass spectrometry (ESI-MS) detection are sensitive and reliable technologies commonly used to determine ASs (**Table 1.2**). The IC-MS method provides AS detection limits at sub to low µg/L levels.[46] Incorporating large volume injection apparatus[70] or solid phase extraction (SPE) preconcentration[48] with LC-MS have lowered detection limits down to sub to low

ng/L. Deuterated internal standards (i.e., acesulfame-d4, sucralose-d6, saccharin-d4, cyclamate-d10) are often used as controls for accurate interpretations of a specific signal. Typically, SPE is used to both clean up complex WW samples and to pre-concentrate the analytes. However, tedious sample preparation or manual injection steps limit sample throughput desired for routine monitoring. Overall, the highly sensitive MS technology is proven to be capable of detecting trace concentrations of ASs in different water sources.

Table 1.2 Selected examples summarizing commonly used analytical methods for the determination of ASs in environmental samples

Analyte LOD (ng/L)				**Analytical Methodology**			**References**
ACE	**SUC**	**CYC**	**SAC**	**Sample Preparation**	**Separation**	**Detection**	
8	21	3	5000	No Sample Prep	IC	ESI MRM	Van Stempvoort *et al*. 2013[47] Snider *et al.* 2017[77]
0.3	10	0.6	1	SPE	HPLC	Z-spray ESI MRM	Tran *et al.* 2013[48] Yang *et al. 2018*[42]
10	200	20	30	2 stacked SPE	HPLC	API SRM	Buerge *et al*. 2009[45]
0.2	5	N/A	N/A	500-µL large volume LC injection	HPLC	ESI MRM	Wu *et al*. 2014[254]
0.2	N/A	N/A	N/A	No Sample Prep	HPLC	ESI MRM	Blackstock *et al.* 2017[78]
N/A	200	N/A	N/A	Online SPE	HPLC	ESI MRM	Prescott *et al*. 2017[71]
3	N/A	N/A	N/A	No Sample Prep	HPLC	MS/MS	Kahl *et al.* 2018[64]
10	50	10	10	SPE	UPLC	ESI MRM	Li *et al.* 2018[58]
0.28	4.2	0.52	0.63	SPE	LC	ESI MRM	Ma *et al.* 2017[255]
0.3	N/A	0.3	N/A	SPE	HPLC	ESI-MS/MS	Zirlewagan *et al*. 2016[256]
Range 0.015-23 ng/L				SPE	HPLC	MS	Watanabe *et al.* 2016[72]

1.2.2.4 Occurrence of ASs in WW and Environmental Water Bodies

To use ASs as quantitative tracers of WW sources, the concentration and profile of ASs must be well characterized both in the monitoring environment as well as at each source. ASs have been found in municipal WWTPs globally, but because both geographical and

temporal variation have been identified, the concentration in any particular WW source cannot be assumed. WW studies in different countries have reported varying AS concentration profiles.[36] In some cases ACE and SUC occur more frequently and in greater concentrations than CYC and SAC in North America.[46,47,71] Whereas in other studies, CYC and SAC have been detected more frequently in higher proportions in some samples from Asian countries.[41,58,72] Differences in ASs approved by regulatory agencies and subsequent consumption habits around the world likely influence this variation.[49,73–75] Temporal variation in WW AS concentrations have been reported in multiple countries. In Indian WWTP influent, daily concentration spikes of ACE in the morning and SUC in the evening corresponded with mealtimes.[76] Seasonally, concentrations of ASs, specifically CYC and ACE were found to decrease in untreated Canadian domestic septic WW during summer months.[77] Similarly, in China, concentrations of ACE, SUC, CYC, and SAC in all five WWTP influents studied were greater in winter.[58] Therefore, care should be taken to ensure that the concentrations of ASs being used to assess impact on affected waterbodies reflect the source and are consistent with the time environmental samples are collected.

An indicator of WW impact is effective only if it is detectable in receiving water bodies. ASs have been classified as environmental contaminants[59] and are continually being reported in new locations.[42,70,72,76,78] For the first time, ASs were reported in South Asian surface and ground waters.[72] ACE, SUC, SAC, and CYC were found in surface waters at the highest concentrations in the Philippines, followed by Vietnam and Myanmar. The highly variable concentrations of ACE observed along closely spaced groundwater sample sites in Vietnam indicated contamination from domestic septic tanks.[72] Furthermore, AS concentrations have been frequently detected at concentrations similar to, or greater than, other proposed WW indicators (i.e., PCPPs).[36,41,44,47,48,76] In South China, of 93 PCPPs and five ASs investigated, the three compounds detected in groundwater with highest occurrence and concentration were ACE, CYC, and SUC.[42] In surface waters, SUC was proposed as the most effective indicator of WW due to its frequent occurrence. Importantly, Yang *et al*. noted that temporal trends in surface water concentrations may be observed in regions with distinct wet and dry precipitation seasons. ASs should still serve as effective WW indicators even

with ongoing use and dispersal through the environment, as long as they continue to occur in WW at concentrations higher than background environmental levels.

1.3 Drinking Water Treatment

In 2007, 95% of Canadians had access to treated municipal water supplies. Of those households, 59% reported to drank water from the tap.[79] Providing clean and safe drinking water of high quality is an ever changing and complex issue for government agencies and drinking water treatment plants (DWTPs). Disinfection is essential to prevent the transmission of waterborne pathogens[80]. The U.S. Environmental Protection Agency (EPA) requires that the concentration of viruses and bacteria are reduced by a factor of 10000. However, disinfectant dosage and contact time varies depending on the mixing efficiency and the number of pathogens present in the source input water.[81] Approximately 57000 of the 63000 total DWTPs in Canada and the United States rely on chlorine chemistry for their operations.[81] Chlorine is inexpensive, effective, and readily available.[82] Chlorine acts as a moderate oxidizer.[83] The lower the pH of the chlorine, the more effective it is at disinfection. Chloramines are often employed as a secondary disinfection step because they provide longer-lasting residual protection against pathogens.

Before disinfection, DWTPs implement a variety of strategies to remove NOM from source water to limit DBP formation.[14,84] A coagulant can be added to source water to remove particulate NOM by colloid destabilization and dissolved organic matter (DOM) by precipitation or coprecipitation.[14] Flocculation is the aggregation to combine small particles together into larger particles called ‘flocs’.[83] Sedimentation occurs when the flocculated particles settle out of the water over time.[85] Filtration physically removes solid material from water by trapping particles in a filtering medium until the medium is saturated and must be cleaned or replaced.[83] Granular and powdered activated carbon (GAC and PAC) can remove dissolved NOM through adsorption.[14] However, current treatment technologies cannot completely remove NOM from source water. Inevitably, a portion of NOM from the raw drinking water source will be present during disinfection.

1.4 Drinking Water Disinfection Byproducts

The disinfectants used to inactivate pathogens react unintentionally with NOM present in source water. This leads to the consequential formation of disinfection byproducts (DBPs).[86] The nature and concentration of DBPs formed is dependent on the composition of source water as well as the disinfection conditions.[87] Water consumption is a major route of exposure to DBPs. Canadian adults reported drinking approximately 970 – 1360 mL of water each day.[88] Similarly, plain water and beverages made up 33% (i.e., 1056 mL) and 48% (i.e., 1536 mL) of the approximate 3.2 L of total water consumed daily by American adults, respectively.[89] Epidemiological studies have consistently identified a potential association between long-term chlorinated water consumption and increased bladder cancer risk.[86,90,91] Bladder cancer is the fourth most common cancer in men and three times more likely to occur in men compared to women in the United States.[92] Bladder cancer risk factors aside from DBPs,[90,93–96] include smoking,[90,97] obesity,[97] and arsenic in drinking water.[98,99] Additional detrimental health effects associated with DBP exposure that have been identified from population level studies, albeit less consistently than bladder cancer, include adverse pregnancy outcomes such as miscarriages, preterm delivery, and low birth weight.[100,101] Mixed evidence for association with colon and rectal cancers has been inconclusive.[101,102]

In efforts to reduce overall DBP exposure, Health Canada regulates the maximum acceptable concentration (MAC) for several organic and inorganic DBPs in drinking water.[103] These include total trihalomethanes (THM4; includes chloroform, bromoform, chlorodibromomethane, and bromodichloromethane): 0.100 mg/L, total haloacetic acids (HAA5; includes monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, monobromoacetic acid, and dibromoacetic acid): 0.08 mg/L, and N-nitrosodimethylamine (NDMA): 0.00004 mg/L. Inorganic DBPs regulated by Health Canada include bromate (0.01 mg/L), chlorate (1 mg/L) and chlorite (1 mg/L). The U.S. EPA has similar guidelines[104] with the exception of NDMA, which is regulated by some, but not all States and is currently included on the fourth Contaminant Candidate List (CCL4).[38,105]

The limited suite of DBPs with maximum contaminant limits in drinking water, regulated by the U.S. EPA, Health Canada, and similar agencies in other regions, do not have the specific toxicity required to account for the observed bladder cancer risk.[91,93] The

regulation of THM4 and HAA5 is intended to serve as indicators of exposure to complex mixtures of DBPs present in drinking waters.[87,91,104] However, due to differences in formation conditions for various DBP classes, it is possible that the toxicological drivers associated with the increased adverse health effects are not being accounted for when only the regulated DBPs are considered.[91] Over 700 halogenated DBPs have been reported in drinking water,[106,107] however approximately 70% of total organic halogenated (TOX) species formed after disinfection remain unknown.[108]

Subsequently, research focus has shifted to identify unknown and unregulated DBPs, and their precursors, of toxicological relevance to better understand the potential increased risks observed in epidemiological studies. Interestingly, *in vitro*[109,110] and *in vivo*[111] studies have identified a trend in the toxicity of halogenated DBPs: iodinated > brominated > chlorinated. Source water containing high levels of bromide and iodide can produce higher proportions of the more toxic Br- and I-DBP analogs.[112,113] New DBP compounds, identified in novel sample types are continuously be reported. Furthermore, investigations continue to determine their precursors and strategies to reduce their formation and subsequent exposure.[107,114]

1.5 Recreational Water Disinfection Byproducts

Recreational water bodies (i.e., swimming pools and hot tubs) shared by the public can lead to transmission of waterborne pathogens. Swimming pools have been implicated in over 11000 cases of illness and over 70 cases of waterborne outbreaks between 1971 and 2000.[115] Various disinfection strategies are employed to keep recreational waters safe from pathogens. Chlorination based disinfectants, including chlorine gas (Cl_2), sodium hypochlorite (NaOCl), and calcium hypochlorite ($Ca(Ocl)_2$) are most commonly used.[116,117] Hot tubs are often managed with bromine based disinfectants[117] due to greater stability under high temperature conditions compared to chlorine.[118] Outdoor pools utilize halogenated organic solvents such as trichloroisocyanuric acid, dichloroisocyanuric acid, and bromochlorodimethylhydantoin to stabilize chlorine under the intense UV exposure from sunlight.[119,120] Finally, alternate non-halogenated disinfectants such as UV or ozone have been combined with traditional disinfectants (e.g., UV + chlorine) to minimize the formation

of DBPs while also providing a disinfectant residual.[121,122] Over the past 20 years, the majority of DBP research has focused on drinking water compared to recreational waters.[117]

When swimming, many different contaminants can be introduced into swimming pool and hot tub waters including: personal care products (e.g., lotions, sunscreens, shampoos and conditioners), bodily fluids (e.g., sweat and excreta (i.e., urine)[123]) as well as organics in outdoor pools (e.g., leaf litter, dirt).[117,124] Contaminants in recreational waters can reach high concentrations due to relatively low dilution factor coupled with recirculation of existing water, with freshwater input only to replace evaporation and splash out.[117,125,126] A 60 second shower has been shown to significantly reduce the amount of pollutants introduced into swimming pools.[127] Additionally, some bodily fluids are an avoidable introduction into a shared water body. Several studies have approximated the amount of urine swimmers contribute to swimming pool waters. The reports have similar volumes ranging from 25-1760 mL/bather).[128–131] High volumes of sweat, ranging from 200-1000 mL/bather[128,129] released at rates from 0.04- 0.8 $L/m^2/h$[132] led to actual pool concentrations approximated as roughly 200 mL of sweat and 50 mL/m^2 of swimming pool water.[133]

DBP concentrations detected in recreational waters are much greater than drinking water,[117,134–136] leading to higher potential exposure levels.[137,138] Three major exposure pathways to DBPs through swimming include inhalation of volatile compounds or aerosolized solutes, dermal adsorption, and oral ingestion of swimming pool water.[117] The physiochemical properties of DBPs impact their major route of exposure. The volatility and skin permeability of HAAs was found to decrease as polarity increased[139]. HAAs can be present on aerosols[140] with concentrations up to approximately 65 $\mu g/m^3$ in indoor swimming pool air.[141] Inhalation and dermal absorption are the major exposure routes of highly volatile THMs.[95,142] Furthermore, the mutagenicity of total extracted halogenated organics is greatest in hot tub waters than swimming pool waters with both significantly higher than corresponding tap water controls[143]. Individual nitrogen containing components in urine and sweat such as urea, ammonia, amino acids and creatinine, have been found to react with disinfectants present in swimming pools to form DBPs including trihalomethanes, haloacetic acids, haloamines and halonitromethanes.[144,145] Exposure to volatile DBPs, specifically

trichloramine, in in-door swimming facilities can lead to eye and respiratory irritation[146–148] and has been linked to the development of occupational asthma.[149]

Unlike drinking water, where the maximum level of specific DBPs have been regulated consistently internationally by various health organizations (e.g., The WHO,[2] U.S. EPA,[104] and Health Canada[150]) the regulation of DBPs in swimming pool waters is limited to a few European countries[117]. Germany[151], Switzerland, and Denmark[136] require THMs or chloroform to stay below MCLs less than 50 µg/L, whereas France,[152] Belgium, Finland and the U.K. limit THMs to less than 100 µg/L.[136] The wide diversity and high concentration of DBPs in recreational waters necessitates increased research focus on both comprehensive detection methods and innovative strategies to monitor overall exposure. Additionally, efforts to reduce the introduction of additional organic materials (e.g., bodily fluids, PCPPs, dirt, etc.) into recreational waters through appropriate swimmer hygiene practices would limit the total DBP precursors available to react with necessary disinfectants.

1.6 Food Preparation Disinfection Byproducts

Food is one of the greatest pathogen exposure routes in the U.S. resulting in an estimated 76 million foodborne illnesses, including 325000 hospitalizations and 5000 deaths, per year.[153] DBP formation during food processing and preparation is an emerging field of significance as an additional, non-conventional route of human exposure.[154] The majority of U.S. foodborne pathogen outbreaks are associated with leafy vegetables.[155] Consequently, processed vegetables often undergo disinfection wash using a high concentration disinfectant,[156,157] resulting in the formation of DBPs.[158] In simulated lettuce wash with chlorinated water, Shen *et al*. reported high concentrations of THM and HAA formation,[159] while Lee and Huang detected formation of THMs, HAAs, C- and N-DBPs (including nitrosamines), and aldehydes in their investigation of hypochlorite and peracetic acid sanitizers.[160] Recent reports have identified 3-chloro- and 3,5-dichlorotyrosine, as well as volatile DBPs in chlorinated spinach and lettuce wash water at concentrations greater than disinfected drinking water.[161] THMs have been found in foods by effect of the accumulation and sorption from packaging materials, from cleaners and disinfectants used on processing equipment, in addition to formation during processing rinses and washes.[162]

DBPs can also be formed in foods and beverages prepared with disinfected water. Residual chlorine (e.g., from hypochlorite (HOCl) or monochloramine (NH_2Cl)) are often added to finished drinking water to ensure that potential contamination does not occur in the distribution system to maintain water quality before it reaches the municipal consumer's tap.[103,104,163] The residual chlorine, up to 4 mg/L in American, or 2 mg/L in Canadian tap water can react with organics during food[162] and beverage preparation.[154,164,165] Coffee and teas are regularly consumed in North America,[166,167] and around the world.[168,169] Wu *et al.* identified elevated TOX formation in instant teas in 1998.[164] A few studies have since determined DBPs in teas and coffees including THMs,[162,170,171] and recently halobenzoquinones (HBQs).[172] Along with the conventionally studied drinking water DBPs,[154] Pan *et al*. determined the formation of emerging iodinated-DBPs (I-DBPs) in simulated chlor(am)ine . Cook water, and highlighted hypoiodous acid production with the addition of iodine from iodized table salt.[173] More recently, Zhang *et al.* detected and characterized 25 brominated-DBPs (Br-DBPs) in cook water prepared with a variety of edible salts.[174] Toxicity attributed to Br-DBPs decreased after cooking preparations (boiling).[174] In contrast, Pan *et al.* reported an increase in polar phenolic I-DBPs with increasing cooking temperature.[173] Pre-treatment of tap water by boiling reduces residual chloramine concentration[175] and has been identified as a simple strategy to reduce volatile DBPs.[57,176]

Huang and Batterman reviewed the occurrence of THMs identified in various prepackaged foodstuffs (e.g., selected baked goods, grains, caned sodas, meats and cooked vegetable dishes, and dairy items).[162] Additionally, Gallego and Jose Cardador detailed available reports of THM and HAA occurrence in commercial beverages (e.g., pure and concentrated juices, nectars, and sodas).[154] Interestingly, the addition of common food additives, ascorbate and sodium carbonate, were found to enhance the reduction of TOX in tap water by an additional 28% and 36% compared to boiling alone.[177]

1.7 Halobenzoquinone Disinfection Byproducts

Halobenzoquinones (HBQs; **Figure 1.2**) are a class of compounds structurally defined as a quinone, an unsaturated six-membered, carbon ring with two carbonyl groups attached either at the 1,2- (i.e., ortho) or 1,4- (i.e., para) positions, with one or more halogen

species (i.e., chlorine, bromine, or iodine) bound on the remaining carbon atoms. HBQs have been identified as unregulated DBPs of toxicological importance that may contribute to the epidemiologically observed adverse health outcomes.[96] Quantitative structure toxicity relationship (QSTR) analysis predicted HBQs to be plausible bladder carcinogens.[96,178] Some HBQs are up to 1000x more cytotoxic than the regulated DBPs (i.e., THM4, HAA5).[110,179,180] Since HBQs were first detected in treated tap water as DBPs in 2010,[181,182] they have remained a relevant group of compounds garnering emerging interest with developments in occurrence and toxicity continuously reported in the literature.

HBQs are not stable, they can undergo oxidation reactions in water to form more stable hydroxyl-halo-benzoquinones (OH-HBQs).[179] In neutral (i.e., pH 7) aqueous solution, 2,6-DCBQ has a half-life of 6-7 hours.[182] The major transformation product was identified as is 3-hydroxy-2,6-dichloro-1,4-benzoquinone (OH-DCBQ). Like 2,6-DCBQ, 2,6-DBBQ, TriCBQ, and DCMBQ were all found to transform to their respective hydroxylated transformation products (i.e 3-hydroxy-2,6-dibromo-1,4-benzoquinone (OH-DBBQ), 3-hydroxy-2,5,6-trichloro-1,4-benzoquinone (OH-TriCBQ), and 3-hydroxy-2,6-dichloro-5-methyl-1,4-benzoquinone (OH-DCMBQ).[179] After reaching equilibrium, the OH-HBQ transformation product was stable for over 60 hours. Decreasing the sample pH (e.g., with formic acid) preserves HBQs in solution.[179,181,183] HBQs also readily transform into HO-HBQs under UV-irradiation.[184] Based on existing literature on BQ phototransformation,[185] Zhao *et al.* proposed the HBQ UV-transformation pathway to include the photo decay of HBQ to halobenzenetriol followed by sequential reaction steps to form hydroxylated quinone, and hydroxy-hydroquinone. Additional extended UV treatment results in OH-HBQ dehalogenation.[184] The rapid transformation of HBQs to OH-HBQs has been supported by subsequent studies in multiple research groups.[183,186–188] Mohan indicated that with increasing pH, [OH^-] catalyzes DCBQ degradation in a first order rate (i.e., > pH 7), and estimated the second order rate constant to be 156$M^{-1}s^{-1}$.[189]

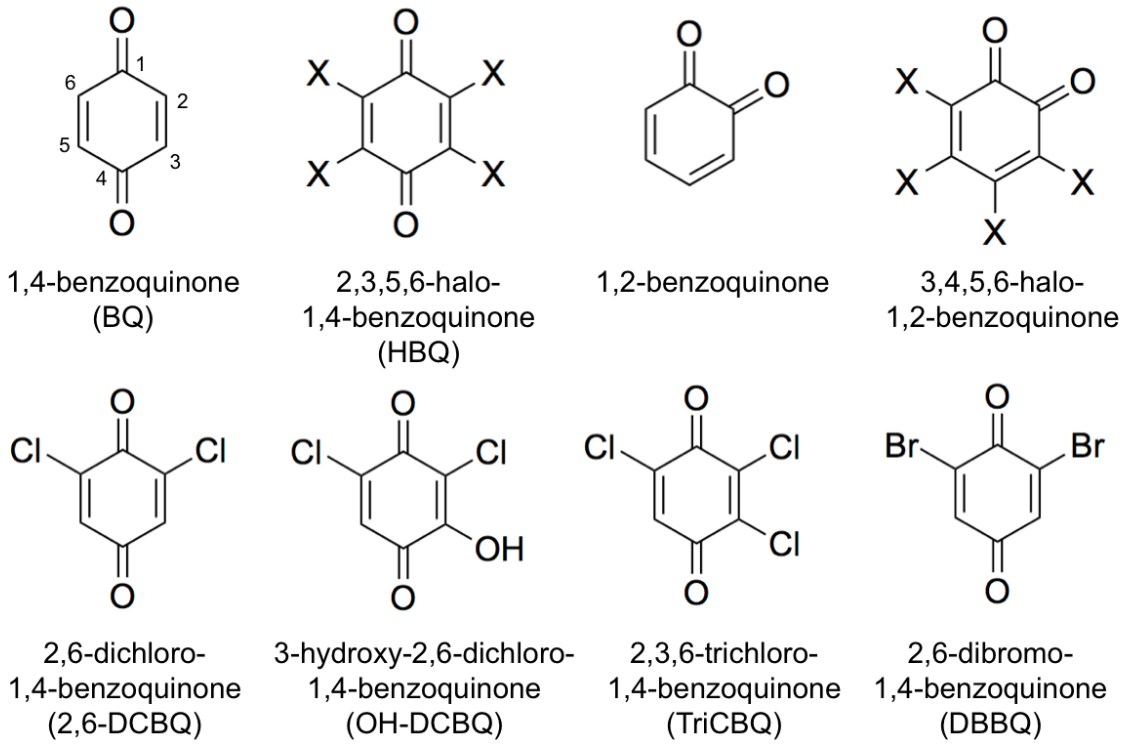

Figure 1.2 General and commonly occurring halobenzoquinone structures

1.7.1 Known Toxicity of HBQs

1,4-Benzoquinone (BQ) is a thoroughly studied metabolite of benzene. Two major molecular pathways have been found to account for the toxicity induced by 1,4-BQ exposure. In the first pathway, 1,4-BQ produces reactive oxygen species (ROS) causing oxidative damage to cellular DNA, proteins, and/or lipids. Alternatively, the second pathway occurs when 1,4-BQ covalently bonds to cellular macromolecules like DNA or protein.[190,191] Oxidative stress can contribute to the development of cancer.[192,193] ROS can be produced in cells from both endogenous and exogenous sources. If ROS species are not counteracted by cellular mechanisms, oxidative stress may lead to DNA, protein or lipid damage, and cell death, or chromosome instability, genetic mutation and even cancer.[192,194] When an exogenous substrate can interact irreversibly (i.e., covalently bound) with proteins or nucleic acids either directly or through metabolic activation, it can alter or inhibit the function of the macromolecule.[195,196] HBQs are structurally similar compounds to 1,4-BQ. Toxicological

assays, both *in vitro* and more recently *in vivo*, continue to provide evidence that HBQs induce cytotoxic oxidative stress and genotoxic effects after exposure.

Acute cytotoxic effects have been observed in different cell lines after being exposed to various concentrations of HBQs. IC_{50} values were determined in Chinese Hamster Ovary (CHO) cells[179] and human urinary bladder cancer (T24) cells.[197] IC_{50} represents the concentration in which the cell index is reduced to 50% of the control. In CHO cells, the 72-hour IC_{50} values for HBQs ranged from 15.9 – 72.9 μM (i.e., 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ) < 2,6-dibromo-1,4-benzoquinone (DBBQ) < 2,6-dichloro-1,4-benzoquinone (DCBQ) < 2,3,6-trichloro-1,4-benzoquinone (TriCBQ)).[179] Comparatively 72-hour IC_{50} values for regulated DBPs are much higher (i.e., less cytotoxic); THMs ranged from 3.96-11.5 mM and HAAs ranged from 8.90 μM to 17.52 mM.[110] In T24 cells, IC_{50} values for HBQs after 24 hours ranged from 94.5-142.0 μM (DCBQ < DMCBQ < DBBQ < TriCBQ)[197] which are greater than the 24 hour IC_{50} values in CHO cells (i.e., 11.4 – 45.5 μM).[197]

Oxidative stress has been observed *in vitro* after exposure to HBQs. In T24 cells, ROS concentration significantly increased with HBQ concentration dose. HBQ cytotoxicity was significantly reduced with the addition of an oxidant scavenger, N-acetylcysteine (NAC),[197] which acts to sequester ROS *in vitro*.[198] Glutathione (GSH) plays a key role in the cellular detoxification of HBQs.[199,200] GSH is an endogenous tripeptide thiol and GSH associated metabolism is a primary intracellular defense against agents that produce oxidative stress. GSH is able to detoxify the cell by scavenging free radicals, reducing peroxidases, and conjugating with electrophilic compounds.[201] The viability of T24 bladder cancer cells was determined when treated with DCBQ or when treated with DCBQ and buthionine sulfoximine (BSO).[199] BSO binds irreversibly with glutamate cysteine synthetase, one of the enzymes responsible for the cellular production of GSH.[202] Cell viability decreased when glutathione production in the cell was inhibited by BSO. This indicates that GSH is essential sin detoxifying the cell from the toxicant. These results were verified when GSH was added exogenously the viability of the T24 cells increased, confirming that GSH is necessary for the detoxification of HBQs.

Li *et al.* proposed a model for the detoxification;[199] when low concentrations of HBQs enter the cell, cellular ROS are produced leading to the depletion of GSH resulting in oxidative stress. This results in the activation of genes that lead to the induction of glutathione S-transferase[199], an enzyme that is responsible for catalyzing the conjunction reaction between GSH and HBQs[201]. To determine the concentration dependent mechanism of GSH reduction after HBQ exposure, Wang *et al*. studied GSH conjugation with HBQs in a human liver carcinoma cell line (HepG2).[200] Intracellular GSH depletion was attributed to the direct conjugation of GSH to HBQs as well as the oxidation of GSH to glutathione disulfide.[200] For example, 11 different conjugates between GSH with 2,6-DCBQ were identified including mono-sulfur-glutathionyl-benzoquinone (mono-SG-BQ), di-SG-BQ, tri-SG-BQ, and tetra-SG-BQ conjugates and isomers. Five of which were confirmed to form *in vitro* when HepG2 cells were treated with HBQs.

Nrf2, the nuclear factor (erythroid derived 2)-like 2, is a transcription factor that regulates the expression of genes through one of the primary signaling pathways that protects cells against oxidative stress.[180] Nrf2 promotes the expression of antioxidant response element (ARE)-mediated antioxidative and detoxifying enzymes. Nrf2-deficient mice have shown significantly increased susceptibility to chemical-induced urinary bladder cancer compared to wildtype mice.[203] HBQ exposure was found to induce significant response from the Nrf2 pathway in both human uroepithelial (SV-HUC-1) cells[204] and epithelial colorectal adenocarcinoma (Caco-2) cells.[180]

The p53 signaling pathway is critical in cellular differentiation and tumor suppression. The p53 tumor suppressor gene preserves the stability of the genome by inducing cell cycle arrest, senescence, and apoptosis. It is activated in response to various events including oxidative stress and DNA damage.[205,206] When Caco-2 cells were exposed to HBQs, increased p53 pathway activity was observed.[180] Additionally, p53 protein expression was significantly increased in CHO cells treated with several HBQs (i.e., 2,5-dibromo-1,4-benzoquinone (2,5-DBBQ), 2,3-diiodo-1,4-benzoquinone (2,3-DIBQ), DCBQ and 2,5-DCBQ) after 24 hours.[207] Here, Li *et al.* compared isomeric structural effects on toxicity and found p53 protein levels were approximately twice as high in 2,5-HBQ treated cells compared to 2,6- isomers.

There is evidence that HBQs are capable of genotoxic effects in cells. A genotoxin is a chemical or agent that can cause DNA or chromosomal damage. Free radicals in cells can potentially act as genotoxins[208] resulting in damage to DNA, lipids and proteins and may eventually lead to cell death either by necrosis or apoptosis.[209] In somatic cells, DNA damage may lead to malignant transformation: cancer.[210] 8-Hydroxy-deoxyguanosine (8-OhdG) is a nucleotide biomarker that is indicative of deoxyguanosine oxidation and is a known sensitive measure of DNA damage.[211] Point mutations in nuclear DNA can be induced by 8-OhdG.[212,213] A foundational bench scale experiment, not conducted in a cell line, found that 8-OhdG was formed in double stranded DNA in the presence of DCBQ and H_2O_2.[208] T24 cells treated with DCBQ showed significantly higher concentrations of 8-OhdG than the control group.[197] More recently, Xu *et al*. found 8-OhdG increased 1.4, 3.2, 8.8, and 9.2 times in T24 cells after treatment with 50 μM tetrabromo-1,4-benzoquinone (TBBQ), tetrachloro-1,4-benzoquinone (TCBQ), 2,6-DCBQ and 2,5-DCBQ, respectively.[214] Contrary to the structural trend observed for p53 protein levels, in CHO cells, 2,6-DCBQ generated nearly double the 8-OhdG *in vitro* compared to the 2,5-DCBQ isomer.[207] Although 5-Hydroxymethyl-2'-deoxycytidine (5HmdC) is not a biomarker of nuclear DNA damage, it is capable of affecting the binding of transcription factors, which can result in errors in subsequent gene expression.[215] In a bench scale experiment, the nucleotide 5-methyldeoxycytidine (5mdC) was oxidized to 5HmdC in the presence of at least 0.1 mM 2,6-DCBQ and H_2O_2.[216]

Stem cells (SCs) are an emerging model used to characterize the toxicity of environmental contaminants and evaluate their potential developmental effects.[217,218] In 2015, Li *et al.* reported that TCBQ significantly induced apoptosis in mouse embryonic SCs in a concentration dependent matter.[219] A recent study investigated the effects of HBQs on human neural stem cells (hNSCs). It was found that 2,6-DCBQ and 2,6-DBBQ influenced the proliferation of hNSCs. After HBQ exposure, a significantly increased proportion were observed in S-phase.[220] Increased p53 protein expression can activate cell cycle arrest.[221] Furthermore, DNA damage checkpoints during S-phase delay cell cycle progression to repair defects.[222]

There are several factors that can introduce uncertainty into the interpretation of toxicological assays. A recent *in vitro* toxicity assessment of HBQs by Hung *et al*. investigated the stability of HBQs in cell culture media and the intra cellular ROS generated by HBQs in normal human colon cells (CCD 841 C*oN*) and human liver cancer cells (HepG2).[186] The rate of DCBQ transformation was investigated for different cell culture media (i.e., HBSS, EMEM, and MEM) and DCBQ exhibited enhanced reactivity in all media types compared to water. DCBQ transformation was accelerated in EMEM and MEM compared to water and HBSS.[186] The results demonstrate that previous *in vitro* toxicity assays may have underestimated the cytotoxicity of HBQs by unintentionally evaluating transformed HBQs which are less toxic.[186]

Many of the HBQ induced cyto- and genotoxic effects observed in vitro have been corroborated in recent *in vivo* research. Zebrafish are advantageous for their rapid lifecycle and development, high number of offspring, transparency of embryos, as well as ease of laboratory maintenance and experimental manipulation. Zebrafish embryos are an established model used to evaluate developmental toxicity for environmental pollutants. [223] Recently, oxidative stress and developmental toxicity in zebrafish embryos was investigated after exposure to HBQs.[224] The LC_{50} values for HBQs (2,5-DCBQ, 2,6-DCBQ, 2,5-DBBQ, TCBQ, and TBBQ) was up to 200 times lower than HAAs: dichloroacetic acid (DCA), dibromoacetic acid (DBA), and iodoacetic acid (IAA). HBQ treatment resulted in significant larval developmental malformations of the heart, gas bladder and spine. These *in vivo* results supported the acute adverse oxidative and genotoxic effects observed *in vitro*. HBQ exposure increased ROS and decreased GSH, which was significantly mitigated by the addition of NAC. Furthermore, evidence of genotoxicity was supported by increased 8-OhdG levels, DNA fragmentation and larval apoptosis.[224] A very similar zebrafish toxicity study replicated these HBQ induced cyto- and genotoxic results.[225]

Caenorhabditis elegans (*C. elegans*), is a small nematode organism, that has been widely used as a toxicological model due to its simple structure, short lifespan, culturability and self-fertilization. Multiple similar biochemical pathways are shared between humans and *C. elegans*, making assay data translatable and comparable for other toxicants.[226] Zuo *et al*. studied the effects of DCBQ on lethality, respiration rate, and DNA damage through an *in*

vivo study on *C. elegans*. Compared to other regulated DBPs (i.e., DCA, trichloroacetic acid (TCA), monobromoacetic acid (MBA), DBA, and NDMA), DCBQ exposure elicited the greatest increase in 24-hour lethality (LC 50) and inhibition of respiration. Additionally, of all studied DBPs, only DCBQ and NDMA exposure lead to DNA damage in *C. elegans.*[227]

The toxicity of the major HBQ transformation products, HO-HBQs is less studied, but existing reports suggest the same trend: the hydrolysis of HBQs to HO-HBQs is a detoxification step.[180,186] Wang *et al*. noted that while HBQ IC_{50} values were 2-fold lower (i.e., more cytotoxic than OH-HBQs) in CHO cells, OH-HBQs were still substantially more cytotoxic compared to regulated DBPs.[179] Further investigation into the molecular mechanisms of toxicity of HBQs and their transformation products, along with a thorough assessment of human exposure are necessary to better understand the health risks associated with HBQs in disinfected water.

1.7.2 Methods for Determination of HBQs

Accurate assessment of HBQ exposure requires the determination of their occurrence in disinfected water using sensitive and specific analytical detection methods. UV-visible absorption spectrometry was an early analytical technique used to monitor HBQs transformation[228] and intermolecular interactions.[229] Wang compiled the available reported characteristic absorption wavelengths of HBQs.[230] The halosemiquinone free radical ($HSQ^{\bullet-}$), is a relatively stable HBQ intermediate in its equilibrium with halohydroquinone.[229] This chemical feature results in a specific line shape, unique to $HSQ^{\bullet-}$, when analyzed with electron paramagnetic resonance (EPR).[200,231] Recently, Wang *et al*. utilized EPR analysis to investigate the semiquinone radical detected in the reaction between GSH and HBQs, which helped elucidate the mechanism of GSH and HBQ conjunction.[200]

Direct gas chromatography mass spectrometry (GC-MS) methods for previously reported HBQ analyses are complicated by their thermal instability. Complex derivatizations were required for detection.[232,233] Detection limits of 10 structurally similar 1-4 ring quinones of 1-2 nmol/mL were achieved in a GC-MS method developed by Lim *et al.* to determine urinary quinone levels in both rats and human urine over time.[234] More relevantly, Heasley *et al.* used GC-MS to identify the formation of 2,6-DCBQ and other chlorinated phenols and

structurally related Cl-DBPs after the mono- and dichlorination of phenol, m-cresol, and 2,4,6-trichlorophenol in aqueous solution.[235]

The high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) method, developed in the Li Group and reported by Zhao *et al.* and Qin *et al*., both in 2010,[181,182] enabled sensitive specific determinations of HBQs with detection limits less than 5 ng/L. The majority of subsequent HBQ determination studies have been accomplished using the same general sample preparation and HPLC-MS/MS analysis workflow, **Table 1.3**.

In general, the workflow can be described as follows: using formic acid (FA) residual chlorine is quenched and samples are acidified to approximately pH 2-3 to stabilize HBQs by preventing hydrolysis to OH-HBQs.[182,183] The aqueous sample is then preconcentrated with a multimode HLB solid phase extraction (SPE) cartridge. Next, the acidified methanol eluate is further concentrated by evaporation under a gentle stream of nitrogen. The concentrated sample is reconstituted in acidified water. The prepared sample is split into 5 portions, for future standard addition quantification before injection for HPLC-ESI-MS/MS analysis.

Reverse phase liquid chromatography columns, specifically C18, has been most commonly applied to separate HBQs (**Table 1.3**). The unique HBQ ionization pathway for stable and intense signal detection under negative ESI during high HPLC-MS analysis was first detailed by Zhao *et al*. in 2010.[181] The method has been expanded to comprehensively include 12 different HBQ analytes[236] and was later optimized to simultaneously detect OH-HBQs.[179] The determination of OH-HBQs is challenging due to the lack of commercial standards. Therefore, pure HBQ standards must first be converted to OH-HBQ transformation products before they can be used as calibration standards to determine the parent and fragment masses for specific and sensitive multiple reaction monitoring (MRM) analysis.[179,230]

Zhao *et al.* first described how monitoring the conventional $[M+1]^+$ ions in positive mode and $[M-1]^-$ ions in negative mode yielded weak and instable HBQ LC-MS signals.[181] Through HPLC-ESI-MS/MS analysis in acidified conditions (i.e., 0.25% FA sample and mobile phase composition) ionization of HBQs form a $[M+H^++2e^-]$ ion (abbreviated as

$[M+H]^-$).[181,182,237] Under negative ESI potential (i.e., -4500 V) HBQs are electron acceptors with high electron affinity.[238] Therefore, HBQs likely directly accept two electrons and one proton. An alternative route to the $[M+H]^-$ ion is through the formation of the intermediate dihydro-halo-benzoquinone followed by a loss of a proton. MRM is then utilized to measure compound specific mass to charge (m/z) ion transitions, increasing raw data accumulation and enhancing detected signals.[239]

Recently, Cuthbertson *et al.* developed an online SPE-LC-MS/MS method, capable of detecting 10 different HBQs with LODs ranging from 0.2 – 166 ng/L. This method is advantageous because it requires less sample volume and minimal sample preparation compared to the traditional analytical sample preparation that relies on benchtop SPE and N_2 evaporation.[183] In innovative analytical approach using a metal organic framework (MOF) to distinguish and determine para (p-) and ortho-tetrachlorobenzoquinone (o-TCBQ) was described by Du *et al.*[240] Both isomers were successfully quantified simultaneously in mixtures, overcoming the common challenge of cross interference from similar signal mechanisms.

Table 1.3 Summary of reported HPLC-MS/MS HBQ detection strategies and corresponding sample preparation details

HBQ	LOD (ng/L)	Recovery %	Sample Pre-treatment	Sample preconcentration	HPLC Separation Summary	MS Detection Summary	Reference
2,6-DCBQ 2,6-DBBQ TriCBQ DCMBQ	0.6-1.9	97-118%	Acidify to 0.5% FA final concentration immediately after collection	SPE HLB oasis cartridge rinse SPE with 6mL MeOH (0.5% FA) after loading wash SPE with 6mL water (0.5% FA) and 6 mL 1:1 MeOH:Water (0.5% FA) Dry 10 min under vacuum Elute with 6 mL MeOH (0.5% FA)	HPLC Agilent 1100 LC Luna C18 column (100x2.0mm i.d. x 3µm) Phenomenex Room Temperature 20µL injection volume Mobile Phase flow rate 150µL/min	MS/MS-MRM API 5000 MS (Sciex) ESI (-) 4500 V 450 °C	Qin *et al.* 2010[182]

Table 1.3 Summary of reported HPLC-MS/MS HBQ detection strategies and corresponding sample preparation details (continued)

				Evaporate to 100 µL under nitrogen Reconstitute to 500 µL with water:MeOH (80:20 0.5% FA)	A: water 0.5% FA B: Methanol	30 minutes	
2,6-DCBQ	1	84 ± 1	Acidify to 0.25% FA	SPE	Agilent 1100 LC	MS/MS-MRM	Zhao *et al.* 2010[181]
2,6-DBBQ	0.5	78 ± 3	immediately after collection	HLB	Luna C18 column (100x2.0mm i.d. x 3µm) Phenomenex	API 5000 MS (Sciex)	
TriCBQ	1.7	59 ± 9		rinse SPE with 6mL MeOH (0.5% FA) and 2 washes of 6mL water (0.25% FA)	Room Temperature	ESI	
DCMBQ	1.9	69 ± 3		after loading wash SPE with 6mL water (0.25% FA) and 6 mL 1:1 MeOH:Water (0.25% FA)	20µL injection volume	(-) 4500 V	
				Dry 10 min under vacuum	Mobile Phase flow rate 150µL/min	450 °C	
				Elute with 6 mL MeOH (0.25% FA)	A: water 0.25% FA		
				Evaporate under N2 (100µL)	B: Methanol 0.25%FA		
				Reconstitute (500 µL) with water:MeOH (80:20 0.25% FA)	40 minutes		
2,6-DCBQ	1.3	95 ± 5 %	Acidify to 0.25% FA	SPE	UHPLC	MS/MS-MRM	Huang *et al.* 2013[236]
2,5-DBCQ	1.3	70 ± 12	immediately after collection. Removed free chlorine and	HLB cartridge	ACQUITY UPLC BEH 1.7µm-C18 column (2.1x100mm; waters)	API 5000 MS (SCIEX)	
DCMBQ	0.6	66 ± 1	HBQs (i.e., eliminate hydrolysis to	rinse SPE with 6mL MeOH (0.25% FA)	50 °C	ESI	
TriCBQ	1.1	88 ± 2	HO-HBQs under neutral aqueous conditions)	after loading wash SPE with 6mL water (0.25% FA) and 6 mL 1:1 MeOH:Water (0.25% FA)	10 µL injection volume	(-) 4500 V	
TetraCBQ	0.9	80 ± 2		Dry 10 min under vacuum			
TetraC-1,2-BQ	2.5	89 ± 2		Elute with 6 mL MeOH (0.25% FA)	Mobile phase flow rate 0.4mL/min		
2,6-DBBQ	0.2	95 ± 5		Evaporate to 100 µL under nitrogen	A: water with 0.25% FA		
2,5-DBBQ	0.3	85 ± 5		Reconstitute to 500 µL with water:MeOH (80:20 0.25% FA)	B: MeOH with 0.25% FA		

Table 1.3 Summary of reported HPLC-MS/MS HBQ detection strategies and corresponding sample preparation details (continued)

DBDMBQ	6.6	90 ± 1			18 minutes		
TetraBBQ	1	81 ± 4					
TetraB-1,2-BQ	4.8	77 ± 0					
DIBQ	0.2	89 ± 2					
2,6-DCBQ	0.07	96 ± 2 %	Acidify to 0.25%.	SPE Waters Oasis HLB Cartridge	UHPLC	MRM	Wang *et al.* 2014[179]
OH-2,6-DCBQ	0.03	92 ± 8	Sample transported to lab on icepacks	condition cartridge with 12 acetic (0.25% FA) MeOH	Agilent 1290	ESI 5600 Q-TOF (IDA Experiments)	
DCMBQ	0.7	96 ± 2	immediately. Analysis within 2 days of sample	Equilibrate with 12 mL acidified water	Luna C18 column (100x2.0mm i.d. x 3µm) Phenomenex	ESI 5500 Q-Trap (MRM Experiments)	
OH-DCMBQ	0.4	76 ± 2	collection	Pump 500 mL sample at 8 mL/min	20 µL injection volume	(-) 4500 V	
TriCBQ	0.8	78 ± 5		Wash with methanol and water	Mobile phase flow rate 0.17 mL/min		
OH-TriCBQ	0.7	79 ± 2		Elute with acidic MeOH	A: Water with 0.1% FA		
2,6-DBBQ	0.05	95 ± 5		Evaporate to 100 µL under nitrogen stream	B: MeOH with 0.1% FA		
OH-2,6-DBBQ	0.04	85 ± 4		Reconstitute in 400 µL acidified water	30 minutes		
	*LOQ (ng/L)	% Recovery (CV)	Sample acidified to 0.1% FA after collection				Cuthbertson *et al.* 2019[183]
2,5-DCBQ	2.5	65 (7)		SPE (Online)	UFLC (Ultra Fast LC, Shimadzu)	MS/MS-MRM SCIEX QTRAP 5500	
2,6-DCBQ	2.5	71 (4)	CV = Coeff of variation				
2,5-DBBQ	2.5	62 (9)		Analysis within 48 hours of collection	C18 (100Angstrom reverse phased Interchim column)	ESI	
2,6-DBBQ	5	117 (6)			30 °C	(-) 4500 V	
TetraCBQ	5	57 (6)		Online SPE with HLB column (2.1 x 20mm x 25 µm)	Autosampler 10 °C	400 °C	
TetraC-1,2-BQ	1000	81 (2)		4 mL sample volume, flow rate 1 mL/min, charge time 11.2 min	Mobile Phase (A and C): water 0.1% FA		
2,6-DBDMBQ	50	71 (4)			B: 0.1% acetonitrile (B)		
2,6-DBCMBQ	100	69 (4)			5 mL sample injected		
TetraBBQ	100	73 (5)			300 µL/ min		
TetraB-1,2-BQ	1000	72 (4)			50 minutes		

Table 1.3 Summary of reported HPLC-MS/MS HBQ detection strategies and corresponding sample preparation details (continued)

	LOD (ng/L)	% Recovery					Wu *et al.* 2019[243]
2,5-DCBQ	1.02	76.5-80.2%	Acidify to pH 2.6-2.8 with FA	SPE	UPLC	MS/MS-MRM	
2,6-DCBQ	0.78	89.9-102.9%		HLB	Thermo Fisher UPLC U-3000	5500 Sciex	
DCMBQ	1.76	62.5-71.0		(Huang 2013 and Zhao 2010)	Acclaim RSLC120 2.2µm C18 column (2.1x100 mm)	ESI	
TetraC-1,2-BQ	0.54	81.0-97.5			2µL injection volume	(-) 4500 V	
2,5-DBBQ	0.66	82.4-83.0			flow rate 0.3 mL/min	550 °C	
2,6-DBBQ	0.38	85.3-90.8			A: water 0.25% FA		
TetraB-1,4-BQ	0.26	72.7-89.3			B: Methanol 0.25% FA 22 minutes		

1.7.3 Occurrence of HBQs in Drinking and Recreational Waters

Continuous development of sensitive and specific detection HPLC-MS/MS methods enabled investigations into the occurrence of HBQs in treated drinking water, recreational water and beverages prepared with treated drinking water. **Table 1.4** summarizes the reports of type and concentration of HBQs detected in disinfected waters. HBQs were first detected in treated drinking water in 2010.[181,182] Since their discovery, 2,6-DCBQ has been determined with the highest frequency and concentrations (i.e., as high as 165 ng/L[181] or 263 ng/L[189] in some cases) in disinfected waters compared to other HBQs. Mohan's 2015 Master's thesis identified greater concentrations of 2,6-DCBQ in treated water from DWTPs using free chlorine as a secondary disinfectant.[189] Source waters containing bromide influence the formation of Br-HBQs.[237] As such, 2,6-DBBQ follows 2,6-DCBQ in HBQ occurrence (**Table 1.4**). Like other DBPs classes, HBQs follow the same toxicological trend: I-HBQs > Br-HBQs > Cl-HBQs.[241] Recently, Cuthbertson *et al*. used their efficient online SPE-HPLC-MS/MS method to detect the highest recorded 2,6-DBBQ concentration in treated drinking water, 254 ng/L.[183] Interestingly, while 2,6-halogen substituted HBQ isomers are exist widely in treated water, the more toxic 2,5-HBQ isomers[207] have not yet been identified in

authentic samples. Wang *et al*. determined the concentration of HBQs and their stable OH-HBQ transformation products in a drinking water distribution system. Over time, as distance from the DWTP increased, the concentrations of HBQs in tap water samples decreased while the concentration of less toxic, OH-HBQs increased proportionally.[179]

HBQs have also been identified in swimming pools in Canada[242] and more recently, in China.[243] Wang *et al*. first noted elevated levels of 2,6-DCBQ in swimming pool water (19-299 ng/L) compared to input tap water control samples (1.1-5.6 ng/L). For the first time, 2,3-dibromo-5,6-dimethyl-(1-4)-benzoquinone (DMDBBQ) was identified as an HBQ-DBP unique to swimming pool water.[242] Wu *et al*. recently observed greater HBQ concentrations in indoor pools, compared to outdoor pools in China.[243] This can be explained by the UV irradiation transformation mechanism of HBQs to OH-HBQs.[184] Additional sunlight exposure to outdoor pools promoted HBQ degradation.

Recently, Lou *et al*. identified the formation of HBQs in tea brewed with disinfected water. The concentration of 2,6-DCBQ decreased in green tea (2.3 ng/L) after preparation with disinfected tap water (0.2 ng/L). Notably, the HBQ, 3,4,5,6-tetrachloro-1,2-benzoquinone (TetraC-1,2-BQ) increased from 0.6 ng/L in tap water to 1.8 ng/L after brewing Green, Oolong, Pu-erh, and Black teas.[172] Characterizing new exposure routes for HBQs enables a more accurate assessment of their risk to human health.

Table 1.4 Summary of reported HBQ occurrences in various disinfected water samples

HBQ Compound	Concentration Range (ng/L)		Frequency	Sample Type	Region	Reference
Disinfected Drinking Water						
2,6-DCBQ	range	14.3-54.6	3 of 3	Treated drinking water, Water distribution system 1	Canada	Qin *et al*. 2010[182]
		5.3-14.4	3 of 3	Treated drinking water, Water distribution system 2		
2,6-DCBQ	average of triplicate	165.1 ± 9.1		Chlorinated drinking water from 12 water purification plants	Canada	Zhao *et al.* 2010[181]
DCMBQ		1.3 ± 0.2				
TriCBQ		9.1 ± 0.6				
2,6-DBBQ		0.5 ± 0.1				
2,6-DCBQ	range	2.5-21.3	2 of 2	Finished drinking water	Canada	Huang *et al.* 2013[236]
2,6-DBBQ	range	7.3-19.4	2 of 2			

Table 1.4 Summary of reported HBQ occurrences in various disinfected water samples (continued)

2,6-DCBQ	range	nd-20	34/37		North America	Wang *et al.* 2014[179]
DCMBQ	n.d. = not detected	nd-4	11/37	Treated water collected from 5 WTPs 2012-2013.		
TriCBQ		nd-20	10/37			
DBBQ		nd-10	6/37			
OH-DCBQ		nd-20	34/37			
OH-DCMBQ		nd-7	12/37			
OH-TriCBQ		nd-20	6/37			
OH-DBBQ		nd-10	6/37			
2,6-DCBQ		< 50	7 of 8	chlorinated and chloraminated drinking water from 8 water treatment plants in the U.S.		Mohan 2015[189]
		~ 263	1 of 8			
2,6-DCBQ	range	8-51	21 of 24 samples	chlorinated drinking water from 12 water purification plants	Japan	Nakai *et al.* 2015[257]
2,6-DCBQ	range	13-14	2 of 4	Finished drinking water samples from four DWTPs using chlorine disinfection	U.S.	Cuthbertson *et al*. 2019[183]
2,6-DBBQ	range	5-254	4 of 4	WTP 1, 2, 3, and 4		
Disinfected Recreational Water						
2,6-DBCQ	range	18.9-299.0	10 of 10	Chlorinated and chlorinated + UV swimming pool water	Canada	Wang *et al*. 2013[242]
TriCBQ	range	7.3-11.3	4 of 10			
DMDBBQ	range	0.6-0.7	2 of 10			
2,6-DBBQ	range	1.6-3.8	2 of 10			
2,6-DCBQ	range	1.1-5.6	10 of 10	chloraminated input tap water control		
2,6-DCBQ	range	4.56-45.30	7 of 7	Indoor and Outdoor Swimming pools	China	Wu *et al*. 2019[243]
2,6-DBBQ	range	<0.38-14.20	2 of 7			
TetraC-1,2-BQ	range	<0.54-2.60	2 of 7			
Disinfected Water Beverage						
2,6-DCBQ	triplicate average	2.3 ± 0.1		Tap Water	China	Lou *et al.* 2019[172]
TetraC-1,2-BQ		0.6 ± 0.0		Tap Water		
2,6-DCBQ	average of all teas	0.2 ± 0.0		Green tea	Prepared with disinfected tap water	
TetraC-1,2-BQ		1.8 ± 0.1		Green tea Oolong Tea Pu-erh Tea Black Tea		

1.7.4 Known Precursors of HBQs

Various strategies have been investigated to reduce exposure to HBQs by limiting their formation during water disinfection. Efforts have been made to identify sources of HBQ precursors in disinfected waters (**Table 1.5**) and design appropriate approaches to reduce those precursors before reacting with disinfectants.[244,245] Multiple individual HBQ precursors including phenol,[235,237] alkyl and carboxyl para-substituted phenolic compounds as well as para-substituted aromatic amines[246] have been identified under chlorination and/or chloramination conditions. Additionally, NOM mixtures with higher aromatic character (e.g., UV_{254}, humic SUVA) have been correlated with increased DCBQ formation after chlorination.[244] Several processes are employed at water treatment plants to remove NOM before disinfection.[245,247] Coagulation,[244,245] granular activated carbon,[247] and ozonation pre-treatments[245] were all found to remove HBQ precursors before chlorination and can subsequently reduce the formation of HBQs in finished drinking water.

Based on HBQ structures, compounds containing aromatic moieties could serve as HBQ precursors. Previous studies have identified phenol,[179,237] and NOM from Otonabee River, Grand River and Lake Ontario as HBQ precursors.[244] NOM in source water is an environment dependent,[248] complex mixture including high aromaticity humic acids,[249] as well as peptides and amino acids. Over 600 distinct peptides have been detected in surface waters[250] and free amino acids have been determined at low μg/L concentrations[82,251] Kosaka *et al*. determined molar yields ranging from 0.0008% to 4.9% for various aromatic DCBQ precursors, including aromatic amino acid tyrosine and dipeptide tyrosyl-alanine, under chlorination conditions.[246] Lignan compounds are present as NOM in source water,[252] and have been shown to be viable precursors of DBPs.[253] Recently, Mohan reported 2,6-DCBQ formation from 10 of 16 lignan compounds, **Table 1.5**, under chlorination conditions ranging from 0.001-0.1% yields.[189]

HBQ precursors in swimming pools have been investigated. Wang *et al*. found that HBQ formation in swimming pools was enhanced with greater levels of DOC, chlorine dose, and increased temperature. Both lotions and sunscreens were found to be precursors of DCBQ and other HBQs (i.e., DCMBQ, TriCBQ, and TetraB-1,4-BQ) under chlorination conditions. More recently, Sun *et al*. specifically identified 2,4-dihydroxybenzophenone, a

component and metabolite of benzophenone type UV filters, present in PCPs (i.e., sunscreen, cosmetics, and shampoos), as a precursor of HO-DCBQ and eight additional aromatic DBPs under chlorination conditions.[187]

The formation of HBQs from phenolic compounds present in tea leaves has been investigated. Overall, both DCBQ and tetrachloro-1,2-benzoquinone (TC-1,2-BQ) were detected in teas. DCBQ was present in the tap water source and TC-1,2-BQ originated from tea leaves' leachate or was generated during the chlorination of tea polyphenols. Four tea polyphenols (i.e., (-)-epigallocatechin gallate, (-)-epicatechin gallate, (-)-epicatechin, and gallic acid) were identified as precursors of both 2,6-DCBQ and TC-1,2-BQ under chlorination conditions at pH 7.[172] Lou *et al.* found that while these HBQs induced cellular reactive oxygen species (ROS) and semiquinone radicals after 24 hours in HepG2 human bladder cancer cells, the resulting oxidative stress could be decreased by the addition of the tea polyphenol (-)-epigallocatechin.[172] Characterizing the types of compounds capable of forming HBQs and their sources is a strategy to control their formation in disinfectant treated waters and mitigate overall human exposure.

Table 1.5 Summary of reported HBQ precursors under chlorination or chloramination conditions

HBQ Compound	Identified Precursor Compound	Yield	Disinfectant	Reaction Details	Reference
2,6-DCBQ	Bisphenol-A	Identified not quantified	Chlorination (sodium hypochlorite) 30-290 mg/L initial Cl	pH 8-9 20-25 °C	Yamamoto *et al.* 2002[250]
2,6-DCBQ	Suwanee Humic River Acid	Identified not quantified	5 mg/L sodium hypochlorite	pH 7.5 in darkness ambient temperature	Zhai *et al.* 2011[251]
2,6-DBBQ	2,4,6-tribromophenol	Identified not quantified			
2,6-DCBQ	**NOM from Canadian surface waters** Lake Ontario Grand River Otonabee River	DCBQ formation correlated with humic substance concentration, UV at 254 nm, SUVA DCBQ formation potential was strongly correlated with the biopolymer fraction of NOM, likely due to do-removal of biopolymer fraction with HBQ precursors during alum treatment.	Chlorination with Sodium Hypochlorite Dosed to achieve residual chlorine concentrations of 3-5mg/L free chlorine after 36 hours	40 hour reaction time	Diemert *et al.* 2013[238]
2,6-DCBQ	**Lignan Compounds** 4-hydroxybenzaldehyde 4-hydroxybenzoic acid Vanilin vanilic acid syringaldehyde syringic aicd p-courmaric acid hydroquinone 3,4-dihydroxybenzoic acid gallic acid	**yield range** range from 0.001-1% formation yield	5 mg/L Cl2 from chlorination	6 hours 25 °C pH 7 0.004-0.009 % 0.02% 0.01%	Mohan 2015[184]
2,6-DCBQ	**Aromatic Compounds** Phenol 2-Chlorophenol 4-Chlorophenol 2,4-Dichlorophenol 2,6-Dichlorophenol 2,4,6-Trichlorophenol *p*-Quinone-4-chloromide 2,6-Dichloroquinone-4-chloromide	**% molar yield** 0.10 ± 5.2 0.12 ± 15 0.03 ± 8.8 0.07 ± 6.4 0.12 ± 14 0.20 ± 6.1 0.0008 ± 32 4.9 ± 13	Chlorination (sodium hypochlorite)	60 minutes	Kosaka *et al.* 2017[240]

Table 1.5 Summary of reported HBQ precursors under chlorination or chloramination conditions (continued)

	p-Cresol	0.016 ± 39			
	4-Methothyphenol	0.0006 ± 24			
	4-Hydroxybenzoic acid	0.14 ± 33			
	Tyrosine	1.5-2.7 ± 28			
	L-Alanyl-*L*-tyrosine	0.11 ± 39			
	4-Hydroxybenzoic acid methyl ester	0.0003 ± 34			
	Bisphenol A	1.6 ± 26			
	4-Nonylphenol	1.8 ± 0.7			
	Analine	2.6 ± 0.4			
	4-Methyl aniline	0.03 ± 10			
	N-Methyl aniline	2.0 ± 1.7			
	N,4-Dimethyl aniline	0.001 ± 11			
	Tinopal AMS-GX	0.02 ± 38			
HBQ Formation Potential	**Teas (=10 mg/L DOC)**	HBQ formation (ng/L)	Excess Chlorination (dose to yield 1-1.5 mg/L free chlorine residual after reaction)	pH 7	Lou *et al.* 2019[166]
TetraC-1,2-BQ	Green Tea (~47 mg/L)	6.2 ± 0.5	~ 35 mg/L	Reaction time 2 h	
	Oolong Tea (~52 mg/L)	7.9 ± 0.5	~ 18 mg/L		
	Pu-erh Tea (~66 mg/L)	23.9 ± 2.0	~ 22 mg/L		
	Black Tea (~39 mg/L)	11.7 ± 0.4	~ 28 mg/L		
2,6-DCBQ	Green Tea (~47 mg/L)	3045.9 ± 60.7	~ 35 mg/L		
	Oolong Tea (~52 mg/L)	160.8 ± 11.7	~ 18 mg/L		
	Pu-erh Tea (~66 mg/L)	1251.7 ± 58.2	~ 22 mg/L		
	Black Tea (~39 mg/L)	1922.6 ± 49.7	~ 28 mg/L		
MCBQ	Green Tea (~47 mg/L)	726.9 ± 11.6	~ 35 mg/L		
	Oolong Tea (~52 mg/L)	102.3 ± 6.8	~ 18 mg/L		
	Pu-erh Tea (~66 mg/L)	462.7 ± 19.1	~ 22 mg/L		
	Black Tea (~39 mg/L)	683.2 ± 10.8	~ 28 mg/L		
TriCBQ	Green Tea (~47 mg/L)	1.8 ± 0.1	~ 35 mg/L		
	Oolong Tea (~52 mg/L)	0.5 ± 0.1	~ 18 mg/L		
	Pu-erh Tea (~66 mg/L)	1.8 ± 0.1	~ 22 mg/L		
	Black Tea (~39 mg/L)	2.3 ± 0.1	~ 28 mg/L		
TetraC-1,4-BQ	Black Tea (~39 mg/L)	0.6 ± 0.1	~ 28 mg/L		
	Tea Polyphenols (10 µM)		500 µM chlorine	pH 7, 2 hours reaction time	
2,6-DCBQ	(-)-epigallocatechin gallate (EGCG)	370.41 ± 10.79 µmol/mol			
	(-)-epicatechin gallate (ECG)	361.07 ± 12.48 µmol/mol			
	(-)-epicatechin (EC)	174.81 ± 4.76 µmol/mol			
	Gallic acid (GA)	3.37 ± 0.21 µmol/mol			

Table 1.5 Summary of reported HBQ precursors under chlorination or chloramination conditions (continued)

TetraC-1,2-BQ	(-)-epigallocatechin gallate (EGCG) (-)-epicatechin gallate (ECG) (-)-epicatechin (EC) Gallic acid (GA)	112.76 ± 6.56 µmol/mol 259.26 ± 10.51 µmol/mol 47.70 ± 3.45 µmol/mol 3.35 ± 0.53 µmol/mol			
	Commercially Available Personal Care Products (PCPs)		Chlorination (sodium hypochlorite) 3 mg/L residual chlorine 10-15 mg/L initial chlorine dose	36 h reaction time 24 ° C	Wang *et al.* 2013[236]
2,6-DCBQ	4 of 4 Lotions studied 4 of 4 Sunscreens studied	Identified not quantified			
DCMBQ	4 of 4 lotions studied 2 of 4 sunscreens studied				
TetraB-1,4-BQ	4 of 4 sunscreens studied				
	UV Filters in sunscreens	formation yield		23 °C	Sun *et al.* 2019[182]
2,6-DC-3-hydroxy-BQ	2,4-dihydroxybenzophenone (BP)	2.70% 5.40% 6.10%	molar ratio free available chlorine (FAC) to (BP-1) pH 10, [FAC]:[BP-1] = 5:1 pH 7.5, [FAC]:[BP-1] pH 5, [FAC]:[BP-1]		

1.8 Rationale and Scope of Thesis

ASs have been demonstrated as effective indicators of waste impact in environmental waterbodies. However, the occurrence and concentration in a given water body must be first determined to interpret the relative waste impact associated with detected increases in ASs. Much of the literature has reported on AS occurrence and successful waste impact indicator use in environmental water bodies (i.e., surface and ground waters) in regions with dense populations (e.g., U.S., Asia, or Europe). Canada has an abundance of freshwater resources, and a comparatively low population density. Few Canadian waters have been investigated for AS occurrence and no surveys of British Columbia's surface waters have been reported. The Thompson River Watershed in B.C. contains major surface water bodies that support several municipalities as a drinking water source. Anthropological wastes from municipal

wastewater as well as agricultural and industrial operations located in the region have the potential to impact surface water quality. This led to the study described in **Chapter 2**.

Chapter 2 research objectives:

- Develop a rapid and sensitive high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) for the detection of ACE and SUC.
- Determine the concentration of ACE and SUC in surveyed surface waters of the Thompson Region Watershed.

Swimming is a popular recreational and exercise activity with known cardiovascular benefits. Disinfection of recreational waters (i.e., swimming pools and hot tubs) is critical to reduce the potential for transmission of waterborne pathogens. Unintentional reactions between organics in swimming pools and chlorine disinfectants can form a variety of DBPs. Trichloramine is a volatile DBP identified as a pulmonary and ocular irritant and long-term exposure has been linked with occupational asthma in pool workers and professional swimmers. A major nitrogenous precursor of trichloramine is urea, excreted in urine and sweat. ASs have never been reported in recreational waters. The potential application of ASs as indicators of human waste has not been investigated in swimming pools or hot tubs. ACE is excreted exclusively in urine; therefore, it is an ideal candidate indictor for urine, as demonstrated in the study of **Chapter 3**.

Chapter 3 research objectives:

- Determine the occurrence of ACE in swimming pool and hot tub samples collected from two cities in Alberta and B.C. using the sensitive and rapid HPLC-MS/MS analysis method.
- Estimate the approximate volume of urine required to account for the average ACE determined in two swimming pools over three weeks as a proof of concept calculation in support of ACE as a urinary indicator.

- Educate the public on the importance of proper swimming pool hygiene practices (i.e., don't pee in the pool, and rinse off in provided showers before jumping in) to reduce unnecessary increases in organic DBP precursors and subsequent DBP exposure.

Long-term exposure to DBPs through chlorinated drinking water has been consistently associated with an increased potential of developing bladder cancer. However, the regulated DBPs (e.g., THMs and HAAs) have been excluded as toxicological drivers capable of inducing these epidemiologically observed adverse health effects. QSTR results first predicted HBQs to be bladder carcinogens based on their chemical structure. HBQs are up to 1000x more cytotoxic than regulated DBP classes and multiple studies have subsequently confirmed HBQs induce oxidative stress both *in vitro* and *in vivo*. HBQ occurrence is frequent in treated drinking water across North America. A fundamental strategy in reducing exposure to HBQs is to identify and remove HBQ precursors from the water source before disinfection (e.g., chlorination, chloramination). Although the number of identified HBQ precursors are limited, they share similar aromatic structural components. Tremendous efforts are employed by DWTPs to remove NOM from source waters to reduce unintentional DBP formation during disinfection treatment. Excess disinfectants are added to the treated water to maintain a residual concentration as it travels through the distribution system to the consumers tap. However, the residual disinfectants can react with organics during food and beverage preparation before consumption. Food and beverage DBPs are a field of emerging importance that may help to identify new DBP precursors and exposure routes that impact human health. Therefore, I studied whether a widely used AS, aspartame can serve as an HBQ DBP precursor in **Chapter 4** and aromatic amino acids (AAAs) as HBQ DBP precursors in **Chapter 5**.

Chapter 4 research objectives:

- Evaluate the artificial sweetener aspartame, and its aromatic structural component phenylalanine, as HBQ precursors under controlled chloramination conditions mimicking tap water parameters.
- Compare the concentration of DCBQ in one cup of authentic chloraminated tap water with and without the addition of one package of Equal artificial sweetener blend 15 minutes after mixing.
- Investigate the impact of aspartame on the stability of DCBQ in pure aqueous solution.

Chapter 5 research objectives:

- Develop a method for simultaneous determination of AAAs (i.e., phenylalanine, tyrosine, and tryptophan) and HBQs
- Evaluate AAAs as HBQ precursors under controlled chlorination conditions.

The anticipated outcomes of my research will provide new analytical methodologies for monitoring of ASs in environmental and recreational waters. Determining the occurrence of ACE and SUC in B.C. surface waters will establish a baseline for future waste contamination investigations. Application of an ACE survey in recreational waters will yield information about swimming pool hygiene practices, potential swimming pool DBP precursors, and provide possible approaches to monitor and prevent urine, and the formation of irritating DBPs, in swimming pools.

Our study contributes understanding to the overall HBQ-DBP exposure pathway by identifying new HBQ precursors with sources ranging from raw water to beverage ingredients. AAAs are ubiquitous components of environmental water NOM and food stuffs. Aspartame is a commonly used AS. This research will add a new perspective to the ongoing discussion weighing the known benefits and potential adverse effects surrounding continued consumption of ASs.

Overall, translating meaningful research outcomes to disciplinary experts, group colleagues and the general public through multiple communication platforms is a rewarding approach to make an impact with each project's results.

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Chapter 2

Occurrence of Artificial Sweeteners Acesulfame and Sucralose in Surface Waters of British Columbia, Canada[†]

"The man who moves a mountain begins by carrying away small stones."

- Confucius

2.1 Introduction

Canada has an abundance of freshwater resources. Geographically, it is the second largest country in the world with the third largest renewable freshwater supply globally yielding 103,899 m^3 fresh water per capita.[1] Canada's population density is low (33,476,688 persons, 3.8 persons/km^2)[1] compared to locations in the United States, Europe, and Asia where many artificial sweetener (AS) occurrence and waste impact evaluation studies are conducted.[2-6] **Table 2.1** presents a summary of the limited reported data for the occurrence and distribution of ASs in Canadian environmental water samples. The majority of studies ASs in Canadian waters investigate water samples collected from Ontario.[7-13] ASs have also been detected in samples collected from Alberta,[7-9, 14] the Yukon,[7] and Saskatchewan.[9] In an effort to support ASs as indicators of waste impact in water systems (i.e., septic fields), groundwater wells are a commonly analyzed sample type among the Canadian studies.[7-9, 14-16] Canadian surface water samples are limited to a few streams in Jasper and Ontario,[7] as well as Grand River[10,13]and Lake Simcoe,[12] both located in Ontario. There is no previously reported data for water bodies located in British Columbia.

[†] Parts of **Chapter 2** (i.e., **Sections 2.3.2, 2.3.3,** and **2.4.2**) have been published as Jmaiff Blackstock, L.K.; Wawryk, N.J.P.; Jiang, P.; Hrudey, S.E.; Li, X.-F. Recent applications and critical evaluation of using artificial sweeteners to assess wastewater impact. Current Opinion in Environmental Health & Science. 2019, 7: 26-33. Reprinted with permission. Copyright 2018 Elsevier.

Table 2.1 Summary of reported non-nutritive artificial sweetener occurrence in Canadian waters

Sample Type	Location	Artificial Sweetener Concentration Range				Ref
		SUC	ACE	CYC	SAC	
Groundwater wells	Whitehorse, Yukon	n.d. - 24 µg/L	n.d. - 9.7 µg/L	n.d. - 0.98 µg/L	n.d. - 2.0 µg/L	(7)
Groundwater wells	Hamilton, Ontario	n.d. - 17 µg/L	0.02 - 8.2 µg/L	n.d. - 0.056 µg/L	n.d. - 0.12 µg/L	
Groundwater along streams	Jasper, Alberta	n.d.	n.d. - 3.5 µg/L	n.d. - 0.046 µg/L	n.d. - 0.15 µg/L	
Stream	Jasper, Alberta	n.d.	n.d. - 0.063 µg/L	n.d.	n.d. - 0.028 µg/L	
Groundwater along streams	Barrie, Ontario	n.d.	n.d. - 33.6 µg/L	n.d.	n.d. - 10.3 µg/L	
Stream	Barrie, Ontario	n.d.	n.d. - 0.088 µg/L	n.d.	n.d. - 0.066 µg/L	
Groundwater along streams	Burlington, Ontario	n.d.	n.d. - 0.36 µg/L	n.d. - 0.038 µg/L	n.d. - 0.12 µg/L	
Stream	Burlington, Ontario	n.d.	0.082 - 0.34 µg/L	n.d.	n.d.	
Grand River	Ontario	n.d. - 21 µg/L	n.d. - 3.6 µg/L	n.d. - 0.88 µg/L	n.d. - 7.2 µg/L	(10)
Groundwater wells	Alberta	n.d. - 541 ng/L	0.9 - 1534 ng/L	-	-	(14)
Sewage Lagoon	Ontario	11.1 - 47.8 ng/L	-	-	-	(8)
Groundwater wells	Regina, Sask.	-	32 µg/L (max)	n.d.	87 µg/L (max)	(9)
Groundwater wells	Cambridge, Ontario	-	12 µg/L (max)	n.d.	25 µg/L (max)	
Groundwater wells	Hamilton, Ontario	-	11 µg/L (max)	0.9 µg/L (max)	250 µg/L (max)	
Groundwater wells	Waterloo, Ontario	-	3.7 µg/L (max)	2.1 µg/L (max)	16 µg/L (max)	
Groundwater wells	Jasper, Alberta	-	0.05 µg/L (max)	14 µg/L (max)	11 µg/L (max)	
Groundwater septic tank / plume effluent	Ontario	-	9.9 - 265 µg/L	-	-	(15)
Groundwater septic effluent	Ontario	< 5 - 98 µg/L	32 - 91 µg/L	-	-	(16)
Groundwater seep Nottawasaga River	Ontario	0.57 µg/L (max)	1.7 µg/L (max)	0.18 µg/L (max)	0.095 µg/L (max)	(11)
Lake Simcoe Wastewater treatment effluent	Ontario	144 - 249 ng/L	4.4 - 32.8 ng/L	-	-	(12)
Grand River	Ontario	-	n.d. - 3500 ng/L	-	-	(13)
Groundwater	Jasper, Alberta	-	2510 ng/L (max)	46 ng/L (max)	35 ng/L (max)	(38)
Groundwater	Barrie, Ontario	-	10 700 ng/L (max)	n.d.	n.d.	

The Thompson River Watershed (TRW) is located in the interior of B.C. and covers approximately 56,000 km^2 (6%) of the province's area.[17] The TRW region has a total Census population of approximately 195,000, encompassing 6 Regional Districts, 16 municipalities, and 29 areas under First Nations governments.[18] The largest ecoregion in the TRW is the Thompson-Okanagan Plateau (TOP).[19] The TOP is a broad forested rolling plateau with low elevation sagebrush-steppe dominated basins. The regions climate consists of warm, dry summers and cool winters, characteristic of the semi-arid plateau region of the B.C. Interior. The Kamloops region specifically has low total precipitation and high evapotranspiration rates which result in overall water deficit conditions.[17, 20] Several large lakes occur in the valley basins with hundreds of small lakes and ponds dispersed across the uplands. The TOP is dissected by the North Thompson, South Thompson and Thompson Rivers. These large rivers flow west until they join the Fraser River.[17]

The Fraser-Lower Mainland drainage region extends 233,104 km^2 from Prince George to Vancouver. Containing the Greater Vancouver Area, the Fraser – Lower Mainland drainage region has the third highest population in Canada. Surface freshwater intake for the Fraser-Lower Mainland region totaled 615.3 million m^3 in 2013 for manufacturing, drinking water plants, irrigation, mining, and thermal electric production.[1] A 2011 report by the British Columbia Ministry of Environment ranked watersheds in the Thompson Region based on spatial data analysis of land use types, water users, and natural watershed characteristics. The three watershed units identified with the highest risk for anthropogenic effects on water quality and its designated users were the South Thompson River from Shuswap Lake to Kamloops, Peterson Creek (Kamloops), and the North Thompson River from Barrier to Kamloops due to their high proportion of urbanization, agriculture, mining, and road and stream crossing density.[21]

Recently, a highly sensitive high performance liquid chromatography – tandem mass spectrometry (Agilent 1100 series system, AB Sciex API 5500 QTrap; HPLC-MS/MS) method was developed capable of detecting ACE (0.2 ng/L) and SUC (5 ng/L) at low part-per-trillion (ppt) levels without any sample pretreatment.[14] This method required 500-µL manual injection with a Rheodyne 6-port valve with a large injection loop. I was able to modify this method to be usable with a commonly available autosampler (Agilent,

Waldbronn, Germany), requiring a volume of only 100-µL for injection, while maintaining a reasonably similar detection limit (ACE: 0.5 ng/L, SUC: 50 ng/L) in the low ppt range. This improved method was used to analyze environmental water samples collected from 12 different surface waters in the Thompson Region surrounding Kamloops, British Columbia.

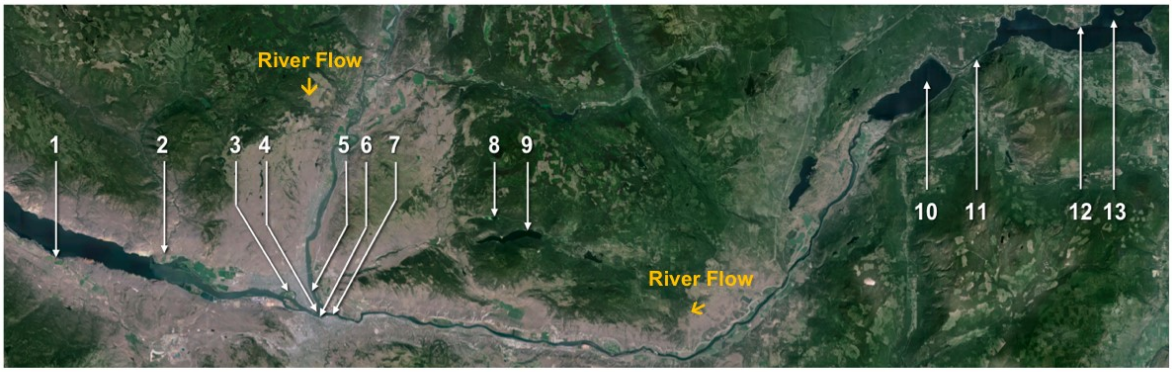
2.2 Materials and Methods

2.2.1 Reagents

The acesulfame-K (ACE) standard was obtained from Supelco (Bellefonte, PA), and the deuterated isotopic internal standard Acesulfame-K-d4 (ACE-d4) was obtained from Toronto Research Chemicals (Toronto, ONT). Working stocks were prepared in methanol and stored in a -20°C freezer. LC/MS grade formic acid (FA, 49−51%) was obtained from Sigma-Aldrich (St. Louis, MO). Water and methanol used in this study were Optima® LC/MS grade, purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2.2 Sample Collection

Water samples were collected from 12 different locations (contributory lakes, creeks, and rivers) from surface water bodies in the Thompson Basin and Shuswap Basin Ecosections[19, 20] of the Thompson River Watershed between July 30 and August 07, 2014, **Figure 2.1**. No specific permissions were required as all locations were publicly accessible. The collection sites include public parks as well as popular tourism destinations with heavy beach usage. The hottest month for the region in 2014 was July with an average daily high temperature of 32°C.[22] Triplicate grab samples were collected by hand from the shoreline at a depth of 10 cm from at least 30 cm deep water.



1. Tobiano Resort Marina, Kamloops Lake
2. Tranquille River
3. McArthur Island Recreational Park
4. Overlander Beach, N. and S. Thompson River
5. River's Trail Beach, N. Thompson River
6. Riverside Park Beach, S. Thompson River
7. Pioneer Park Beach, S. Thompson River
8. Louis Lake
9. Paul Lake
10. Little Shuswap Lake
11. Scotch Creek
12. Shuswap Lake Beach
13. Shuswap Lake Centre

Figure 2.1 Map of sites for environmental samples collected in the Thompson Okanagan region of British Columbia

2.2.3 HPLC-MS/MS Analysis of ACE and SUC

Samples were stored at 4°C after collection. Before analysis samples were filtered through disposable 0.45-µm Millipore filters. The triplicate samples were split into duplicates before analysis for a total of n=6. A 10-ng/L spike of the deuterated internal standard, ACE-d4, or a 200-ng/L spike of SUC-d6, prepared in LCMS grade methanol, was added to every sample and standard before analysis for a final composition of 9:1 H_2O:MeOH. The peak area ratio between ACE and ACE-d4, or SUC and SUC-d6 primary transitions were used for quantification, while secondary transition peaks were used to confirm analyte identity. A calibration curve was prepared with each batch to account for variation in instrument signal intensity. Outliers were removed using the Q-test with an n=6 modified Thompson Tau value. An analysis blank was injected into the LC-MS/MS after each set of six samples to detect and avoid any carryover or contamination during sequential analysis. No ACE or SUC was detected in any of the analysis blanks.

100µL of each filtered (0.45µm, Millex) water sample was injected directly onto an Inspire C18 (100 x 3.0 mm, 3µm, Dikma) HPLC column at room temperature, using the

Agilent 1100 G1329A Autosampler and 2LC system. The LC mobile phase consisted of solvent A: 0.1% formic acid (v/v) in water (Optima Grade). Solvent B is 0.1% formic acid (v/v) in MeOH. The gradient program is listed in **Table 2.2.** A triple quadrupole tandem mass spectrometer (5500 QTRAP, AB Sciex, Concord, ON, Canada) was used in negative multiple-reaction monitoring (MRM) mode for the determination of ACE, SUC, and their corresponding deuterated internal standards. The MS instrument operating conditions are provided in **Table 2.3** and the MRM transitions and parameters are listed in **Table 2.4**. ACE and SUC were quantified using internal standard peak area ratios (ACE-d4 and SUC-d6) and positive identifications were made by confirming peak area ratios between MRM transitions and by matching retention times with authentic standards.

Table 2.2 HPLC mobile phase gradient program

Time (min)	Solvent A (%)	Solvent B (%)	Flow Rate (µL/min)
0.00	95.0	5.0	500
2.00	5.0	95.0	500
4.00	5.0	95.0	500
4.10	95.0	5.0	500
11.00	95.0	5.0	500

Solvent A: Optima H2O (0.1% FA)
Solvent B: Optima MeOH (0.1% FA)

Table 2.3 MS instrument operating conditions

Collision Gas	High
Curtain Gas	10 psi
Ion Source Gas 1	50 psi
Ion Source Gas 2	40 psi
Ion Spray Voltage	-4500 V
Temperature	500 °C
Entrance Potential	-10
Dwell Time	100 (msec)

5500 Sciex QTrap Mass Spectrometer

Table 2.4 Multiple reaction monitoring mode analyte ion transitions and detection parameters

Name	Rt (min)	Q1 Mass (Da)	Q3 Mass (Da)	DP Declustering Potential	CE Collision Energy	CXP Collision Cell Exit Potential
ACE	4.11	162.0	81.9	-60	-18	-6
	4.11	162.0	77.9	-60	-18	-6
ACE-d4	4.11	166.0	85.9	-60	-18	-6
	4.11	166.0	77.9	-60	-18	-6
SUC	4.41	441.0	395.0	-70	-12	-15
	4.41	443.0	396.9	-70	-12	-15
SUC-d6	4.41	447.0	401.0	-70	-12	-15
	4.41	449.0	403.0	-70	-12	-15

2.3 Results and Discussion

2.3.1 Concentration of ACE and SUC in Environmental Samples from the Thompson River Region of British Columbia

Figure 2.2 and **Table 2.5** show the concentrations of ACE and SUC detected from surface waters collected from the Thompson Region. ACE was detected in every sample with averages ranging from 1 to 17 ng/L and the majority (10/12) of percent relative standard deviation (%RSDs) less than 20% (2/12, %RSD > 35%). For ACE, 10/12 samples had concentrations less than 5 ng/L, whereas samples collected from popular provincial parks had concentrations of 16 and 17.8 ng/L. There were no differences between the range of average ACE determined in rivers (1.0 – 16.1 ng/L) and lakes (1.5 – 17.8 ng/L). Location 3 yielded exceptional results; average ACE was 133 ng/L but had %RSD of 83.6% between the triplicate collected samples. This could be due to the water stagnation in the collection area for location 3, which was a recreational park island river mote. During collection the water level was low. McArthur Island Sport and Event Centre includes 3 NHL sized arenas, 12 baseball diamonds, 9 soccer fields, playgrounds and outdoor fitness equipment, BMX and biking paths, and a disk golf course among other facilities.[23] Due to the wide variety of use in the area, waters with high ACE concentration may be impacted by leaching from food wrap and refuse. Addition contamination sources of the low flow mote could include the local population of homeless who set up shelter in Kamloops' riverbanks.[24] SUC was only

quantifiable in 2 of 12 samples both of which were Lakes (i.e., Louis Lake and Paul Lake). This could be due to the higher limit of detection compared to ACE. SUC may be a more successful indicator of human waste impact in environmental waters impacted by much larger populations, as the largest city in the region, Kamloops, has a population under 86 000 residents.[25]

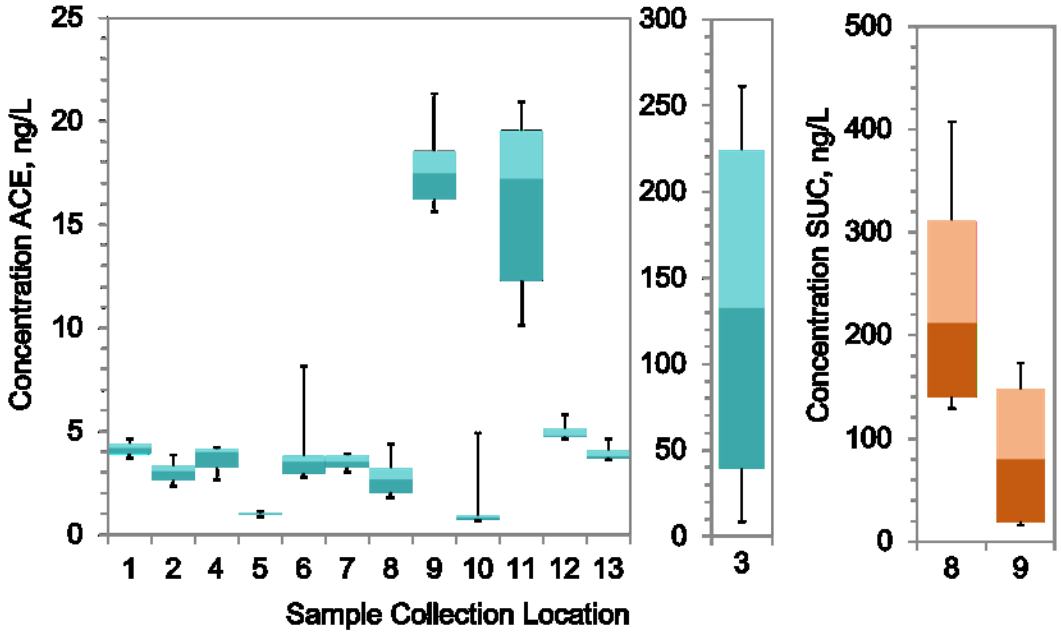


Figure 2.2 Box and whisker plots of ACE and SUC determined in environmental water samples from each collection location. No SUC was detected in samples 1-7, 10-13

Table 2.5 Average ACE and SUC concentrations determined in environmental water samples corresponding to **Figure 2.2**, collected from locations indicated in **Figure 2.1**

		ACE, ng/L	
Location	**Average**	**St. Dev**	**% RSD**
1	4.2	0.4	8.6
2	3.1	0.6	18.1
3	133	111.2	83.6
4	3.7	0.6	17.5
5	1.0	0.1	8.9
6	4.1	2.0	49.1
7	3.5	0.4	10.3
8	2.8	1.0	34.8
9	17.8	2.1	11.9
10	1.5	1.7	113.0
11	16.1	4.6	28.7
12	5.0	0.4	8.9
13	4.0	0.4	9.4
Location		**SUC, ng/L**	
	Average	**St. Dev**	**% RSD**
8	239.8	130.3	54.3
9	87.1	80.5	92.4

There are several potential sources of ASs in the collection region. The South Thompson Rivers flows through several settled areas including downtown Kamloops. The small town of Pritchard, east and upstream of Kamloops, has a sewage treatment plant discharge. This section of the South Thompson River is adjacent to several working farms, as well as the Lafarge gypsum mine, and multiple sand and gravel pits. The North Thompson River is impacted by high road density, as well as urban and agricultural uses.[21] The Kamloops Wastewater Treatment Plant is located on the south side of the Thompson River, west of Kamloops. In 2015, the facility processed a total of 10 billion litres of wastewater, approximately 30 million litres per day. The collection system consists of 525 km of sewer mains with 22,511 service connections.[26] The Paul Lake Community Sewer System services approximately 105 customers by processing 35 septic tanks per year through a septic tank effluent system consisting of one pumping station, Rotating Biological Contactor, and septic

field disposal.[27] Paul Lake hosts a popular Provincial Park site. SUC was only detected in Paul Lake and its neighbour Louis Lake. It is possible that the nearby septic field is the unique source of SUC in the area. The Scotch Creek community located on Shuswap Lake is one of several popular tourist destinations on the Lake. The water quality can be impacted by domestic household sewage disposal systems, septic fields, and some smaller community-based disposal systems.[28]

The concentrations of ACE determined in the surface water samples collected from the Thompson Region of British Columbia are in the low ng/L, with the majority of samples containing less than 20 ng/L ACE. This is similar to ACE reported in streams from Jasper and Barrie ranging from not detected up to 90 ng/L[7] and Lake Simcoe ranging from 4.4 to 32.8 ng/L. However, the surface water samples in the Thompson River region had far less ACE than maximum values determined in samples from Grand River (3500 ng/L)[10, 13] and Nottawasaga River (1700 ng/L)[11]. In the two samples in this study where SUC was detected, the average concentrations (i.e., 87 and 240 ng/L) were accordant with maximum SUC determined in other Canadian surface water samples (e.g., Nottawasaga River, 570 ng/L[11]; Lake Simcoe, 249 ng/L[12]).

2.3.2 Potential Attenuation of ASs in the Environment

As persistent environmental contaminants, the potential toxicological impact of ASs in aquatic ecosystems has been investigated. Toxicity studies with exposures up to 1000 mg/L found none of the ASs, ACE, CYC, SUC or SAC, induced any significantly adverse effects in the *Daphnia magna* water flea or the freshwater plant, *Lemna minor*.[29] Likewise, ACE induced no significantly adverse effects on Zebrafish embryos at exposure concentrations up to 1000 mg/L.[28] With reported concentrations in waterbodies well below 1 mg/L, ASs have not been demonstrated to pose a risk to aquatic ecosystems.

Ideally, chemical tracers undergo negligible attenuation under environmental processes. If unknown routes for losses of ASs exist in the environment, assessment of the WW impact may be impaired. The fate and transformation of ASs in the environment is a topic of continuous development; however, distinct transformation pathways will not be discussed in detail here. Several environmental transformation processes such as UV irradiation, sorption, and microbial degradation have been reported to decrease ASs. For

example, simulated realistic solar radiation levels were found to degrade ACE, but not SUC, SAC, or CYC.[6] Evidence showing variable sorption of ASs to different organic materials have been reported in detail.[31-33] Li *et al*. found that for granular activated carbon (GAC), SAC was absorbed to the highest degree followed by SUC > CYC > ACE.[31] Consequently, some fraction of AS may sorb to organic materials suspended in water bodies or surrounding sediments and soils.

Buerge *et al*. found that under aerobic soil conditions, CYC, SAC, ACE and SUC had half-lives of 0.8, 3.3, 6.1 and 9.0 days, respectively.[34] Interestingly, ASs have been found to transpire into vegetation grown in AS-rich soils. Ma *et al*. determined uptake of ASs by plants grown in soils enhanced with pig manure fertilizer,[35] and Riemenschneider *et al*. detected ACE in field-grown vegetables irrigated with treated municipal WW.[36] If notable proportions of ASs are removed by environmental transformation processes, WW contributions may be unintentionally underestimated.

2.3.3 Recent Applications using ASs as WW Indicators

ASs have been demonstrated as effective indicators of WW contribution to water bodies.[37] For example, ACE and SUC have received attention as being stable indicators of municipal WW effluent, while CYC has proven to be a suitable indicator of untreated sewage.[2,6] Zirlewagen *et al*. investigated ACE and CYC as indicators of WW contamination in the rural catchment area of a German karst spring and found CYC/ACE ratios approximately three orders of magnitude higher in untreated vs treated WW.[6] CYC spikes were observed exclusively after combined sewer overflow events and correlated with breakthrough of fecal indicator bacteria.

Septic waste can contribute to nitrogen and/or phosphorus contamination of nearby waterbodies. Unlike municipal WWTP effluent, AS concentrations and distributions vary between different septic system sites because they collect WW from significantly fewer sources than WWTPs and do not represent integration across a population source.[38] In authentic ground and surface water applications, total inorganic nitrogen (TIN)/ACE ratios were used to differentiate septic tank leachate from other waste sources as the cause of nitrogen contamination in groundwater in agricultural land use areas.[15] Additionally, both ACE and SUC were found to correlate with TIN and soluble reactive phosphorus in a study

of 19 single and multi-dwelling domestic septic systems in Canada. Even though SUC was found to be susceptible to slow degradation, it could be used to fingerprint recent sources of WW contamination of groundwater.[16]

ASs have been demonstrated as indicators of domestic WW impact on surface and ground waters that serve as source waters for drinking water. Recently, ASs have been applied to study the impact of WW and subsequent formation of disinfection by-products (DBPs) in treated drinking water.[3] Reactions between disinfectants (e.g., chlorine, chloramine) and organic matter in source water forms DBPs.[39] Formation of toxic DBPs, like *N*-nitrosodimethylamine (NDMA), have been found to increase with WW contamination of source water.[40-47] In a study of 19 North American DWTPs, Prescott investigated the relationship between SUC in source water and NDMA formation potential (FP), which represents NDMA precursor loading in the source water. This study found a correlation between NDMA FP and SUC concentrations in source water. Prescott cautioned that the SUC and NDMA FP relationship may be confounded when assessing water sources impacted by non-domestic WW (e.g., agricultural or industrial) that contain other NDMA precursors.[3]

Using the same principles for ASs to trace WW contamination, my **Chapter 3** research[48] proposed the use of ACE, excreted exclusively in urine[49] to approximate its presence in recreational waters. For the first time, ACE load was found to be up to 570 times greater in swimming pool and hot tub samples than input control samples. By quantifying the average ACE in 20 urine samples as well as two swimming pools sampled over three weeks, the study estimated the approximate volume of urine required to account for the average ACE concentration observed.[48] While more controlled studies in swimming pools are needed to clarify this relationship, it may be useful for future assessment of pool water age or FP of swimming pool irritant DBPs (e.g., trichloramine) with known precursors in urine.[50]

Studies that quantitatively assess WW discharge into a waterbody using detected AS concentrations are limited in comparison to AS occurrence surveys. This is likely because WW impact quantitation requires comprehensive knowledge of the waterbody being investigated coupled with specific mathematics to determine the level of AS dilution based on water flow.

A one-dimensional deterministic non-equilibrium advection dispersion equation was used to model a breakthrough event that determined a total of 262 m^3 of combined WW discharge, corresponding to 1.1 g of CYC, had entered a karst spring.[6] Lee *et al*. estimated approximately 20-60% WW effluent in Sacramento River samples based on the SUC concentrations detected.[51] Calculations regarding this complex hydraulic system were made possible with the Delta Simulation Model II. Similarly, measured SUC concentrations along with water flow rates allowed Prescott *et al*. to estimate up to 37% WW-impacted DWTP influents.[3] Using ASs to quantify WW impact on drinking water supply is a unique tool that has limited studies and is a logical next step in future AS investigations in this field.

2.4 Conclusion

2.4.1 Occurrence of ACE and SUC in B.C. Surface Waters

For the first time, ACE and SUC concentrations were reported for surface waters in the Thompson River region of British Columbia. Compared to the limited data of other non-nutritive artificial sweeteners in Canadian environmental waters, these samples had ACE concentrations in the low part-per-trillion (ng/L) range compared to reports ranging from low parts per trillion in streams to low parts per billion (µg/L) in wells and high ppb in sewage effluent. Studies on ASs on Canadian water bodies are scant. It is possible the low concentration of ACE in the Thompson region is due to a relatively low population density impacting the water bodies compared to the populous southern Ontario region, and other global locations studied (e.g., California, U.S., Europe, China, India). The Grand River is known for being heavily impacted by human activity,[52] rationalizing high ACE occurrence. The low ACE background levels in the waterbodies would make ACE suitable to detect a nearby waste discharge event, however, unlike CYC, ACE is not specific to untreated waste. SUC was only detectable in two lakes of all 12 locations surveyed. The higher detection limit (50 ng/L) compared to ACE (0.5 ng/L) make SUC a poor choice as an indicator in this region.

2.4.2 Perspectives on ASs as WW Indicators

The characteristics of ASs align with ideal indicators of WW impact. Early literature consisting of surveys and proof of concept studies were fundamental in generating new hypotheses to validate ASs as WW indicators. However, recent literature has suggested that

some general assumptions made in previous studies should be re-assessed to ensure that ASs are appropriately monitored to estimate WW contributions. Domestic waste may not be the only source of ASs; landfill leachate and manure fertilizer were identified as alternate contamination sources of ASs in groundwater. Although increasing evidence indicates biologically mediated removal of ASs by different treatment technologies, AS effluent concentrations remain useful for assessment of downstream WW impact. AS transformation pathways under various environmental conditions[53-55] is a topic of continuous investigation that exceeds the scope of this discussion. With careful design of experiments and controls, ASs can serve as effective indicators of WW impact in water bodies. Continuous advancement of analytical tools providing low detection limits will enhance the understanding of fate and behavior of ASs in environmental waters, increasing the accuracy of WW impact assessments.

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Chapter 3

Artificial Sweetener Acesulfame as an Indicator of Urine in Swimming Pools and Hot Tubs ‡

"A little more persistence, a little more effort,
and what seemed hopeless failure may turn to glorious success."
– Elbert Hubbard (1856-1915)

3.1 Introduction

The recent news article "Chemical reactions taking place in your pools"[1] and the overnight colour change of the water from blue to green in the 2016 Rio Olympic pools[2] highlights the need to monitor water quality in swimming pools. A variety of chemicals can be introduced into recreational waters via body fluids[3] that can react with disinfectants. Recently, a study identified over 100 disinfection byproducts (DBPs) in swimming pools and hot tubs, and found that organic extracts from those samples were more mutagenic than corresponding tap water extracts.[4] Epidemiological studies have found a potential association of increased risk of bladder cancer with long-term DBP exposure via drinking water,[5] but association via exposure through swimming pools has been inconsistent.[6,7]

Human urinary input into swimming pools is a public health concern although urine itself is sterile. Urine contains many nitrogenous compounds such as urea, ammonia, amino acids, and creatinine. These compounds can react with disinfectants (e.g., chlorine) in swimming pools to form DBPs including trihalomethanes, haloacetic acids, haloamines, and halonitromethanes.[8,9] Exposure to volatile DBPs, specifically trichloramine, in indoor swimming facilities can lead to eye and respiratory irritation[10-12] and has been linked to occupational asthma.[13] Although considered a taboo, 19% of adults have admitted to having

‡ **Chapter 3** has been published as Jmaiff Blackstock, L.K.; Wang, W.; Vemula, S.; Jaeger, B.T.; Li, X.-F. Sweetened swimming pools and hot tubs. Environmental Science & Technology Letters. 2017, 4(4): 149-153. Reprinted with permission. Copyright 2017 American Chemical Society. Parts of **Section 3.5** have been published as Zheng, Q.; Jmaiff Blackstock, L.K.; Deng, W.; Wang, H.; Le, X.C.; Li, X.-F. Keep swimming but stop peeing in the pools. Journal of Environmental Sciences. 2017, 53: 322-325. Reprinted with permission. Copyright 2017 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences.

urinated in a swimming pool at least once.[14] The average urine excretion per swimmer in pools is approximately 70 mL.[15] Dissolved organic carbon (DOC) in swimming pools has been associated with both bather load and formation of trihalomethanes.[16,17] The potential negative health effects associated with DBPs led us to investigate a marker for urine in swimming pools and hot tubs.

Artificial sweeteners are consumed in large quantities due to their negligible calories and low impact on blood sugar.[18] Found pervasively in natural water bodies, [19-23] they have been recognized as emerging environmental contaminants.[23,24] Non-nutritive artificial sweeteners including acesulfame-K (ACE), [25,26] sucralose (SUC), saccharin (SAC), and cyclamate (CYC) have been recognized as indicators of wastewater in environmental waters.[27,28] ACE, used in prepackaged foods,[18,29,30] is not metabolized by humans; it is completely absorbed and excreted exclusively in the urine,[29,31] whereas SUC is excreted mainly in feces.[32] The average concentration of ACE in urine is approximately 4000 ng/mL.[33] Several studies have shown that ACE is stable at varying pH and high temperatures[30] and is resistant to microbial action in aerobic soils.[34] Furthermore, ACE is much more resistant to wastewater treatment processes compared to SAC or CYC.[25,35,36] Because of its widespread consumption, stability, and persistent nature, I hypothesized that ACE may serve as an indicator of urinary input in swimming pools. The occurrence of any artificial sweetening agents in swimming pools has not been studied previously. The objective of this study was to determine the occurrence of the artificial sweetener ACE in swimming pools and hot tubs compared to input tap water.

The determination of ACE in water typically includes separation by ion or liquid chromatography (LC) and detection using electrospray ionization mass spectrometry (ESI-MS), with[37] or without[27] solid phase extraction (SPE). Recently, a method capable of detecting ACE at 0.2 ng/L without preconcentration was developed.[38] However, the need for specialized equipment or sample preconcentration makes these methods impractical for monitoring studies. To enable the determination of ACE in the complex matrices of pool and hot tub water samples, without preconcentration, I have developed a rapid, high throughput method using high performance liquid chromatography (HPLC) with tandem mass

spectrometry (MS/MS). The method was applied to determine ACE in over 250 samples collected from 31 pools and hot tubs and over 90 corresponding input tap water samples.

3.2 Materials and Methods

3.2.1 Reagents

The acesulfame-K (ACE) standard was obtained from Supelco (Bellefonte, PA), and the deuterated isotopic internal standard acesulfame-K-d4 (ACE-d4) was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). Working stocks were prepared in methanol and stored in a -20 °C freezer. LCMS-grade formic acid (FA, 49−51%) was obtained from Sigma-Aldrich (St. Louis, MO). Water and methanol used in this study were Optima LCMS-grade, purchased from Fisher Scientific (Fair Lawn, NJ).

3.2.2 HPLC-MS/MS Analysis of ACE

An Agilent 1100 HPLC system (Agilent, Santa Clara, CA) was used with an Inspire C18 column (100 x 3.0 mm, 3 µm particle size; Dikma Technologies, Lake Forest, CA) at room temperature. The flow rate was set to 0.5 mL/min with the autosampler injection volume set to100 µL. Solvent A was acidified water [0.1% formic acid (FA)] and solvent B was acidified methanol (0.1% FA).

A triple quadrupole tandem mass spectrometer (5500 QTRAP, AB Sciex, Concord, ON, Canada) with multiple-reaction monitoring (MRM) mode was used for the detection and quantification of ACE and the internal standard, ACE-d4. For ACE, the Q1 and Q3 mass-to-charge ratios for primary and secondary transitions were 162.1 > 81.9 Da and 162.1 > 77.9 Da; for ACE-d4, transitions of 166 > 85.9 Da and 166 > 77.9 Da were used. Retention times were matched with authentic standards.

ACE-d4 was prepared in LCMS-grade methanol. Each sample was spiked with ACE-d4 at a concentration of 10 ng/L. All samples had a final composition of 9:1 H_2O:MeOH. The relative peak area of the primary transition for ACE to ACE-d4 was used for quantification. The secondary transition peak confirmed the identity of ACE in the samples. A calibration curve was prepared with each batch to account for variation in instrument signal intensity.

3.2.2.1 Optimized HPLC-MS/MS Conditions

The mobile phase gradient program linearly increased B from 5% to 95% in 2 min; maintained B at 95% until 6 min; returned to 5% B between 6.0 and 6.1 min; and remained at 5% for column equilibration until run completion at 15 min.

Optimized conditions for negative electrospray ionization (ESI) were: curtain gas, 45 psi; collision gas, high; gas 1, 50 psi; gas 2, 80 psi; ion spray voltage, -4500 V; temperature, 400ºC; and dwell time, 75 msec. The optimized declustering potential (DP), collision energy (CE), and cell collision exit potential (CXP) for all transitions are listed in **Table 3.1**.

Table 3.1 Optimized parameters for MS/MS (MRM) ion pair transitions

MRM Ion Pair Transition	Declusterization Potential (DP)	Collision Energy (CE)	Cell Exit Potential (CXP)
162.1 > 81.9	-60	-19	-10
162.1 > 77.9	-60	-44	-7
166 > 85.9	-53	-20	-10
166 > 77.9	-54	-46	-10

3.2.3 HPLC-MS/MS Method Validation

3.2.3.1 Limit of Detection Calculation

The instrument limit of detection (LOD) was calculated as three times the standard deviation of a 0.5 ng/L standard divided by the slope. This agrees with the observed chromatographic signal-to-noise ratio of 4.3 obtained for a 0.5 ng/L ACE standard. The method LOD was calculated as three times the standard deviation of the method blank signal divided by the slope. LCMS-grade water was filtered through 0.45-µm PVDF filters and analyzed as method blanks. Typical chromatograms displaying signal response from the method blank, LOD standard, and the ACE-d4 sample spike are shown in **Figure 3.1**. The linear range for ACE was 0.5 to 1000 ng/L; a typical calibration curve is shown in **Figure 3.2**.

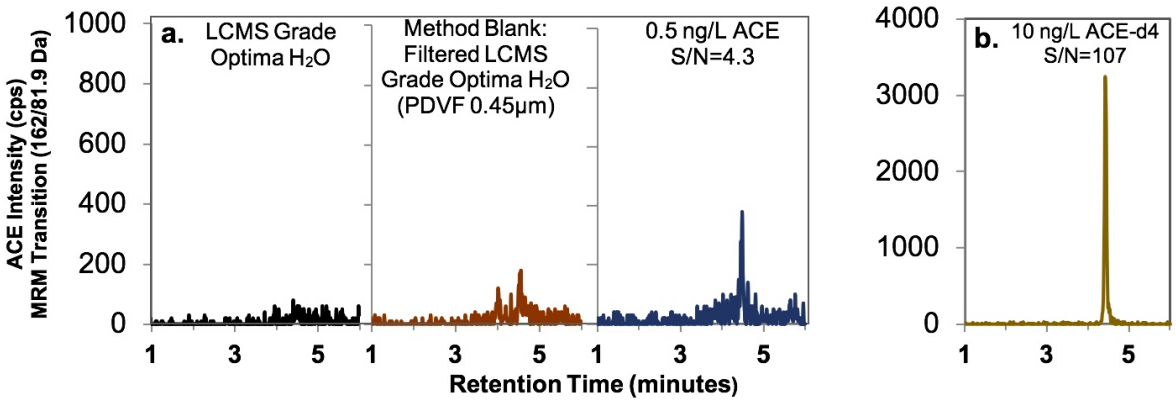

Figure 3.1 Typical chromatograms showing the signal intensity of (**a**) ACE in a blank sample, method blank sample, and a 0.5 ng/L ACE standard and (**b**) a 10 ng/L ACE-d4 internal standard

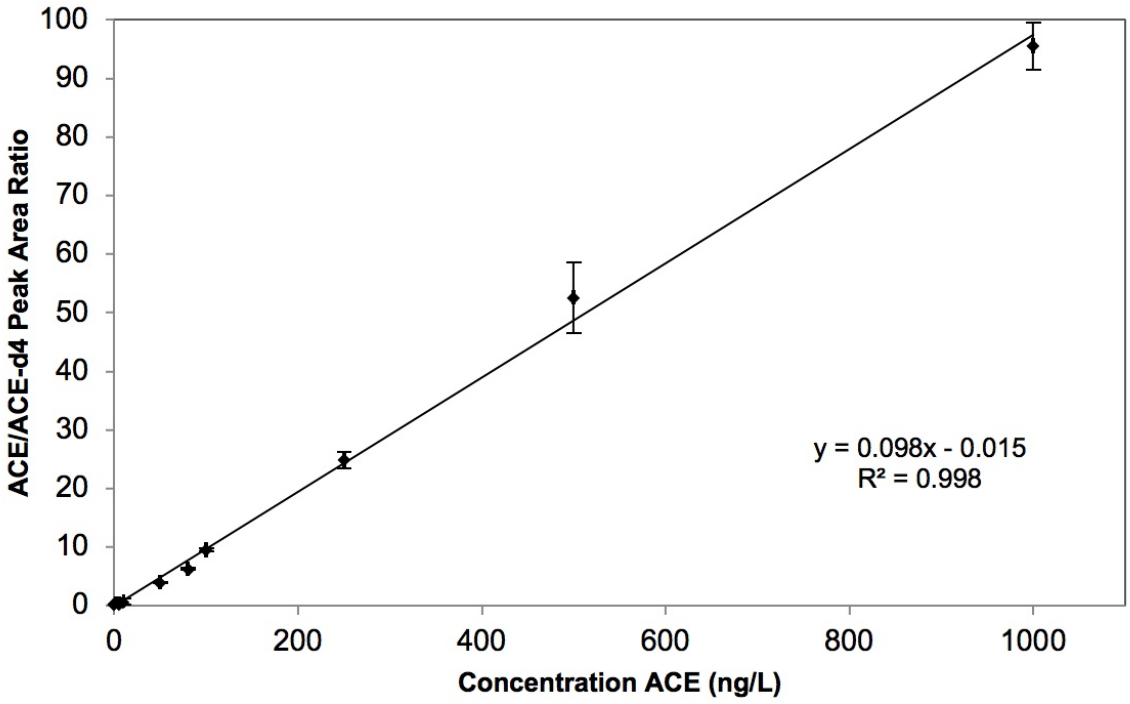

Figure 3.2 A typical ACE calibration curve prepared with 10-ng/L ACE-d4 spiked ACE standards ranging from 0.5 to 1000 ng/L in 9:1 LCMS H_2O:MeOH

3.2.3.2 Reproducibility

Intraday and interday reproducibility was evaluated for a 5.0-ng/L standard prepared in LCMS grade Optima water (**Figure 3.3**). Within the same day, the %RSD for the determined concentrations from triplicate analyses was less than 2%. Over four non-consecutive days, a one-way ANOVA test determined that p=0.564, indicating no significant difference between the mean concentrations reported on different days. The retention time for ACE and ACE-d4 in LCMS-grade, tap, and recreational waters were 4.4 ± 0.1 minutes (n=558 over 4 days), respectively. Therefore, this method is stable over a multi-day analysis on a shared instrument.

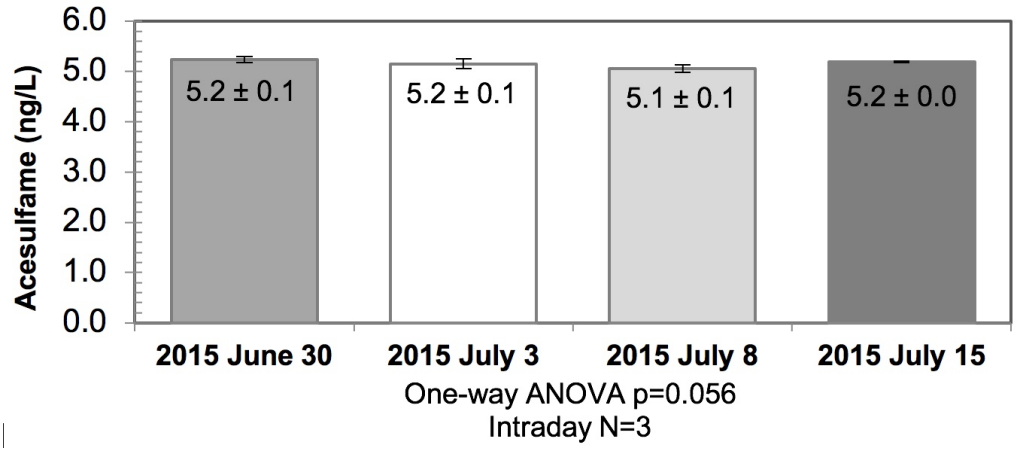

Figure 3.3 Interday and intraday variation of a 5-ng/L ACE standard prepared in LCMS grade water (10% MeOH with 10-ng/L ACE-d4 spike)

3.2.3.3 Matrix Effects

The matrix effects for recreational waters were evaluated in two ways. First, HT5 was used as a representative complex sample to determine whether recreational water matrix effects had an impact on signal intensity. The sample was diluted to 1/5, 1/10, 1/20, 1/40, and 1/80 and analyzed. The concentration of ACE was plotted against the dilution factor resulting in an R^2 value of 0.994 (**Figure 3.4**), indicating that the matrix did not affect the analyte intensity. Based on the concentration range of ACE in swimming pool and hot tub samples

obtained from preliminary screening, I chose a dilution factor of 1/20 to be used for all samples to ensure the analyte was within the linear range, to eliminate carry over, and to reduce residue buildup in the LC column and ESI source. Second, a spike recovery study was conducted with three representative samples: SP2, SP4, and HT8 (**Table 3.2**). Each sample was diluted to 1/20 and split into six portions; three were spiked with 50 ng/L ACE. The non-spiked samples represented the matrix. Recoveries ranged from 86 to 91% indicating that the matrix did not affect the recovery of ACE at 1/20 dilution.

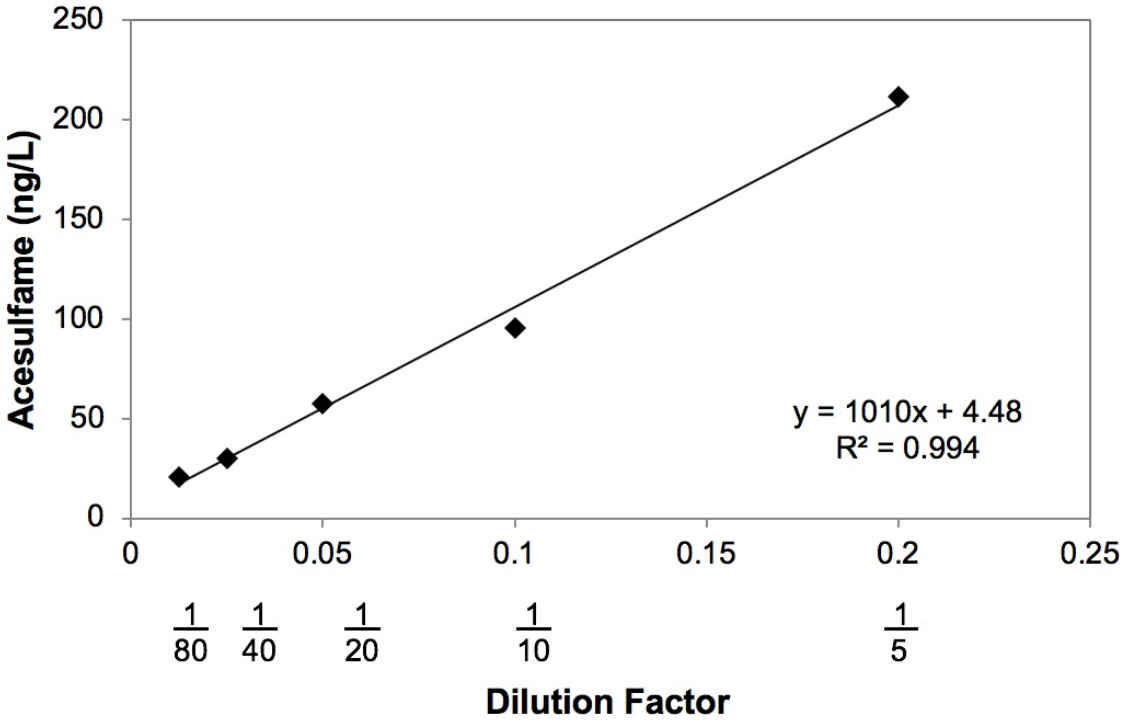

Figure 3.4 Recreational water matrix dilution and corresponding ACE response, the sample HT5 was diluted to 1/5, 1/10, 1/20, 1/40, and 1/80 and the concentration of ACE was determined

Table 3.2 Percent spike recovery for ACE in three representative recreational sample matrices

Sample	% Spike Recovery ϕ	% RSD
RF1 SP2	87%	4%
RF2 SP4	86%	5%
RF12 HT8	91%	8%

%R=((F-I)/A)*100%

Where: F=[Matrix+Spike]; I=[Matrix]; A=[Spike]

ϕ Each sample was diluted to 1/20 and then split into 6 portions. Three portions were spiked with 50 ng/L ACE; all portions were spiked with 10 ng/L ACE-d4 internal standard for quantification.

3.2.4 Dissolved Organic Carbon Analysis

Swimming pool, hot tub, and input tap water samples collected from City 1 were analyzed for their DOC content. DOC was measured at the Biogeochemical Analytical Service Laboratory of the University of Alberta. The US EPA Method 415.1 for Determination of Total Organic Carbon in Water was used with a Shimadzu TOC-5000A Total Organic Carbon Analyzer (Shimadzu, Japan).

3.2.5 Sample Collection

3.2.5.1 Collection of Swimming Pool and Hot Tub Samples

I collected samples from two Canadian cities between May and August 2014. In City 1, samples were collected from 10 swimming pools (SP) and 5 hot tubs (HT) from 5 recreational facilities (RF) and 3 hotels (H). In City 2, samples were collected from 11 SPs and 3 HTs from 8 RFs and one private pool (P). All facilities used municipal tap water as the input source. Triplicate grab samples were collected using new, sterile, 15-mL polystyrene vials. In swimming pools and hot tubs, samples were collected away from the jets, approximately 30 cm from the edge and 15 cm below the surface. Municipal tap water was collected on the same day, in triplicate, at each site.

Samples were stored at 4 °C until analysis. ACE is stable and resistant to decomposition, showing no detectable decrease in concentration after 10 years of storage at room temperature.[39] Samples were filtered through disposable 0.45-µm Millipore filters (PVDF, 25-mm). An analysis blank was injected into the HPLC-MS/MS after each set of

samples to detect and avoid any carryover or contamination during sequential analysis. No ACE was detected in the analysis blanks.

3.2.5.2 Case Study Sample Collection

Samples were collected over three weeks from two swimming pools, SPx and SPz, in City 2 in August 2016. SPx and SPz have volumes of 110000 and 220000 US gallons (roughly 420000 and 840000 L). Both SPs are on a closed water filtration system with new water only being added to replace losses due to evaporation or splash out. Both pools are disinfected with Cl_2 gas and shocked with $CaOCl_2$. Each day, 6 SP samples and 3 tap water samples were collected from the same locations at the same time. Human urine samples (n=20) with equal volume were pooled and homogenized. The mixture was diluted 100000 times with Optima water through serial dilution. The diluted sample was analyzed in triplicate to obtain the average concentration of ACE.

3.3 Results and Discussion

3.3.1 Concentration of ACE in Swimming Pools and Hot Tubs

Figure 3.5 shows the concentrations of ACE determined in the pools and hot tubs. In City 1, the concentration of ACE in the pool samples ranged from 30 ng/L in SP10 to 2110 ng/L in SP8 (**Figure 3.5(a)**). In City 2, ACE ranged from 90 ng/L to 580 ng/L in all the pools except SP20, where 1070 ng/L of ACE was determined (**Figure 3.5(b)**). ACE concentrations in all hot tub samples ranged from 70 to 100 ng/L (HT3, HT4, HT6, and HT7) and from 2220 to 7110 ng/L (HT1, HT2, HT5, and HT8). HT5 contained the highest ACE concentration (7110 ng/L), more than double any other sample. These samples were collected at one time and represent only a snapshot in time. The large variation of ACE in the pools and tubs may be explained by the water change cycling time point, the number of users and events, and facility management practices. Typically, fresh water is only added to swimming pools to maintain water levels, whereas hot tub water in community facilities is replaced frequently to prevent health issues associated with heavy use.[40,41]

ACE was detected in all tap water samples at significantly lower concentrations than those in the pools and tubs in both cities. ACE in tap water samples ranged from 6 to 12 ng/L (**Figure 3.6(a)**) in City 1 and 12 to 15 ng/L (**Figure 3.6(b)**) in City 2. The difference in ACE

concentration in the two cities' tap waters is statistically significant ($p<0.001$; unpaired *t*-test). This is expected, as the source water for each city is unique. The ACE concentrations in swimming pools and hot tubs were 4 (SP10) to 571 (H4) times greater than the corresponding input tap water (**Table 3.3**). The ACE concentrations determined in the tap water samples in this study are comparable to some Albertan well water samples (0.9–1530 ng/L ACE)[38] and lower than those in Swiss tap waters (20–70 ng/L ACE).[18,42]

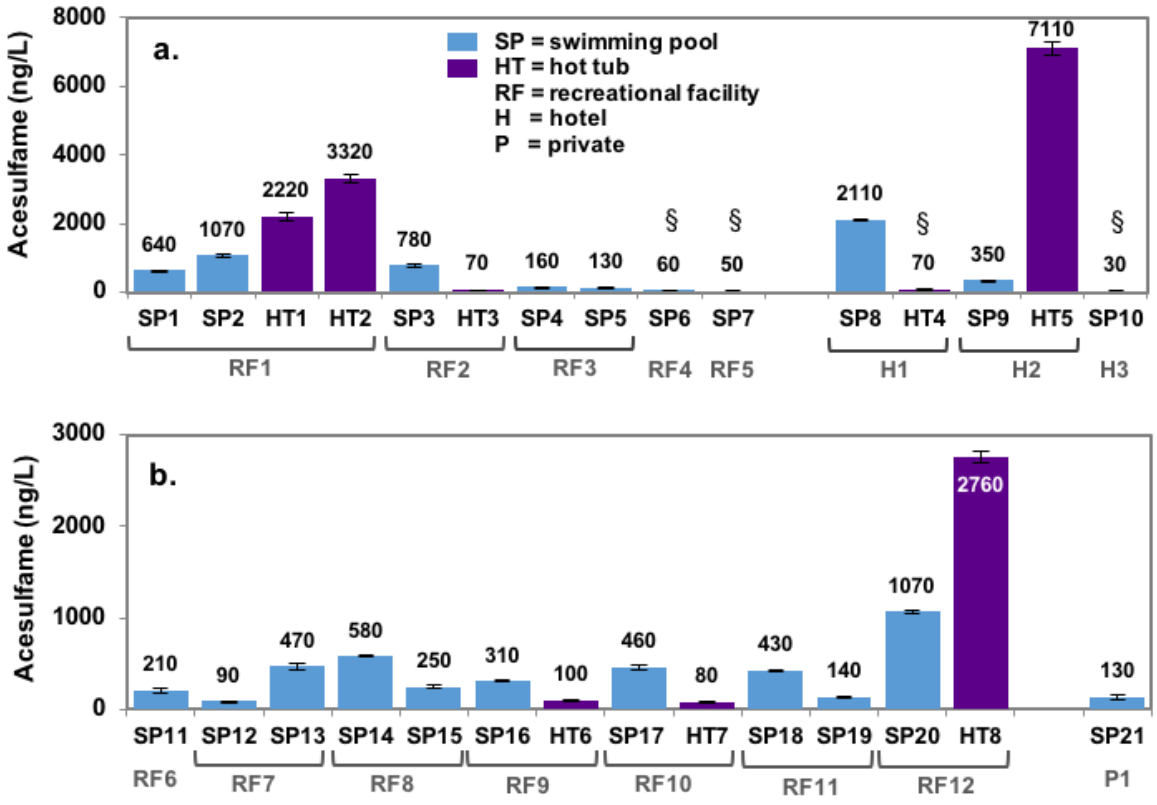

Figure 3.5 Average ACE concentration (n=3) detected in swimming pool (SP) and hot tub (HT) samples collected from public recreational facilities (RF), hotels (H), and a private residence (P) in (**a**) City 1 and (**b**) City 2. Samples indicated by the silcrow (§) were analyzed at 1/10 dilution, rather than 1/20, due to their low ACE concentration

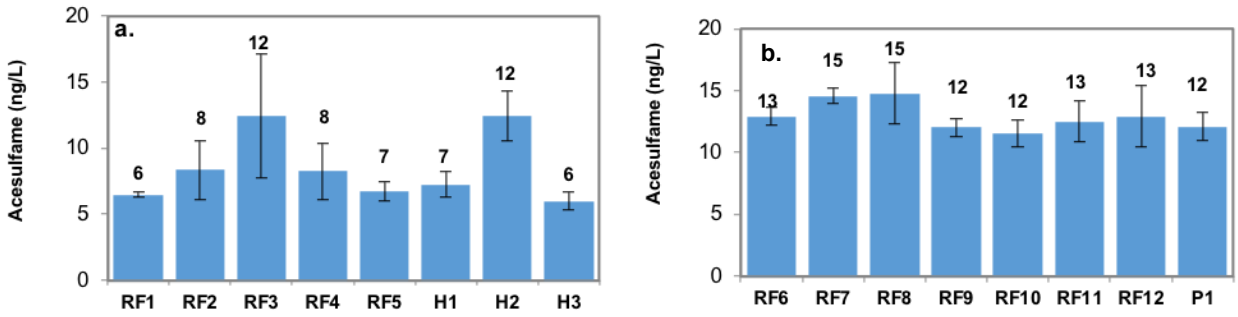

Figure 3.6 Average ACE concentration (n=3) detected in tap water samples collected from each sampling location in (**a**) City 1 and (**b**) City 2. The combined average of ACE concentration in each city's tap water was found to be statistically different using an unpaired Student's *t*-test ($p<0.001$)

Table 3.3 Average ACE fold increase in swimming pools and hot tubs compared to input tap water concentration; fold increase = (A-B)/B, where A = SP or HT, B = T.

City 1			City 2		
RF1	SP1	98	**RF6**	SP11	14
	SP2	165	**RF7**	SP12	5
	H1	343		SP13	36
	H2	513	**RF8**	SP14	36
RF2	SP3	93		SP15	15
	HT3	7	**RF9**	SP16	12
RF3	SP4	12		HT6	7
	SP5	9	**RF10**	SP17	39
RF4	SP6	6		HT7	5
RF5	SP7	6	**RF11**	SP18	34
H1	SP8	290		SP19	10
	HT4	9	**RF12**	SP20	75
H2	SP9	27		HT8	199
	HT5	571	**P1**	SP21	10
H3	SP10	4			

3.3.2 Concentration of Dissolved Organic Carbon in City 1 Samples

To examine the potential human impact on the water quality in the pools and hot tubs, I determined DOC in all samples collected in City 1 (**Figure 3.7**). The average DOC ranged from 4.8 to 6.3 mg/L in the tap waters and from 6.7 to 40.5 mg/L in the pools and tubs. For controls, DOC of input tap waters was significantly greater ($p<0.001$, unpaired *t*-test) than the blanks (LCMS Optima water). In the paired samples, DOC of all SP and HT samples, except HT4, was significantly greater ($p<0.01$ or $p<0.001$, unpaired *t*-test) than the respective input tap water samples. The increase of DOC in the pools and hot tubs suggests human impact on the water quality. Previous studies have observed the association of increasing DOC in swimming pools with increasing bather load.[16,17] Increased DOC is linked with enhanced formation of DBPs (e.g., THMs and HBQs).[16,17,43] Natural organic matter (NOM) is the primary source of DOC in tap water, whereas human inputs such as personal care products and body fluids introduced by swimmers (e.g., urine, sweat) may contribute to DOC in recreational waters.

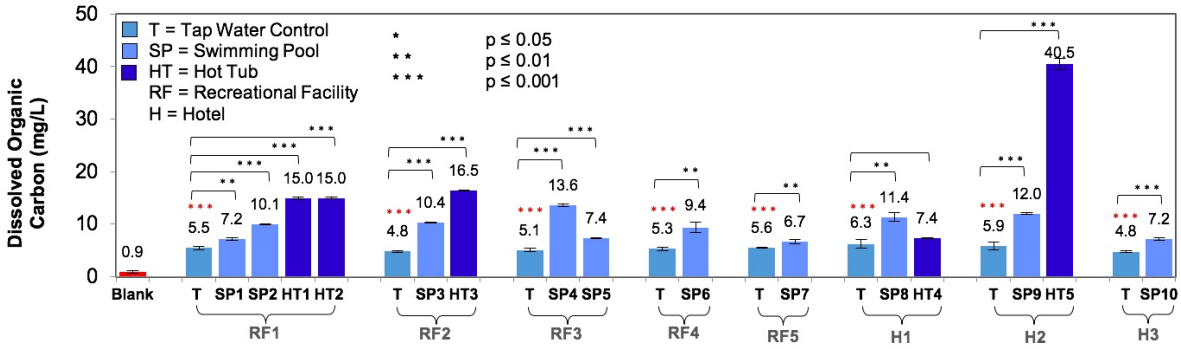

Figure 3.7 Average dissolved organic carbon (DOC) concentration detected* in tap and recreational water samples (n=3) collected from City 1. The average ACE in each sample type was compared using an unpaired Student's *t*-test

(*) Total Organic Carbon in Water EPA Method 415.1. https://www.epa.gov/sites/production/files/2015-06/documents/415_1dqi.pdf (accessed Feb 15, 2017)

3.3.3 Case Study – ACE Variability in Swimming Pools Over Time

To investigate the degree of variation of ACE in pool water, samples were collected from two different sized swimming pools, SPx (110 000 US gal.) and SPz (220 000 US gal.), over three weeks. ACE in the tap water control samples ranged from 12 to 20 ng/L (**Table 3.4**) during the collection period (n=45). The average ACE in SPx and SPz was 156 and 210 ng/L, respectively (**Figure 3.8**). The concentration of ACE in both pools varied similarly. The percent relative standard deviation (%RSD) for ACE determined in SPx and SPz was 18% and 15%, respectively. Based on the volume of each pool, the total mass of ACE present was estimated to be 65 mg in SPx and 176 mg in SPz (see **Calculation 3.1** and **Table 3.5**).

Table 3.4 Average concentration of ACE in SPx, SPz, and corresponding input tap water samples over 15 non-consecutive days

Sample Day #	Acesulfame, ng/L								
	Tap Water (n=3)			SPx (n=6)			SPz (n=6)		
	Avg	± St Dev	%RSD	Avg	± St Dev	%RSD	Avg	± St Dev	%RSD
1	16	± 0.5	3	133	± 5	4	173	± 13	8
2	16	± 0.8	5	135	± 3	2	180	± 13	7
3	17	± 0.5	3	125	± 7	5	240	± 10	4
4	16	± 0.4	3	134	± 4	3	242	± 13	5
5	17	± 1.9	11	127	± 8	6	251	± 9	3
8	18	± 3.6	20	114	± 21	18	221	± 16	7
9	15	± 0.1	1	165	± 4	2	202	± 8	4
10	16	± 0.4	2	174	± 8	4	258	± 24	9
11	15	± 0.9	6	150	± 27	18	242	± 9	4
12	14	± 0.7	5	154	± 12	8	220	± 7	3
15	16	± 0.6	4	173	± 9	5	186	± 19	10
16	17	± 1.0	6	181	± 8	4	191	± 3	2
17	20	± 1.4	7	183	± 6	4	186	± 13	7
18	16	± 0.7	4	181	± 6	3	178	± 5	3
19	12	± 0.4	3	202	± 8	4	182	± 7	4

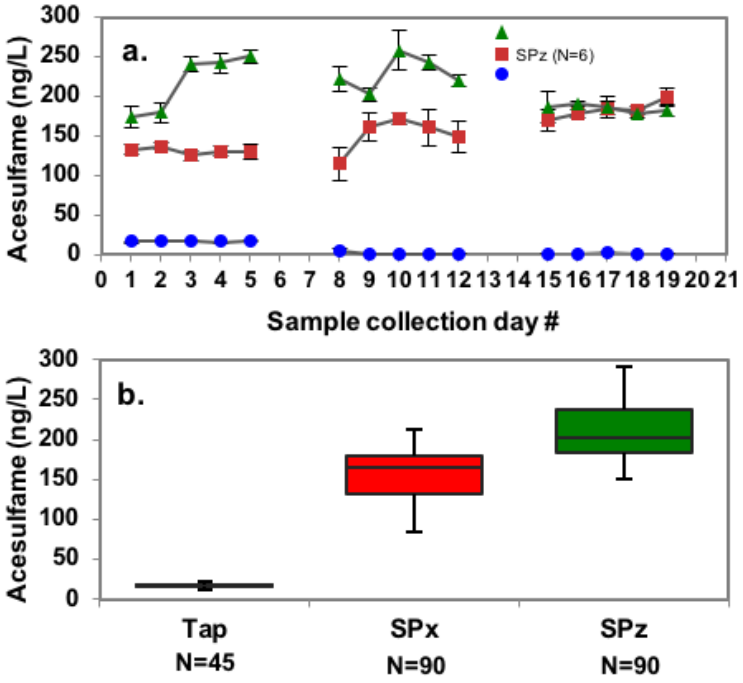

Figure 3.8 (**a**) Average ACE concentration detected in SPx, SPz (n=6), and tap water (n=3) samples per day over three weeks. (**b**) Box and whisker diagram for average ACE concentration detected in SPx, SPz (n=90), and tap water (n=45) over three weeks

Calculation 3.1 Total ACE present

The following calculation estimates the total mass of ACE in SPx. The values used were the known pool volume (V1) and average concentration of ACE determined over all 15 days of collection (C1).

V1 = Swimming Pool Volume = 110 000 US Gal ≅ 420 000 L

C1 = Average ACE in pool = 156 ng/L

$$156\ \frac{ng}{L} \times \frac{1\ \mu g}{1000\ ng} \times \frac{1\ mg}{1000\ \mu g} \times \frac{1\ g}{1000\ mg} = 1.56E^{-7}\frac{g}{L}\ of\ ACE$$

$$1.56E^{-7}\frac{g}{L} \times 420\ 000\ L = 0.0655\ g \cong \mathbf{65\ mg\ ACE}$$

To estimate the total urinary input in SPx and SPz, I determined the average ACE concentration in a pooled Canadian human urine sample (n=20) to be 2360 ng/mL with 4% RSD (**Figure 3.9**). Although this is less than the mean concentration of ACE in Chinese human urine samples (n=54), reported as 4070 ng/mL,[33] both values have the same order of magnitude. Using approximate pool volumes and the ACE concentration determined in Canadian urine samples, I estimated the total urinary input in SPx and SPz to further illustrate the feasibility of ACE as an indicator (see **Calculation 3.2** and **Table 3.5**). I calculated the volume of urine to be approximately 30 and 75 L in SPx and SPz, respectively.

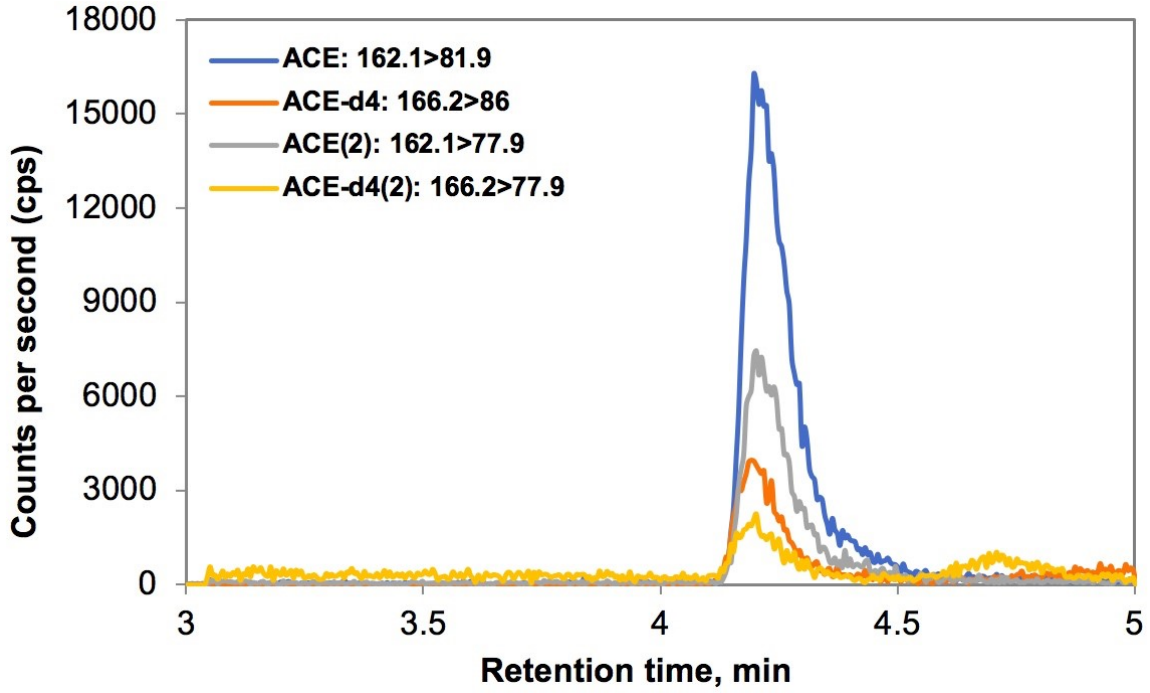

Figure 3.9 A sample chromatogram showing ACE and ACE-d4 internal standard transitions detected in a pooled human urine (n=20) sample at 100000 times dilution

Calculation 3.2 Total volume of urine

The following calculation estimates the volume of urine in SPx. The values used were the known pool volume (V1) and average concentration of ACE determined on the final day of collection (C1), along with the determined average concentration of ACE in Canadian urine (C2, **Figure 3.9**).

V1 = Swimming Pool Volume = 110 000 US Gal ≅ 420 000 L

C1 = Average ACE in pool = 156 ng/L

C2 = Average ACE in Adult Urine = 2 360 ng/mL ≅ 2 360 000 ng/L

$$C1V1 = C2V2$$

$$V2 = \frac{C1V1}{C2}$$

$$V2 = \frac{156\,\frac{ng}{L} \times 420\,000\,L}{2\,360\,000\,\frac{ng}{L}}$$

$$\boldsymbol{V2 \cong 30\,L\ \text{Urine}}$$

Note: **Calculations 3.1** and **3.2** were repeated for SPz, which had twice the total volume of SPx (220 000 US Gal) and an average ACE concentration of 210 ng/L over the collection period (**Table 3.5**).

Table 3.5 Average concentration of ACE in SPx and SPz over three weeks of sample collection as well as the corresponding estimated total mass of ACE and total urine content

Pool	ACE Average (ng/L, N=90)	ACE Standard Deviation (ng/L, N=90)	ACE % Relative Standard Deviation	ACE Estimated Total Mass (mg)	Estimated Total Urine (L)
SPx	156	28	18	65	28
SPz	210	32	15	176	75

3.3.4 HPLC-MS/MS Method Development and Optimization

The successful and rapid determination of ACE in more than 350 samples is due to the new method I developed. This method eliminates the need for preconcentration, and provides sensitive and rapid analysis for a wide range of concentrations of ACE in swimming pool, hot tub, and tap water samples. The method can achieve an instrument limit of detection (LOD) of 0.5 ng/L and method LOD of 2.2 ng/L. Compared to previous methods requiring manual injection of 500-µL or SPE,[37,38] the new method, with a 100-µL autosampler injection volume enabling high throughput analysis, makes the analysis of many samples feasible.

3.4 Conclusion

This is the first reported occurrence study of ACE in swimming pools and hot tubs. The high concentration of ACE with 100% occurrence in pools and hot tubs demonstrates human impact on recreational water quality. The association of occupational asthma in swimmers with volatile N-DBPs (e.g., trichloramine) highlights the need to control the water quality of swimming pools. Several studies have reported that increased DOC in swimming pools results in enhancement of DBP formation. To reduce exposure to N-DBPs and their negative health impacts in swimming pools, I should monitor and control water quality. Public education on personal hygiene in the pools is important as demonstrated in the C&EN cover story.[1]

3.5 Media Platform and Public Education

3.5.1 Media Impact

The results from **Chapter 3** were published online as "Sweetened Swimming Pools and Hot Tubs" [44] in the Journal *Environmental Science & Technology Letters (ES&TLett)* on March 1st, 2017. Within one day, the research was covered by more than 100 news stories of multiple media and languages.[45] The coverage ranged from scientific press, such as Science Daily,[46] to popular press, such as The Guardian,[47] BBC News,[48] CBC News,[49] and CBS News.[50] Since December 26, 2019, Altmetric (a website that ranks publications by collecting metrics for attention) has documented over 250 news stories from 211 news outlets around

the world. [45] Currently, the publication ranks at #1055 out of the 14,220,799 research outputs tracked by Altmetric, **Figure 3.10**.

Many news stories of the popular press have such headlines as “how much pee is in our swimming pools?” or “Yes, there is a lot of pee in that public pool”, emphasizing 75 liters of urine in a swimming pool smaller than one-third of an Olympic-size pool. “Buckets of urine in swimming pools” presented “gross” and “scary” images in the minds of some readers/viewers, which could discourage people from swimming. This was not the purpose of the study. An appropriate public education and public health message should not dwell on how much urine is in the pools, but rather how to promote swimming hygiene and encourage people to enjoy swimming for its health benefits.

When interviewed by the media, both Dr. Xing-Fang Li and I repeatedly emphasized that the benefits of swimming far outweigh the risk of urine in the pools.[51-53] The importance of public awareness and education to stop peeing in the pools was stressed.[53] The new research findings on the evidence of urine in swimming pools should be used to promote good swimmer hygiene practice.

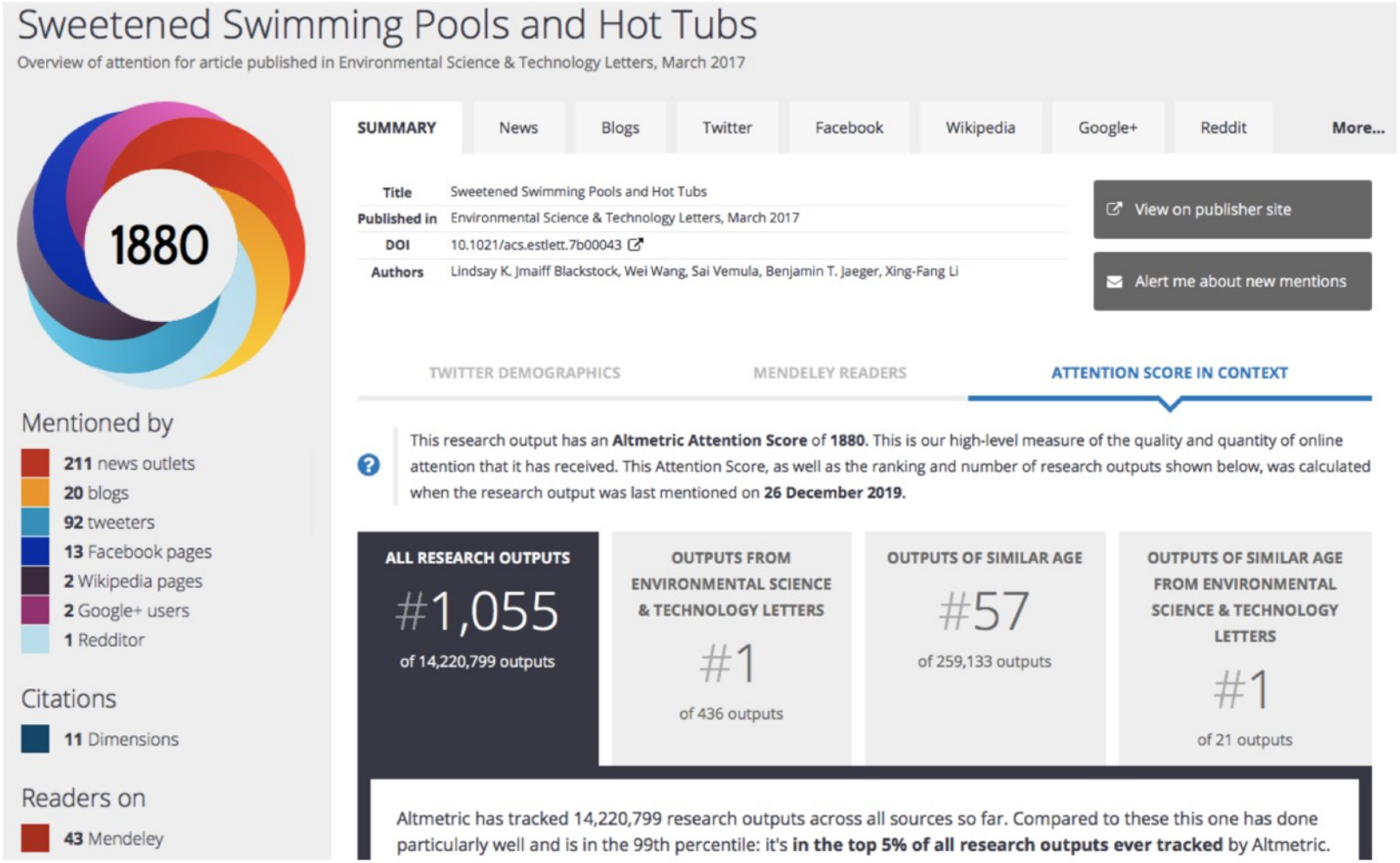

Figure 3.10 Summary of Altmetric data[45] collection for scoring the online attention for the research article, "Sweetened Swimming Pools in Hot Tubs"[44]

3.5.2 Public Education Perspective

Following the widespread social impact of the publication, "Sweetened Swimming Pools and Research", I collaborated with Mark Rober, a science education content creator on YouTube for the video "How to measure HOW MUCH PEE IS IN YOUR POOL" to properly explain the research and correct the negative narrative propagated to the public by the media at large. To date, the video has amassed over 20.7 million views and garnered over 25000 comments of discussion.[54]

Several key messages were emphasized in the video. ACE was measured in the pools, not urine. Swimming and other water-related activities are excellent ways to engage in physical activity to achieve wellness benefits and lead a healthy life.[55, 56] Disinfection is an essential strategy to maintain safe water and prevent the transmission of waterborne illness. The U.S. Center for Disease Control and Prevention (CDC) encourages swimmers to practice good hygiene. "Keep the pee, poop, sweat, blood and dirt out of the water." "Shower before you get in the water. Rinsing off in the shower for just 1 minute removes most of the dirt or anything else on your body".[55] Consistent with these recommendations, studies on personal care products in swimming pool water have shown that chemicals in sunscreen and lotions can be precursors to the formation of toxic DBPs.[43] An important measure to minimize the formation of DBPs is to control and reduce DOC.[57] Thus, showering to remove excess debris before going into the pools and keeping body fluids out of the pools it is critical to minimize the formation of DBPs.

3.6 References

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Chapter 4

Chloraminated Water Sweetened with Aspartame Increases Disinfection Byproduct: Dichlorobenzoquinone [§]

"Success is not final, failure is not fatal: It is the courage to continue that counts."
– Winston Churchill (1874-1965)

4.1 Introduction

Total daily intake of water by American adults is approximately 3.2 L, with roughly half that amount as prepared beverages (1536 mL).[1] Sugar sweetened beverages are the largest source of beverage calories.[2] Thus, for the management of caloric intake and blood sugar levels, sugar is often replaced with artificial sweeteners.[3,4] Aspartame is an intense artificial sweetener, approximately 200 times sweeter than sucrose,[5] and is found in a variety of products. This includes general tabletop sweeteners (Nutrasweet®, SugarTwin®, Equal®), processed foodstuffs such as dry bases for certain foods, and carbonated beverages and their syrup bases.[6,7] With its popular use as a beverage sweetener, aspartame is often added to drinks prepared with disinfected drinking water.

Drinking water disinfection is essential to prevent the transmission of waterborne pathogens.[8-12] To prevent microbial contamination within a distribution system, residual disinfectants must be maintained.[8-12] However, residual chlorine or chloramine can react with organics in food and beverages,[13-16] resulting in formation of disinfection byproducts (DBPs). DBPs have long been a human health concern in treated drinking water due to the potential epidemiologically observed association between increased bladder cancer risk and long-term chlorinated water consumption.[17] Because regulated DBPs do not have sufficient potency to account for this observed risk, research is shifting to identify toxicologically relevant unknown and unregulated DBPs.[18,19] Recent studies identified emerging iodo-DBPs in simulated tap water containing iodized salt after boiling[20] and demonstrated the formation of

[§] The results in **Chapter 4** are unpublished. The instrumental analysis described in **Section 4.2.3.3** was completed by the University of Alberta Department of Renewable Resources Natural Resources Analytical Laboratory

selected DBPs in a variety of brewed tea and coffee.[21] To date, the formation of DBPs in beverages from the use of artificial sweeteners prepared from authentic tap water has not been investigated.

Halobenzoquinones (HBQs) are a class of unregulated DBPs detected frequently in drinking water[22,23] and have up to 1000× higher cytotoxicity than regulated DBPs.[24] The most commonly detected HBQ in tap water is 2,6-dichloro-1,4-benzoquinone (DCBQ). Several studies have identified DCBQ precursors under chlorination and/or chloramination conditions including phenol,[22,26] alkyl and carboxyl para-substituted phenolic compounds, and para-substituted aromatic amines.[27] Natural organic matter (NOM) mixtures of higher aromatic character have also been correlated with increased DCBQ formation after chlorination.[28] A recent study demonstrated phenolic compounds present in tea leaves as HBQ precursors and antioxidents.[29] Because aspartame is a dipeptide consisting of phenylalanine and aspartic acid, the presence of its aromatic component (i.e., phenylalanine) makes it a plausible HBQ precursor.

The predominant use of aspartame in the US is sweetening low calorie drinks.[30] However, it remains unknown whether aspartame may react with residual chloramine in tap water to form HBQs during beverage preparation. In this study, aspartame and its aromatic component phenylalanine were investigated as DCBQ precursors, **Figure 4.1** under controlled conditions mimicking chloraminated tap water. Next, DCBQ concentrations were examined in one cup of authentic chloraminated tap water with and without the addition of one Equal packet. Finally, the impact of aspartame on DCBQ stability in Optima water was evaluated.[23] This study highlights the potential indirect impact of artificial sweetener use on DBP exposure, adding a new perspective when weighing the known benefits of reduced blood sugar and weight management against potential adverse effects surrounding long-term consumption of artificial sweeteners.

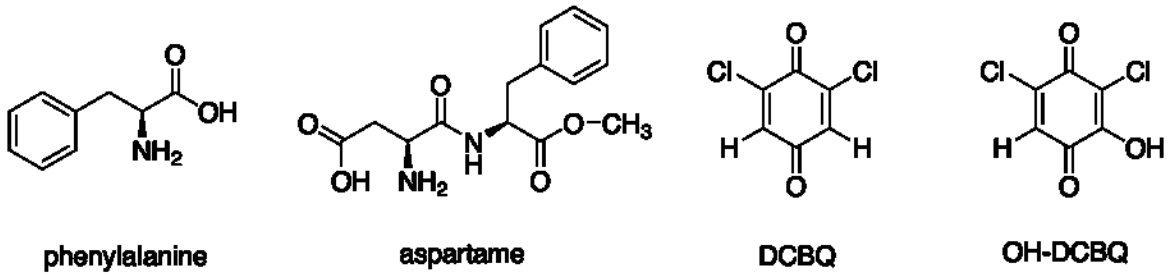


Figure 4.1 Chemical structures of experimentally relevant precursors (phenylalanine and aspartame) and products (DCBQ and OH-DCBQ)

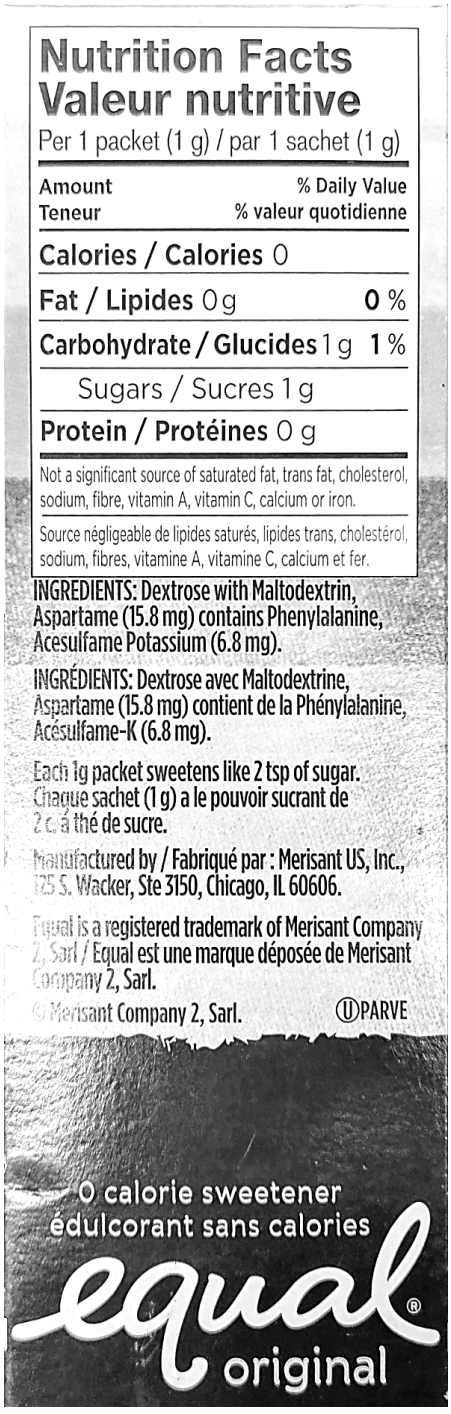
4.2 Materials and Methods

4.2.1 Chemicals, Materials and Instrument Details

High performance liquid chromatography-tandem mass spectrometry (HPLC-MS) grade formic acid (FA, 98%), sodium hypochlorite solution, ascorbic acid (AA), phenylalanine, and 2,6-dichloro-1,4-benzoquinone (DCBQ) were obtained from Sigma-Aldrich (St. Louis, MO). Optima LC-MS grade methanol, water, and hydrochloric acid, as well as ammonium chloride, aspartame, anhydrous dibasic potassium phosphate, and sodium bicarbonate were obtained from Thermo Fisher Scientific (Fair Lawn, NJ). Equal Original Sweetener was purchased from a local grocery store, **Figure 4.2**. The concentration of free chlorine in the sodium hypochlorite solution was determined to be 120 mg/mL (as Cl_2) with a chlorine amperometric titrator (Autocat 9000, HACH, Ontario, Canada). A pH meter (Model 15, Accumet, Fisher Scientific, Ontario, Canada) was used to monitor pH. A nitrogen evaporator (TurboVap LV Concentration Workstation, Caliper Life Sciences, Massachusetts, United States) was used to preconcentrate SPE eluate. A HPLC system (Agilent 1290 HPLC, Waldbronn, Germany) was coupled with a tandem mass spectrometer (MS/MS) (5500 QTRAP System; Sciex, Concord, Ontario, Canada) for sample analysis.

The investigated authentic chloraminated tap water (pH 7.7; residual chloramine 1.8 mg/L = 34.8 μM) was surface water sequentially treated by a DWTP through coagulation, flocculation, filtration, UV disinfection, and chlorination, followed by the addition of ammonium to form chloramine before leaving the plant. Therefore, the residual chlorine in the distribution system is primarily chloramine.

Figure 4.2 Equal® Original Sweetener blend package nutritional information and ingredient list.



Calculation 4.1 Molar concentration of aspartame in one package of Equal® Original Sweetener blend

aspartame molar mass = 294.3 g/mol

aspartame in 1g packet of Equal® Original = 15.8 mg

15.8 mg dissolved in 1 cup (i.e., 250 mL) of water:

$$\frac{15.8 \text{ mg aspartame}}{0.250 \text{ L}} = 63.2 \frac{\text{mg}}{\text{L}} \text{ aspartame}$$

Molarity of aspartame:

$$63.2 \frac{\text{mg}}{\text{L}} \text{ aspartame} = 0.0632 \frac{\text{g}}{\text{L}} \times \frac{1 \text{ mol}}{294.3 \text{ g}}$$

$$= 0.0002147 \frac{\text{mol}}{\text{L}} \text{aspartame}$$

$$= 214.7 \frac{\mu\text{mol}}{\text{L}} \text{ aspartame}$$

4.2.2 Chloramination of Phenylalanine and Aspartame

Individual reaction solutions (n=3) containing 10 µM of aspartame or phenylalanine were prepared in 250 mL of 10 mM phosphate buffer (prepared in Optima water). The pH was adjusted to 6.5, 7.5, or 8.5. Next, freshly prepared monochloramine[32] was added into reaction solutions for a final concentration of 80 µM (4.1 mg/L). The molar ratio of monochloramine to aspartame or phenylalanine was 8:1, near the previously reported optimum DCBQ formation conditions between monochloramine and phenol (i.e., 10:1).[23] Caution is necessary because preparation of monochloramine at high concentrations can be very reactive. Use adequate personal protective equipment and safety measures within a fume hood.

After 24 hours in the dark at 24 ºC, samples were quenched with excess ascorbic acid (AA, 100 µM) to ensure a complete quench of free chlorine the reaction solution (µM AA = 1.3 x µM monochloramine). After 10 minutes, a 625-µL aliquot of formic acid (FA; 0.25% v/v, final) was added to stabilize DCBQ. Sample preparation, analysis, and quantification procedures were adapted from previously described methods,[22,23] with specific high-performance liquid chromatography tandem mass spectrometry with multiple reaction monitoring HPLC-MS/MS (MRM) method parameters detailed in **Section 4.2.2.1**, and **Table 4.1**. Before HPLC-MS/MS (MRM) analysis, the samples underwent solid-phase extraction (SPE), nitrogen evaporation, and reconstitution (**Section 4.2.2.2**). The DCBQ concentration (mean ± SD) was quantified using standard addition (**Section 4.2.2.3**). Controlled experiments (**Section 4.2.2.4**) confirmed addition of AA or AA/FA did not affect DCBQ signal, **Figure 4.3**.

Using the same procedures, DCBQ (mean ± SD) was determined after addition of 10 µM aspartame in chloraminated tap water (pH 7.7, residual chloramine 1.8 mg/L) at 24 ºC after 24 hours.

4.2.2.1 HPLC-MS/MS (MRM) Method Details

The HPLC-MS/MS (MRM) analysis was adapted from previously described methods[23,33] An Agilent 1290 series LC system consisting of an autosampler with temperature control and a binary pump (Agilent, Waldbronn, Germany) was used with a Luna C18(2) column (100 × 2.0 mm i.d., 3 µm; Phenomenex, Torrance, CA). The mobile phase

was comprised of solvent (A), water containing 0.1% FA; and solvent (B), methanol containing 0.1% FA. The flow rate of the mobile phase was 300 μL/min, and the injection volume was 20 μL. A gradient program was performed as follows: linearly increased B from 20% to 90% in 15 min; kept B at 90% for 1.5 min; changed B to 20% for column equilibration at 16.51 until 20.00 min. The column oven was set at 40 ºC, and the autosampler was kept at 4 ºC.

HPLC-MS/MS with MRM mode was performed using a triple quadrupole ion-trap tandem mass spectrometer (Sciex QTRAP 5500) to confirm the identity of DCBQ and HO-DCBQ in all samples. The optimized MS instrumental parameters were as follows: ion-spray voltage, -4500 V; source temperature, 450 °C; gas 1, 50 arbitrary units; gas 2, 60 arbitrary units; curtain gas, 30 arbitrary units; entrance potential (EP), -10 V; accumulation time for each ion pair, 250 ms. The MRM ion pairs and the optimized values of declusturization potential (DP), collision energy (CE), and cell exit potential (CXP) are listed in **Table 4.1**. Analyst software version 1.5.2 for Sciex QTRAP 5500 was used for data analysis.

Table 4.1 MRM parameters for HPLC-MS/MS analysis (positive ESI mode) of DCBQ and OH-DCBQ

Compounds	Protonated molecule $(M+2H-H)^+$	Product ion	DP (V)	EP (V)	CE (V)	CXP (V)
DCBQ	177.0	113.0	-100	-10.0	-24	-13.0
		141.0	-100	-10.0	-20	-13.0
HO-DCBQ	191.0	83.0	-50	-10.0	-33	-10.0
		163.0	-90	-10.0	-26	-6.0

4.2.2.2 Solid Phase Extraction and Nitrogen Evaporation

A Waters Oasis HLB cartridge (6 mL, 200 mg) mounted in a VISIPREP SPE manifold (Supelco, Bellefonte, PA) was used to desalt and concentrate the quenched reaction solutions. The HLB cartridges were first activated with two 6 mL washes of methanol (0.25% FA, v/v), then rinsed twice with 6 mL of Optima water (0.25% FA, v/v). The quenched reaction solution was drawn through the cartridge under vacuum at a flow rate of 8 mL/min. Next, the cartridge was washed with two 6 mL portions of Optima water (0.25% FA, v/v),

and the analytes were finally eluted with 10 mL of methanol (0.25% FA, v/v). The eluate was evaporated down to 100 μL under a gentle (< 5 psi) nitrogen stream, reconstituted with Optima water (0.25% FA, v/v) to a final volume of 500 μL water/methanol (v/v, 4/1).

4.2.2.3 Standard Addition Calibration and Quantification Details

For standard addition, four 80 μL aliquots were removed from the 500 μL reconstituted sample. Each aliquot was spiked with 20 μL of DCBQ methanol solution yielding 2.5, 5, 10 or 25 μg/L additional DCBQ (100 μL total). The peak areas of the spiked samples, determined with HPLC-MS/MS (MRM), were used to calibrate against the spiked concentrations. The resulting standard addition calibration curves, with linear coefficients ranging between 0.989−0.999, were used to determine the concentrations of DCBQ in each sample by solving for the absolute value of the X-intercept.

4.2.2.4 Controlled Ascorbic Acid Experiments

To evaluate ascorbic acid (AA) and/or formic acid (FA) incubation time on the stability of DCBQ, I prepared identical DCBQ solutions by spiking 100 μL of DCBQ standard (500 ppb in methanol) into 1.9 mL of Optima water (final, 25 μg/L). Next, AA (32.5 μL of 2 mg/L standard) was added (final, 32.5 μg/L) and the triplicate solutions were kept in the dark for 0, 10, or 20 min. Finally, at the end of each time interval, 5 μL of FA was added (0.25% FA, final) to stabilize DCBQ. The final solutions were analyzed by HPLC-MS/MS. The variation of DCBQ with different AA incubation times was calculated based on the DCBQ peak area ratio of each incubation time to that of time zero. In **Figure 4.3a**, DCBQ was still detected (97% of time zero signal) after 20 min incubation with AA, demonstrating that DCBQ is stable in the presence of ascorbic acid for 20 minutes.

Identical DCBQ solutions were prepared with AA exactly as described above and were kept in the dark for 10 minutes. Next, 5 μL of FA was added to the DCBQ and AA solutions (0.25% FA, final) and the triplicate samples were kept in the dark for 0, 2, 4, or 24 h, and analyzed by HPLC-MS/MS. Similarly, the response of DCBQ to different AA plus FA incubation times can be calculated from DCBQ peak area ratio of a particular time to that of time zero. As seen in **Figure 4.3b,** 92% DCBQ remained in the solution after 24 hours incubation with AA and FA, which demonstrated that DCBQ can be stable once in the presence of AA and FA, for 24 hours before analysis. Since all our sample pre-treatment

(SPE and nitrogen evaporation) was completed in less than 24 hours, no loss of DCBQ was expected after quenching.

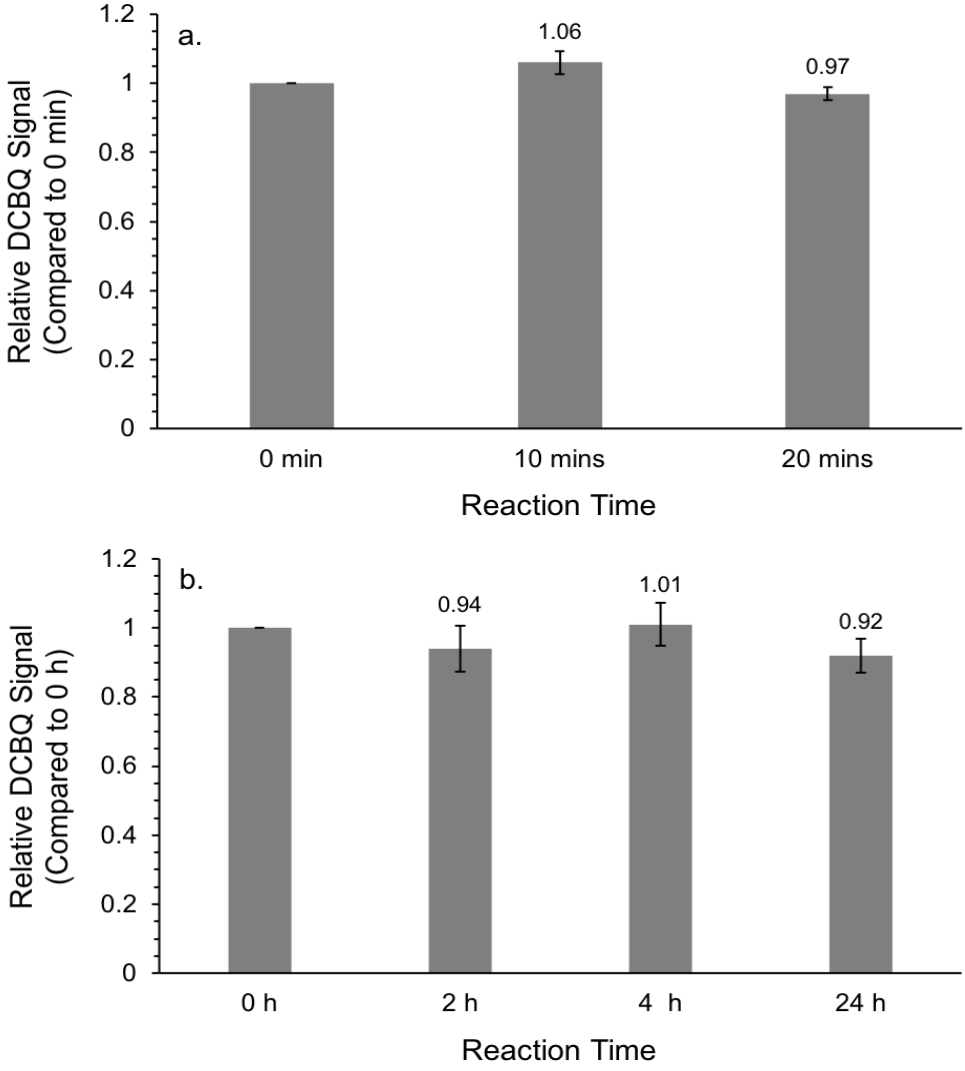


Figure 4.3 Relative DCBQ signal detected after different incubation times with AA (**a**), or AA and FA (**b**). Data represents the average and standard deviation of triplicate experiments (n=3).

4.2.3 Determination of DCBQ in an Authentic Tap Water with and without Equal

A 1-g packet of Equal Original contained aspartame (15.8 mg) (**Figure 4.2)**. To mimic realistic household beverage preparation, one packet of Equal was dissolved into 250-mL of authentic tap water at 24 ºC. Authentic tap water samples with and without the addition of Equal were prepared in triplicate. Samples were quenched with excess AA (45 µM) after 15 minutes reaction time in the dark at 24 ºC. Finally, FA was added (0.25% FA, final) to stabilize DCBQ before sample preparation and analysis.[22,23] Quenched reaction solutions underwent extraction, detection, and quantification identical to controlled formation solutions (**Sections 4.2.2.1 to 4.2.2.3**). DCBQ recovery with and without the presence of Equal using the SPE method was 89% and 73%, respectively (**Section 4.2.3.1**). Experiments were repeated with the use of boiled authentic tap water (**Section 4.2.3.2**).

4.2.3.1 Recovery of DCBQ in the Presence of Equal using an HLB Cartridge

Samples were prepared in duplicate (n=2). First, 250 mL solutions of DCBQ (40 ng/L, final) were prepared in Optima water with or without one Equal packet (63.2 mg/L aspartame, final, **Figure 4.2**). Then, AA (50 ng/L) was spiked into the solution to mimic the NH_2Cl quenching step for authentic tap water sample pre-treatment. After 15 minutes in the dark, 625 µL FA (0.25% FA, final) was added to quench and stabilize the DCBQ present in solution. The recovery solutions underwent the same SPE, nitrogen evaporation, and reconstitution protocol described in Materials and Methods. Similarly, the final extracts were analyzed by HPLC-MS/MS in MRM mode and DCBQ was determined using an external calibration curve of 1, 5, 10, 25, and 50 µg/L). The recovery of DCBQ in Optima water with and without the Equal Original package was 89% and 73%, respectively. After obtaining the data for the standard curve, the concentration of DCBQ in the recovery solution was compared to the known initial concentration (i.e., 100% recovery = 40 ng/L initial DCBQ x 500 concentration factor = 20 µg/L). The recovery was determined as the determined DCBQ concentration after sample preparation and analysis, divided by the initial DCBQ concentration, multiplied by 100%.

4.2.3.2 Boiling Pre-treatment of Authentic Tap Water Before Addition of Equal

Each experimental condition was examined in triplicate (n=3). Freshly collected authentic chloraminated tap water was brought to a boil (90 ºC) using an electric kettle. The concentration of DCBQ was determined in the boiled tap water under three different conditions: 1, boiled tap water without Equal; 2, addition of one Equal packet immediately after boiling; and 3, addition of one Equal packet 1 hour after boiling (34 ºC). All samples were quenched after 15 minutes reaction time. The quenching, sample preparation and analysis details are the same as described for the experiment described in **Section 4.2.1**. The pH and monochloramine concentration were determined in the authentic tap water before and after boiling. To maintain instrument integrity, measurements were only taken on the pre-boiled samples after cooling for 1 hour.

4.2.4 Stability of DCBQ in Optima Water Containing Aspartame

DCBQ solutions (100 μg/L = 0.56 μM) were prepared by spiking a 1-mL aliquot of 2.0 mg/L DCBQ methanol standard into 19 mL of Optima water containing increasing concentrations of aspartame: 0, 0.57, 5.70, 28.50, and 209.8 μM (n=3). The molar ratio of aspartame to DCBQ in each solution was 0:1, 1:1, 10:1, 50:1, and 368:1. The highest concentration condition is comparable with the approximate aspartame concentration in one serving size of Equal (i.e., 215 μM, **Figure 4.2**). A 2-mL aliquot was removed at 0, 1, 2, 8, 24 and 53 hours and FA was added immediately (0.25% FA, final) to stabilize DCBQ. The high initial DCBQ concentration in the prepared solutions allowed for direct analysis with HPLC-MS/MS (MRM) without SPE and nitrogen evaporation. Control experiments detailed in **Section 4.2.4.1** confirmed that aspartame does not interfere with DCBQ detection in a pure solution, **Table 4.2.** All Optima water solutions had pH 5.0-5.4 (**Table 4.3**). DCBQ concentration was determined using freshly prepared external calibration standards, analyzed immediately before the experimental sample.

Table 4.2 Peak area ratio of DCBQ in Optima water (0.25% FA, v/v) in the presence of increasing aspartame concentrations to DCBQ only in Optima water (0.25% FA, v/v). The molar ratio of aspartame to DCBQ in each solution was 0:1, 1:1, 10:1, 50:1, and 368:1

DCBQ concentration in Optima water samples (μg/L)	Peak area ratio of DCBQ + Optima water to:			
	DCBQ + Optima water + 0.6 μmol/L aspartame	DCBQ + Optima water + 5.7 μmol/L aspartame	DCBQ + Optima water + 28.5 μmol/L aspartame	DCBQ + Optima water + 209.8 μmol/L aspartame
2	1.06	0.96	0.97	0.89
10	0.97	0.95	0.94	0.90
50	0.91	0.92	0.95	1.01
100	0.98	0.91	0.98	0.93
160	0.89	0.92	0.89	0.97

Table 4.3 pH of Optima water solutions with increasing aspartame concentrations

Aspartame in Optima Water (μg/L)	pH
0	5.0 ± 0.1
0.6	5.3 ± 0.1
5.7	5.4 ± 0.1
28.5	5.4 ± 0.1
209.8	5.4 ± 0.1

4.2.4.1 Investigating Interference of Aspartame on DCBQ Detection

To evaluate the matrix effect of aspartame on DCBQ detection, a set of solutions containing 40, 200, 1000, 2000 and 3200 ng DCBQ in 20 mL Optima water containing aspartame of 0, 0.6, 5.7, 28.5, and 209.8 μmol/L were prepared. The final concentration of DCBQ in the Optima water solutions were 2, 10, 50, 100, and 160 μg/L, respectively. Immediately after vortexing, a 2 mL aliquot was removed from the 20 mL solution and quenched with 5 μL FA (0.25% FA, final). This solution was analyzed by HPLC-MS/MS (MRM). The DCBQ signal of Optima water sample containing DCBQ and increasing concentrations of aspartame was compared to the Optima water sample containing DCBQ only, **Table 4.2**. All conditions had ratios between 0.89 and 1.1. If the ratio of [DCBQ with aspartame]/[DCBQ only] is close to 1, it indicates negligible matrix effects.[34] It was

determined that aspartame had negligible interference on DCBQ detection after comparing the peak areas of a range of DCBQ concentrations with and without increasing aspartame in Optima water.

4.2.5 DOC in Tap Water with and without Aspartame or Equal

The concentration of dissolved organic carbon (DOC) was determined for each of experimental and control conditions including the authentic tap water and Optima water, both with and without 10 μM aspartame or one package of Equal.

4.2.5.1 DOC Analysis Method

An OI Analytical Aurora 1030W TOC Analyzer (OI Analytical, Xylem Inc, College Station TX, U.S.A.) was used to determine the non-purgeable organic carbon (NPOC) in each sample. The dissolved organic content was determined using the persulfate oxidation method.[35] Briefly, the instrument mixes phosphoric acid (5% w/v) and sodium persulfate (10% w/v) with the aqueous sample in a heated glass reaction vessel to chemically oxidize carbon into CO_2. The evolved CO_2 gas is then passed through a non-dispersive infrared (NDIR) detector to determine NPOC (carrier gas: helium, purge gas: nitrogen). Total organic carbon calibration and quality control check certified reference standards were purchased from SCP Scientific.

4.3 Results and Discussion

4.3.1 Formation of DCBQ from Chloramination of Aspartame or Phenylalanine

Tap water pH ranges between 6.5-8.5 in the US[31] (7.0-10.5 in Canada)[36] and the EPA maximum residual chlorine level is ≤ 4.0 mg/L (≤ 2.0 mg/L by Health Canada). Therefore, DCBQ formation was evaluated at 24 °C under the conditions of 4.1 mg/L (80 μM) monochloramine and 10 μM aspartame or phenylalanine in phosphate buffered Optima water at pH 6.5, 7.5, and 8.5. **Table 4.4a** presents the concentration of DCBQ detected after 24 hours reaction time under each testing condition.

Chloramination of aspartame at pH 6.5 and 7.5 formed 0.6 ± 0.04 and 2.0 ± 0.4 ng/L DCBQ, respectively. Chloramination of phenylalanine yielded DCBQ only at pH 6.5 (9.8 ± 1.0 ng/L). Our results are consistent with previous reports that found DCBQ was not stable at pH 8.5.[19] The molar yields for DCBQ from aspartame (pH 6.5: 0.00004%, pH 7.5:

0.0001%) and phenylalanine (0.0006%) were low under mimicked tap water conditions, but are comparable with other studies.[27] Chlorination of the aromatic amino acid tyrosine and the dipeptide alanyl-tyrosine formed DCBQ with molar yields ranging from 0.0008-4.9%.[27] DCBQ and other halogenated phenolic and quinone compounds have been identified as intermediates in the formation of halomethanes.[37] The minor could be due to further transformation to other DBPs not included as analytes in this study.

Adding aspartame (10 μM) directly into authentic tap water containing 1.8 mg/L residual monochloramine increased the concentration of DCBQ to 9.0 ± 1.2 ng/L from 8.0 ± 0.3 ng/L (**Table 4.4b**) after 24 hours (24 °C). It is likely due to the low concentrations of the reactants that did not yield statistically different DCBQ. This led to examine formation of DCBQ in a cup of tap water when a common artificial sweetener package containing a large amount of aspartame was added.

Table 4.4 DCBQ detected (**a**) after 24 hours reaction time under controlled conditions between monochloramine and phenylalanine or aspartame at different pH, (**b**) after 15 minutes reaction time after the addition of aspartame to authentic chloraminated tap water. Data represents the average and standard deviation of triplicate samples (n=3)

	pH	Reactants	DCBQ in 250 mL original sample (ng/L)	DCBQ Molar formation yield (%)
(a)	6.5	monochloramine control	N.D.	-
		+ 10 μM phenylalanine	9.8 ± 1.0	0.000 6
		+ 10 μM aspartame	0.6 ± 0.04	0.000 04
	7.5	monochloramine control	N.D.	-
		+ 10 μM phenylalanine	N.D.	-
		+ 10 μM aspartame	2.0 ± 0.4	0.000 1
	8.5	monochloramine control	N.D.	-
		+ 10 μM phenylalanine	N.D.	-
		+ 10 μM aspartame	N.D.	-
(b)	7.7	Authentic chloraminated tap water control	8.0 ± 0.3	N.A.
		Authentic chloraminated tap water + 10 μM aspartame	9.0 ± 1.2	
			Difference ≈ 1.0	0.000 5

Where N.D. stands for not detected; N.A. stands for not applicable
[monochloramine] = 4 mg/L;
authentic tap water [residual chloramine] = 1.8 mg/L

Determination of experimental evidence for the transformation reaction mechanism of phenylalanine or aspartame to yield DCBQ was not an aim of this project. However, this is an important question to investigate as the existing literature focuses on describing the formation of DCBQ from phenolic precursors. In general, phenolic compounds can react with halogens (i.e., chlorine and bromine) through either electrophilic aromatic substitution (e.g., phenol) or by oxidative electron transfer processes (e.g., hydroquinone). Some compounds show both partial oxidation and electrophilic aromatic substitution reaction pathways depending on pH.[38] 2,4,6-Trichlorophenol is rapidly and quantitatively produced from chlorination of phenol.[37] Heasley *et al*. found chloramines (i.e., mixture of NH_2Cl and $NHCl_2$ at pH 6.5) were capable of chlorinating all activated positions (i.e., 2, 4, and 6; *ortho* and *para*) of the phenol ring.[39] They presented an ion radical reaction mechanism for the formation of DCBQ from 2,4,6-trichlorophenol under chloramination conditions, adapted as **Figure 4.4**.

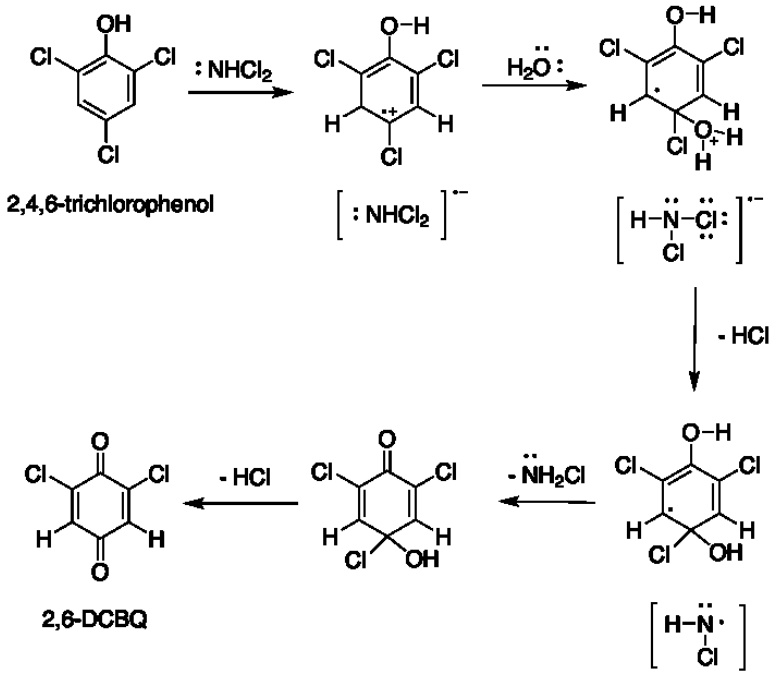


Figure 4.4 Formation of DCBQ from 2,4,6-trichlorophenol via an ion radical pathway reaction with chloramines. Adapted with permission from Heasley V.L., *et al.* Investigations of the reactions of monochloramine and dichloramine with selected phenols: examination of humic acid models and water contaminants. *Environ Sci Technol*. 2004, 38(19), 5022-5029. Copyright (2004) American Chemical Society.

Heasley *et al.* described a strategy to include a radical inhibitor 2,2,6,6-tetramethyl-1-piperdinyloxy to differentiate between radical mechanisms and ion radical mechanisms.[39] Their study ultimately provided evidence suggesting an ion radical mechanism in the formation of DCBQ from 2,4,6-trichlorophenol, **Figure 4.4**, was involved in the pathway to form DCBQ from phenol in the presence of chloramines at pH 6.5. For DCBQ to be formed through reactions with chloramine or free chlorine, halogenation and oxidation of a phenolic precursor is likely required. However, neither aspartame nor phenylalanine contain a phenolic moiety, and the methylated phenyl group may not react directly with chloramines to yield a phenol group. To determine if the formation of DCBQ from aspartame and phenylalanine, here, includes a radical pathway, the same strategy could be employed. The absence of DCBQ formation in the presence of a radical scavenger could provide solid support for a radical-driven pathway to DCBQ.

4.3.2 DCBQ in Equal Sweetened Authentic Chloraminated Tap Water

Equal Original contains aspartame and acesulfame as its primary sweetening components. In tap water at room temperature, acesulfame is highly stable, making it an unlikely DCBQ precursor.[40] Therefore, DCBQ formation will most likely be due to aspartame and its aromatic component, phenylalanine, which are supported by the formation results (**Table 4.4a)**.

Figure 4.5a shows that after the addition of one packet of Equal the concentration of DCBQ was significantly greater ($p < 0.01$) in one cup of authentic tap water containing 1.8 mg/L residual monochloramine. The average concentration of DCBQ detected in the authentic tap water control was 8.0 ng/L, consistent with concentrations previously determined in tap water collected from the same treatment system. [23,33] Just 15 minutes after dissolving one Equal packet in tap water, 11.2 ng/L of DCBQ was detected. No other di-halogenated HBQs, including 2,6-dibromo- or 2,6-diiodo-1,4-benzoquinone (DBBQ or DIBQ), were detected. The concentrations of Br^- and I^- in the tap water were too low (below 0.01 mg/L bromide and 0.25 Bq/L iodine-131, respectively) to yield detectable levels of either DBP, consistent with our previous studies. [23,33]

Figure 4.5b shows the average peak areas of DCBQ and OH-DCBQ in the same samples as **Figure 4.5a**. Because an OH-DCBQ standard is not commercially available, the

exact concentration of OH-DCBQ in each sample was not quantified. However, it is clear in **Figure 4.5b** that the peak areas of OH-DCBQ were substantially higher than those of DCBQ. **Table 4.5** compared the MS-MRM peak area signal for DCBQ and OH-DCBQ in tap water with and without Equal. While the raw signals for each compound individually did not show statistically significant differences, the total signal (i.e., DCBQ+OH-DCBQ) was significantly increased in tap water after the addition of Equal. A previous study has shown that as finished drinking water moves through the distribution system over time, OH-DCBQ concentrations increase as DCBQ concentrations decrease, leading to higher OH-DCBQ concentrations relative to DCBQ in authentic tap water samples.[23] The increased OH-DCBQ concentration with distribution distance was correlated with the transformation of DCBQ to OH-DCBQ in water. Because this transformation (**Figure 4.5b**) may have influenced the detectable concentration of DCBQ in our sweetened cup of tap water, it was important to examine how aspartame may influence the stability of DCBQ in aqueous solution.

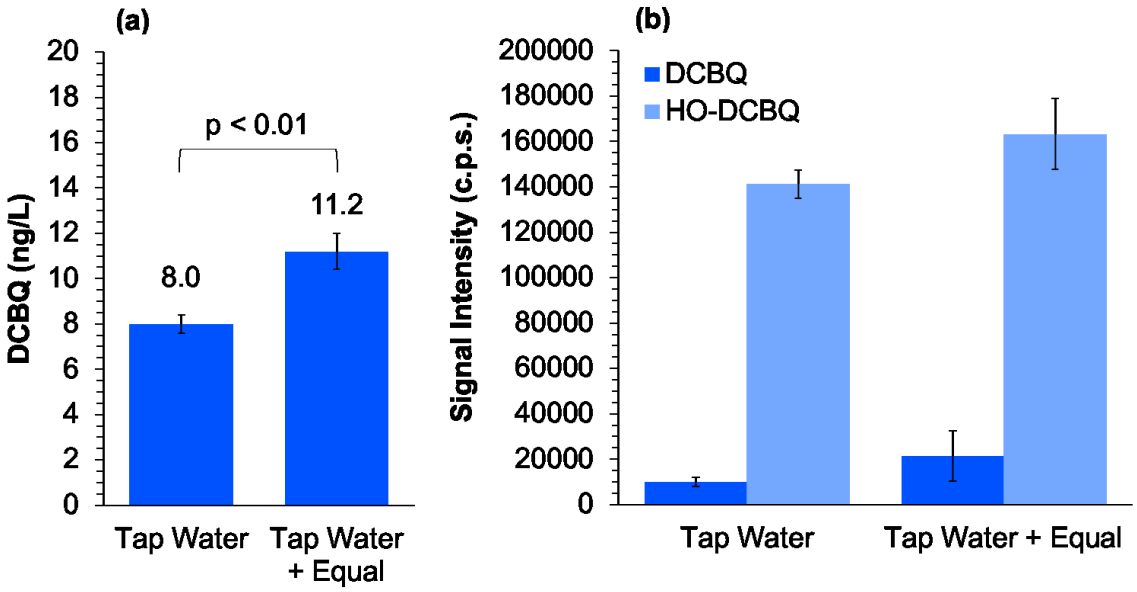


Figure 4.5 (a) DCBQ detected 15 minutes after dissolving one Equal® packet in 250 mL of authentic chloraminated tap water at room temperature (24 ºC); (**b**) Total DCBQ and OH-DCBQ peak area signal detected in the same samples. Data displayed (mean ± SD) is the average of triplicate samples (n=3). Significance evaluated with one-tailed paired Student's T test.

Table 4.5 DCBQ, OH-DCBQ, and total (DCBQ + OH-DCBQ) signal in authentic chloraminated tap water with and without Equal

Compound	**DCBQ**		**OH-DCBQ**		**Total (DCBQ + OH-DCBQ)**	
Sample	TW	TW+Equal	TW	TW+Equal	TW	TW+Equal
MS-MRM peak area (c.p.s.); n=3	12200	12800	110000	148000	122200	160800
	8840	17500	120000	119000	128840	136500
	9010	33800	109000	125000	118010	158800
Student unpaired T-test one-tailed p-value	0.0767		0.0684		0.0129	
Conclusion	p>0.05; Not-Significant		p>0.05; Not-Significant		**p<0.05; Significant**	

While the reaction mechanism describing the transformation of DCBQ to OH-DCBQ under UV conditions has been described in the literature,[41] the hydrolysis of DCBQ to OH-DCBQ in simple aqueous conditions has not. After critical review of previously published literature,[42-44] a potential pathway was proposed, **Figure 4.6**. First, the lone pair of electrons on oxygen (from water), provided by the aqueous solution, will attack DCBQ via Michael addition to intermediate 2 (**Eq. 1**).[42,43] Next, intermediate 2 will undergo keto-enol tautomerization yielding intermediates 3 and 4. Since the reaction solution was kept under darkness, the 2,6-dichloro-3-hydroxy-1,4-semibenzoquinone free radical (intermediate 5) was generated through subsequent oxidation pathways of intermediate 4 by oxygen (O_2) (**Eq. 2**), superoxide ($O_2^{\bullet -}$) (**Eq. 3**), or DCBQ in an excited state (intermediate 6) (**Eq. 4**).[44] HO-DCBQ was formed through oxidation (**Eq. 5** and **6**), and/or comproportionation (**Eq. 7**) of intermediate 5.[44]

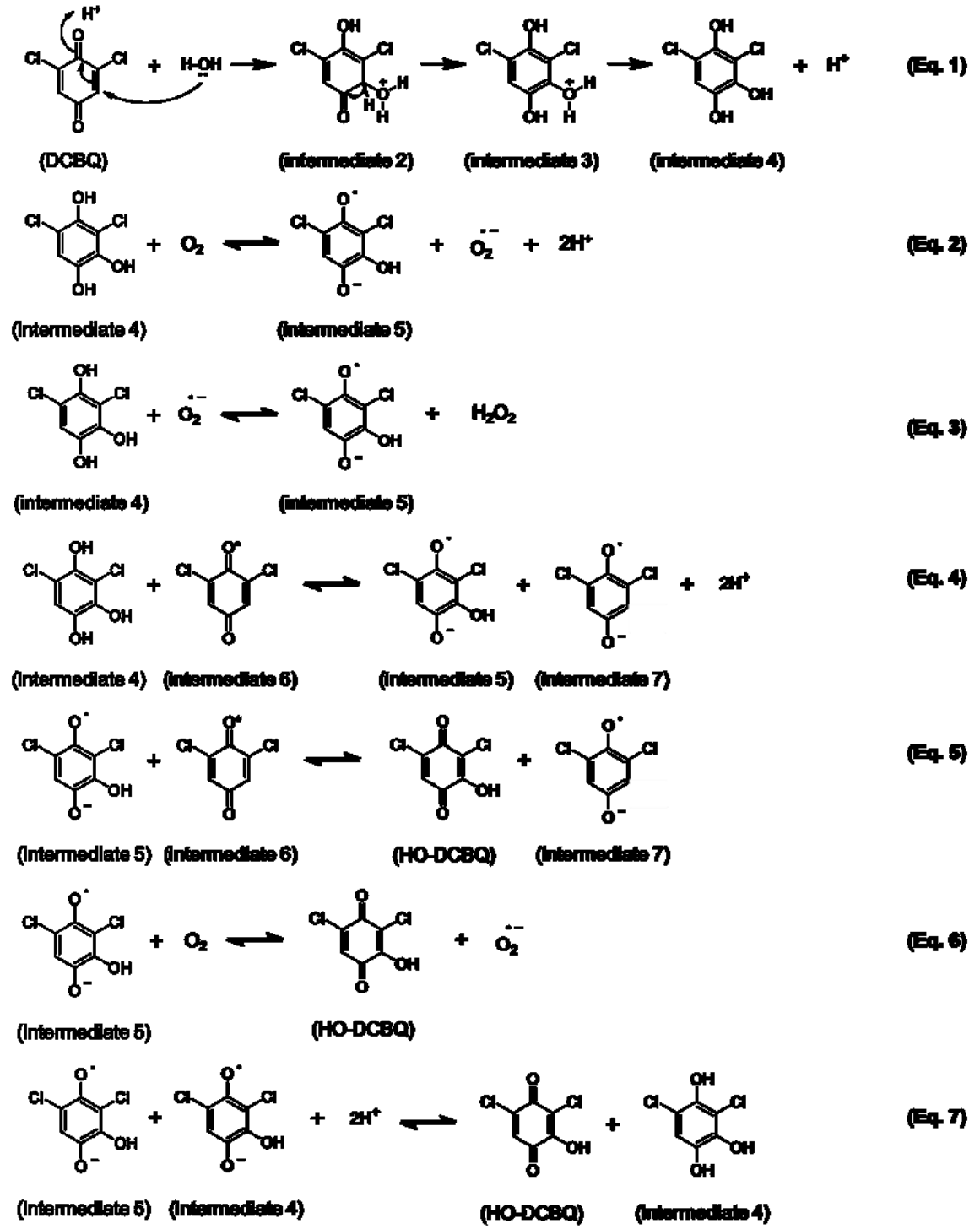


Figure 4.6 The proposed transformation mechanism of DCBQ to HO-DCBQ in aqueous solution

4.3.3 Aspartame Enhanced Stability of DCBQ in Optima Water

Controlled and simplified laboratory experiments were used to eliminate confounding variables. DCBQ at 100 μg/L was spiked into Optima water samples containing increasing aspartame concentrations (0-210 μM). **Figure 4.7** represents the peak areas of DCBQ and OH-DCBQ, detected under each condition. **Figure 4.7** shows that DCBQ stability in aqueous solution increases in the presence of aspartame. By 53 hours, the DCBQ signal completely disappeared in the Optima water control (**4.7a**). Conversely, 26.7 % (**4.7b**), 27.3% (**4.7c**), 54.5% (**4.7d**), and 73.9% (**4.7e**) of the initial DCBQ remained in the solutions with increasing aspartame concentrations. Thus, the increased DCBQ concentration detected in our cup of Equal sweetened tap water may be due to a combination of increased stability and formation. Nevertheless, it is important to note that the use of a single packet of Equal in tap water resulted in an increase in DCBQ concentration, suggesting that artificial sweetener use can result in increased human exposure to DBPs.

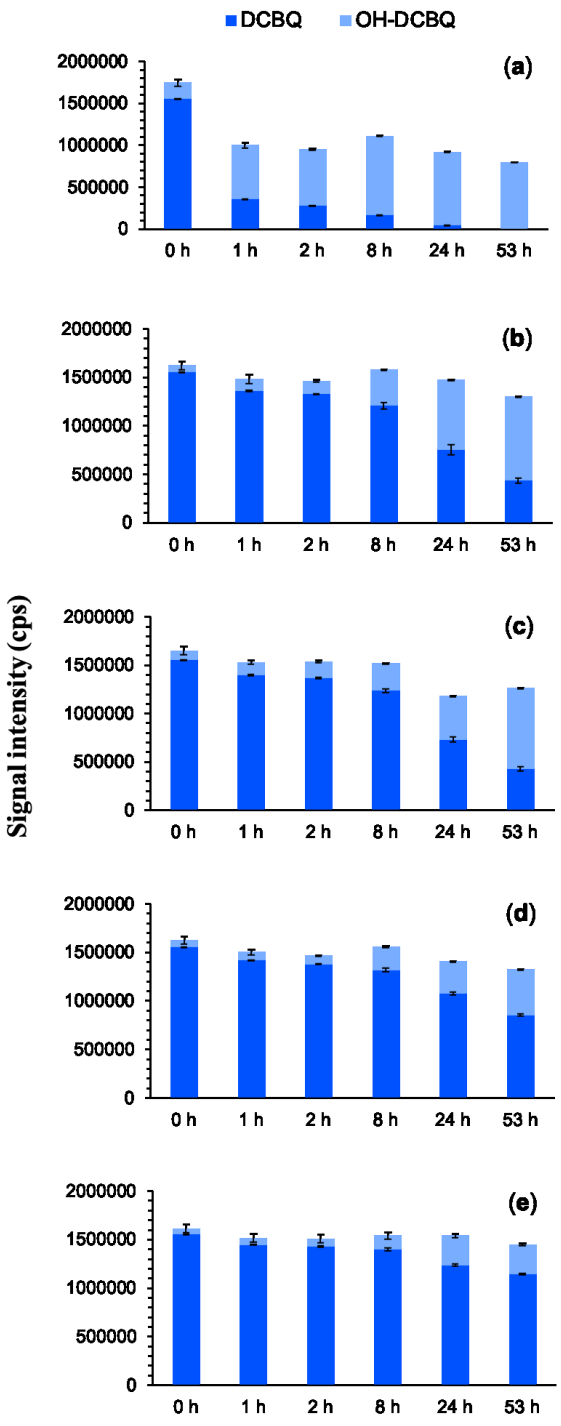


Figure 4.7 Relative proportion of DCBQ and its transformation product OH-DCBQ at different time intervals in Optima water at room temperature (24 ^{o}C) with increasing aspartame concentration (i.e., Optima water control (**a**), + 0.57 µmol/L aspartame (**b**), + 5.7 µmol/L aspartame (**c**), + 28.5 µmol/L aspartame (**d**), and + 209.8 µmol/L aspartame (**e**)). Data displayed (mean ± SD) is the average of triplicate samples (n=3).

4.3.4 Perspectives

Overall, residual chloramine (1.8 mg/L) in authentic tap water was found to be sufficient to significantly increase the concentration of DCBQ after the addition of one Equal packet. Both aspartame and its aromatic amino acid component, phenylalanine, were identified as DCBQ precursors under controlled chloramination conditions. Furthermore, the presence of aspartame enhanced the stability of DCBQ in aqueous solution, preventing its transformation to the less cytotoxic product OH-DCBQ.[23,45] Our findings indicate a potential increased risk of human exposure to DCBQ with aspartame use. Although DCBQ is a known cyto- and genotoxicant *in vitro*, it is not known whether the low, but elevated concentrations detected here pose a risk to human health.

An HBQ occurrence survey across nine water treatment plants in North America detected DCBQ in finished water in 100% of the samples tested at a range from 4.5-274.5 ng/L.[22] The 8.0 ng/L of DCBQ detected in the authentic tap water tested here is on the lower end of this range. The addition of Equal to tap waters containing DCBQ at the highest range detected in occurrence studies (e.g., 274.5 ng/L)[22] may have a greater impact on the exposure dose in terms of human health risk, especially if the presence of aspartame prevents its transformation and subsequent detoxification. Furthermore, the sampled tap water contained negligible levels of free bromide or iodide, preventing formation of the more cytotoxic brominated and iodinated HBQs.[45] It is important that future studies consider the impact of source water characteristics when examining the influence of artificial sweetener use on DBP formation, as the potential increase in HBQ concentration following addition of Equal may pose a more significant risk at other utilities.[46] While the focus of this study was DCBQ, the formation of other DBPs in prepared beverages from artificial sweetener use is likely. The DOC content in Optima and tap water with and without the addition of 10 μM aspartame or 1 Equal package was determined, **Table 4.6**. The persulfate oxidation method detected 1.73 mg/L DOC from 10 μM aspartame in Optima water, corresponding closely (i.e., within 3% difference) to the theoretically calculated value (i.e., 1.68 mg/L), **Table 4.6(c)**. The addition of one packet of Equal into the cup of authentic tap water increased DOC levels from 1.8 mg/L to nearly 1500 mg/L (**Table 4.6(b)**), suggesting a rich precursor pool for DBP formation. Using the same theoretical DOC estimate, the proportion of aspartame in one

Equal package (i.e., 214 μM or 63.2 mg/L, **Calculation 4.1**) could contribute 36.11 mg/L DOC, which was approximately 2.5% of the DOC concentration detected in the Optima water sample, **Table 4.6(c).**

The most efficient means to reduce risk is to eliminate exposure. Previous studies have shown that boiling can detoxify treated water by reducing residual chloramine concentration as well as volatile and non-volatile DBPs in tap water.[47-52] Five minutes of boiling was sufficient to reduce brominated and chlorinated DBPs in simulated tap water by 63% and 61%, respectively,[51,52] resulting in a reduction in CHO cell cytotoxicity of the boiled water by 77%.[47] Boiling tap water was evaluated as a method to remove DCBQ or prevent its formation during use of the artificial sweetener Equal. After boiling, DCBQ concentration was reduced from 8.0 to 1.6 ng/L (80% reduction; **Figure 4.8**). Furthermore, DCBQ was not detectable after a packet of Equal was added to hot boiled tap water (90 °C) or to boiled tap water cooled for 1 hour (34 °C). This is likely due to the reduction in residual chloramine from 1.7 to 0.8 mg/L and the increase in pH from 7.5 to 8.0 after boiling (**Table 4.7)**. The reduction of residual chloramine limited DCBQ formation, while the higher pH increased DCBQ hydrolysis.

Investigations into the occurrence and formation of DBPs during food and beverage preparation is an emerging field of importance. This study contributes understanding to DBP exposure pathways by identifying a new DCBQ precursor and the potential to increase DCBQ exposure by addition of aspartame to beverages prepared with chloraminated tap water. Applying new approaches to identify toxicity forcing agents will help to better understand exposure and health risks.[18,53-55]

Table 4.6 (**a**) Aspartame characteristics, (**b**) Average DOC in each sample determined with the persulfate oxidation method, and (**c**) Comparison between theoretical and determined DOC concentrations

(a)	**Aspartame Characteristics**			
	Molecular Formaula	**Molecular Mass**	**Carbon Mass**	**% Carbon**
	C14H18N2O5	294.3 g/mol	168.1498 g/mol	57.14%
(b)	**Sample**	**Average NPOC (mg/L), [n=3]**	**Standard Deviation (± mg/L)**	**%RSD**
	Optima Water (OW)	0.42	0.25	60
	OW + 10 µM aspartame	1.73	0.02	1.1
	OW + Equal	1398	21.49	1.5
	Authentic Tap Water (TW)	1.77	0.09	5.1
	TW + 10 µM aspartame	3.33	0.06	1.9
	TW + Equal	1453	56.93	3.9
(c)	**Sample**	**Theoretical Calculated DOC**	**Measured DOC (in OW)**	**% Difference**
	10 µM aspartame (2.94 mg/L)	1.68 mg/L	1.73 mg/L	2.7%
	1 Equal package (63.2 mg/L)	36.11 mg/L	1398 mg/L	97.4%

Table 4.7 pH and residual chloramine concentrations measured 15 minutes after dissolving one Equal package in authentic chloraminated tap water without boiling pre-treatment and 1 h after boiling pre-treatment. Data represents the average of triplicate (n=3) experiments

Sample	**Residual Chloramine (mg/L)**	**pH**	**Sample**	**Residual Chloramine (mg/L)**	**pH**
Tap Water	1.8 ± 0.09	7.7 ± 0.2	**Cooled Boiled Tap**	0.8 ± 0.01	8.5 ± 0.1
Tap Water + Equal	1.7 ± 0.07	7.5 ± 0.1	**Cooled Boiled Tap + Equal**	0.8 ± 0.01	8.0 ± 0.1

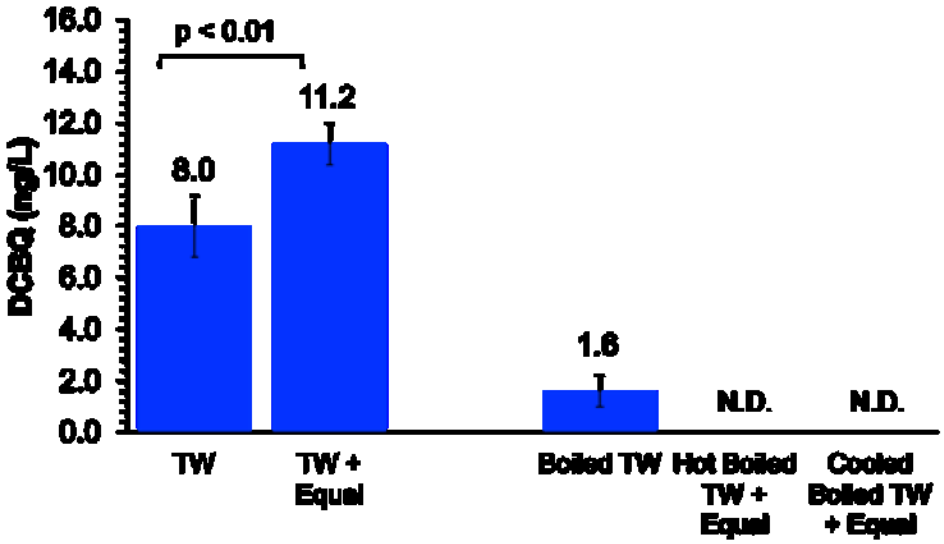


Figure 4.8 Concentration of DCBQ in authentic chloraminated tap water (TW) after boiling pre-treatment alone, and with the addition of one Equal packet immediately after boiling (Hot Boiled, 90 °C) or 1 hour after cooling (Cooled Boiled, 34 °C), compared to TW without boiling pre-treatment (left two columns, **Figure 4.3a)**

4.4 Conclusion

Investigations into the occurrence and formation of DBPs during food and beverage preparation is an emerging field of importance. This study contributes understanding to the overall DBP exposure pathway by identifying a new DCBQ precursor and the potential to increase DCBQ exposure by addition of the AS, aspartame, to beverages prepared with disinfected tap water. Aspartame could react with monochloramine to form 2,6-dichloro-1,4-benzoquinone (DCBQ), an emerging DBP more cytotoxic than regulated DBPs.[56] Under controlled reaction conditions, DCBQ was formed (0.6-9.8 ng/L) from aspartame (pH 6.5 and 7.5) and its aromatic component phenylalanine (pH 6.5) in the presence of 4 mg/L monochloramine. The minor DCBQ yield from chloramination of aspartame and phenylalanine indicate other major products could be formed in this reaction. In a cup of authentic chloraminated tap water, the concentration of DCBQ increased significantly from 8.0 to 11.2 ng/L, after adding one packet of Equal artificial sweetener. The presence of aspartame in Optima water solutions of DCBQ reduced transformation of DCBQ to its less cytotoxic product, 3-hydroxy-2,6-dichloro-1,4-benzoquinone (OH-DCBQ).[56] This study

identified aspartame as both a new precursor and stabilizer of DCBQ in chloraminated tap water.

Reductions in residual chloramine and DCBQ concentration in authentic tap water after boiling pre-treatment provides additional evidence in support of boiling to improve water quality before consumption or beverage preparation. However, adding a packet of Equal to one cup of tap water increased the dissolved organic content (DOC) by 800 times, suggesting the need to understand the formation and subsequent exposure of DBPs via consumption of sweetened beverages. Future work investigating DBP formation from residual chloramine in tap water during beverage preparation should consider high precursor to low disinfectant ratios in their experimental designs.

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Chapter 5

Aromatic Amino Acids as Halobenzoquinone Precursors[**]

"Our greatest weakness lies in giving up.
The most certain way to succeed is always to try just one more time."
– Thomas Edison (1847-1931)

5.1 Introduction

Free aromatic amino acids (AAAs) are present in raw water at low nmol/L concentrations.[1] Studies on amino acids as disinfection by-product (DBP) precursors found nitriles, aldehydes and chloroaldimines to be the major products under chlorination at neutral pH.[2,3] More recently, an investigation on the chlorination of adenine and cytosine found multiple transformation products with chlorine additions on the heterocyclic ring and the aliphatic amine. N-chloramine was formed through chlorine substitution on the primary amine functional group.[4] Additionally, simple tyrosyl-dipeptides have been identified as precursors of chlorinated, brominated, iodinated, and mixed halogenated N-chloramine dipeptides.[5-7] Due to the structural similarity to halobenzoquinones (HBQs) (**Figure 5.1**), AAAs (i.e., phenylalanine, PHE; tyrosine, TYR; and tryptophan, TRP) are plausible precursor candidates. PHE contains a phenyl group, TYR contains a hydroxy phenyl group, while TRP is aromatic due to its heterocyclic indole ring.

The bromide concentration in 23 difference source waters across Canada and the United States ranged from 0.024 to 1.12 mg/L.[8] Several groundwater sources in Alberta are high in Br^- or I^- due to geomorphological deposits.[9,10] Hypochlorous acid (HOCl) can oxidize Br^- and I^- to form reactive intermediates including hypobromous acid (HOBr) and hypoiodous acid (HOI)[11-13] which have a higher substitution reactivity toward NOM compared to HOCl.[14] Raw water containing high levels of bromide and iodide can produce higher proportions of Br- and I-DBP analogs.[15-17] Increasing the bromide concentration in

[**] The results presented in **Chapter 5** are unpublished. The experiment described in **Section 5.2.2.2** and corresponding **Figure 5.5** was executed and prepared in 2014 by Dr. Yichao Qian as a post-doctoral fellow in Li Research Group.

drinking water samples resulted in all polar halogenated DBPs (including four newly identified groups of aromatic halogenated DBPs, dihalo-4-hydroxybenzaldehydes, dihalo-4-hydroxybenzoic acids, dihalo-salicylic acids, and trihalophenols), from being less brominated to more brominated.[18] Phenol, a known HBQ precursor, was found to produce higher proportions of DBBQ to DCBQ under high bromide source water concentrations compared to normal conditions.[19]

In vitro[20,21] and *in vivo*[22] studies have identified a trend in the toxicity of halogenated DBPs: iodinated > brominated > chlorinated. This halogen substitution trend resulted in a reduction in cell density for Chinese Hamster Ovary (CHO) cells after a 72 hour exposure period[23] for many different DBP classes including: halo acetic acids,[8,23,24] halomethanes,[8,24] haloacetamides,[25] haloacetaldehydes,[26] haloacetonitriles,[27] halonitromethanes,[28] and cyanogen halides[29] among others.[21] HBQ cyto- and genotoxicity generally follows the similar trend: I > Br >> and Cl-HBQs.[30] Here the formation of Br- and Cl-HBQs from AAAs was investigated under chlorination conditions in the presence of bromide.

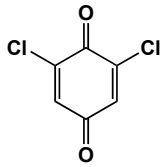
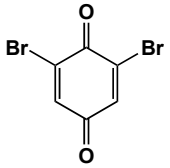
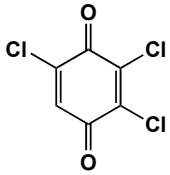
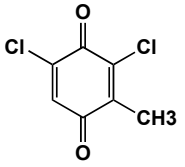
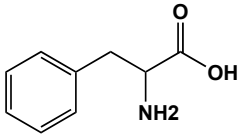
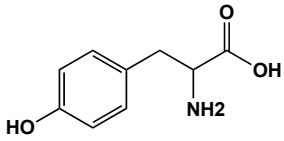
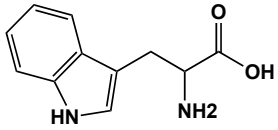
Halobenzoquinones (HBQs)			
Dichlorobenzoquinone	Dibromobenzoquinone	Trichlorobenzoquinone	Dichloromethylbenzoquinone
			
2,6-DCBQ	**2,6-DBBQ**	**TriCBQ**	**DCMBQ**
Aromatic Amino Acids (AAAs)			
Phenylalanine	Tyrosine	Tryptophan	
			
PHE	**TYR**	**TRP**	

Figure 5.1 Structures of experimentally relevant HBQs and AAAs: 2,6-DCBQ, 2,6-DBBQ, TriCBQ, and DCMBQ; and PHE, TYR, and TRP, respectively

5.2 Materials and Methods

5.2.1 Chemicals and Materials

High performance liquid chromatography-tandem mass spectrometry (HPLC-MS) grade formic acid (FA, 98%), phenylalanine (PHE), tyrosine (TYR), and tryptophan (TRP) and sodium hypochlorite solution (reagent grade, available chlorine 10−15%) were obtained from Sigma-Aldrich (St. Louis, MO). Optima LC-MS grade methanol and water, hydrochloric acid, and anhydrous dibasic potassium phosphate, were obtained from Thermo Fisher Scientific (Fair Lawn, NJ). 2,6-Dibromo-(1,4)-benzoquinone (DBBQ) was purchased from Indofine Chemical Company (Hillsborough, NJ). 3,5-Dichloro-2-methyl-(1,4)-benzoquinone (DCMBQ) and 2,3,6-trichloro-(1,4)-benzoqui- none (TriCBQ) were synthesized by Shanghai Acana Pharmtech (Shanghai, China); 2,6-dichloro-(1,4)-benzoquinone (DCBQ) was purchased from Sigma-Aldrich (St. Louis, MO). A pH meter (Model 15, Accumet) was used to monitor pH.

5.2.2 Sample Preparation

5.2.2.1 Chlorination of PHE at Increasing Molar Ratios for 1 and 24 Hours

A stock solution of PHE (50 mM) standard was prepared on the day of the experiment. Working solutions of 5 mL of PHE (1 mM, final) each were used for chlorination reactions with varying free Cl_2 (0, 15, 30, 150, 750, 1500, 3000 μL of 10% Cl_2 from NaOCl). Each reaction mixture had a total final volume of 250 mL Optima water and all reactions took place in 500 mL amber glass bottles. In these reaction mixtures, the molar ratios of PHE:Cl_2 were 1:0, 1:0.1, 1:0.2, 1:1, 1:5, 1:10, and 1:20, respectively. The reaction mixtures in the bottles were covered with aluminum foil and left to react for 1 hour. The same procedures were repeated with a reaction time of 24 hours. The solutions were quenched with FA (0.25%, final) and a 1-mL aliquot was immediately analysed with a HPLC-MS/MS (MRM) method,[31] described below. After the method was optimized, HBQs were quantified using an external calibration curve that was obtained prior to the sample run on the same day.

5.2.2.2 Chlorination of PHE and TYR Over Time

Reaction solutions containing 1 mM of PHE or TYR with free Cl_2 from NaOCl at 5 and 10 mM were freshly prepared, corresponding to molar ratios of Cl_2:AAA of 5:1 and 10:1 mM, respectively. Aliquots of each reaction mixture were collected and quenched with FA (0.25%, final) at given time points ranging from 0.5 to 72 hours, and immediately analysed using the HPLC-MS/MS (MRM) method, and quantified with an external calibration curve that was obtained in the same day prior to the sample analysis.

5.2.2.3 Chlorination of PHE, TYR and TRP with Br- for 24 Hours at pH 7.5

The details of the workflow for chlorination, sample preparation, and the HPLC-MS/MS analysis have been described in **Figure 5.2**. High concentration standard solutions of PHE (50 mM), TRP (50 mM), and TYR (2 mM), and Br^- (100 mM), were prepared the day of the experiment. Triplicate AAA reaction solutions were prepared with 10 mL of PHE or TRP, or 250 mL of TYR (1 mM, final), 1 mL of Br^- (0.2 mM, final), and 3 mL of 10% free Cl_2 solution (from NaOCl; 10 mM, final). Samples were diluted to a final volume of 500 mL with phosphate buffer (i.e., 20 mM at pH 7.5, prepared with Optima water) in amber glass bottles and left to react in the dark (i.e., wrapped in foil) for 24 hours at room temperature. Samples were quenched with FA (0.25%, final) immediately before sample clean up and preconcentration with solid phase extraction (SPE) and nitrogen evaporation (N_2).

A 625-µL aliquot of FA (0.25% v/v, final) was added to acidify the sample to stabilize DCBQ before solid phase extraction (SPE). A Waters Oasis HLB cartridge (6 mL, 200 mg) mounted in a VISIPREP SPE manifold (Supelco, Bellefonte, PA) was used to desalt and concentrate the quenched reaction solutions. First, the SPE cartridges were activated with two, 6 mL washes of methanol (0.25% FA, v/v), then rinsed twice with 6 mL of Optima water (0.25% FA, v/v). The quenched reaction solution was drawn through the cartridge under vacuum at a flow rate of 8 mL/min. Next, the cartridge was washed with two 6 mL portions of Optima water (0.25% FA, v/v), one 6 mL portion of 1:1 Optima: MeOH (0.25% FA, v/v) and finally the analytes were eluted with 10 mL of methanol (0.25% FA, v/v) into graduated test tubes. A TurboVap LV Concentration Nitrogen Evaporation Workstation (Caliper Life Sciences, Massachusetts, United States) was used to evaporate the eluate down

to 100 µL under a gentle (< 5 psi) nitrogen stream and reconstituted with Optima water (0.25% FA, v/v) to a final volume of 500 µL water/methanol (v/v, 4/1).

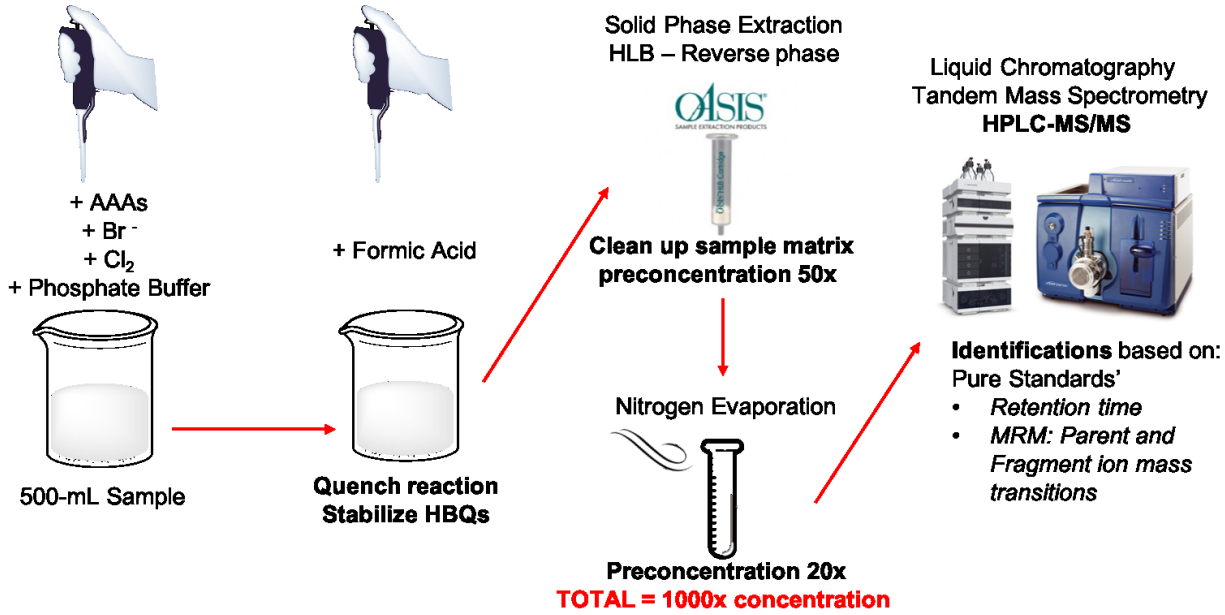


Figure 5.2 Quantitative chlorination experiments are conducted as follows. Aromatic amino acid reactants are prepared individually in optima water, with or without excess bromide, and phosphate buffer in an amber glass bottle. The reactant solution is spiked with free chlorine (from NaOCl) and left to proceed in darkness. Formic acid (final concentration 0.25%) quenches the chlorination reaction and stabilizes HBQs at low pH. Solid phase extraction (HLB, reverse phase) cleans up the sample matrix and nitrogen evaporation is employed for a final sample pre-concentration of 1000x. Samples are analyzed with high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). Identifications are confirmed by comparing retention time and parent and fragment ion mass transitions with pure standards. HBQ concentrations were quantified using standard addition calibration.

For standard addition, four 80 µL aliquots were removed from the 500 µL reconstituted sample. Each aliquot was spiked with 20 µL of HBQ methanol solution (e.g., yielding 2.5, 5, 10 or 25 µg/L additional HBQ (100 µL total)). Samples were analyzed by HPLC-MS/MS (5500 QTRAP System; Sciex, Concord, Ontario, Canada) operating in multiple reaction monitoring (MRM) mode. The peak areas were used to calibrate against

the spiked concentrations. The resulting standard addition calibration curves with linear coefficients ranging between 0.989−0.999, were used to determine the concentrations of HBQs in each sample by solving for the absolute value of the X-intercept.

5.2.3 HPLC-MS/MS Method

The high-performance liquid chromatography tandem mass spectrometry with multiple reaction monitoring (HPLC-MS/MS (MRM)) analysis was adapted from previously described methods.[31] An Agilent 1290 series LC system consisting of an autosampler with temperature control and a binary pump (Agilent, Waldbronn, Germany) was used with a Luna C18(2) column (100 × 2.0 mm i.d., 3 μm; Phenomenex, Torrance, CA). The mobile phase was comprised of solvent (A), water containing 0.1% FA; and solvent (B), methanol (MeOH) containing 0.1% FA. The gradient program is described in **Table 5.1**. The sample injection volume was 20 μL. The autosampler was kept at 4 ºC and the column was at room temperature.

Analyte detection by MRM mode was performed using a triple quadrupole ion-trap tandem mass spectrometer (Sciex QTRAP 5500, Ontario, Canada). The optimized MS instrumental and MRM ion transition detection parameters are listed in **Table 5.2** and **Table 5.3**. Analyst software version 1.5.2 for Sciex QTRAP 5500 was used for data analysis. An example chromatogram for a 60 ppb HBQ standard mixture is shown as **Figure 5.3.**

Table 5.1 HPLC mobile phase gradient program

Time (min)	Solvent A (%)	Solvent B (%)	Flow Rate (μL/min)
0.00	80	20	170
50.00	10	90	170
55.00	10	90	170
55.10	80	20	170
60.00	80	20	170

Solvent A: Optima H2O (0.1% FA)
Solvent B: Optima MeOH (0.1% FA)

Table 5.2 MS instrument operating conditions

Collision Gas	Medium
Curtain Gas	30 psi
Ion Source Gas 1	50 psi
Ion Source Gas 2	60 psi
Ion Spray Voltage	-4500 V
Temperature	450 °C
Entrance Potential	-10
Dwell Time	200 (msec)

5500 Sciex QTrap Mass Spectrometer

Table 5.3 MRM mode analyte ion transitions and detection parameters

Name	Rt (min)	Q1 Mass (Da)	Q3 Mass (Da)	DP Declustering Potential	CE Collision Energy	CXP Collision Cell Exit Potential
DCBQ	*	177	113	-100	-24	-13
	*	177	141	-100	-20	-13
DCMBQ	19.7	191	127	-85	-24	-11
	19.7	191	155	-85	-20	-11
TriCBQ	20.3	211	175	-80	-18	-11
	20.3	211	35	-80	-30	-15
DBBQ	16.5	267	79	-100	-50	-10
	16.5	267	81	-100	-50	-10

* DCBQ isomers are both detected with the same MRM parameters but elute at different retention times. 2,5-DCBQ (12.9 min) and 2,6-DCBQ (13.3 min)

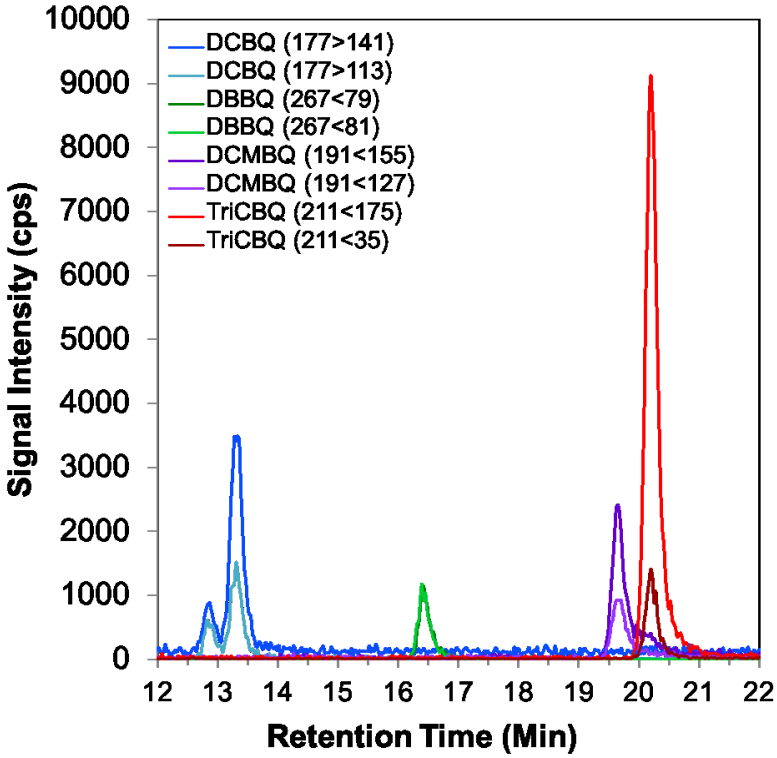


Figure 5.3 Example chromatogram displaying a 60-ppb standard mixture of HBQs, 2,5-DCBQ and 2,6-DCBQ (blue), DBBQ (green), DCMBQ (purple), and TriCBQ (red) detected using MS/MS in MRM mode

5.3 Results and Discussion

5.3.1 Identification of Aromatic Amino Acids as HBQ Precursors

HBQs are a class of highly cytotoxic DBPs,[32, 33] detected frequently in North American tap water.[31] Characterizing HBQ precursors is essential to reduce their formation, yet this area is not well studied. AAAs are ideal HBQ precursor candidates because of their inherent ring structure. First, I optimized a high-performance liquid chromatography mass spectrometry (HPLC-MS) method capable of low µg/L detection of HBQs. With this method preliminary experiments have confirmed that AAAs can serve as HBQ precursors.

My initial experiments used relatively high concentrations of PHE and free chlorine to directly monitor the formation of HBQs from PHE without extraction. PHE (1 mM) was

reacted with increasing concentrations of free chlorine (from NaOCl) ranging from 0.1 to 20 mM. Direct analysis of the reaction mixtures at 1 and 24 hours after chlorination showed HBQ formation from PHE. Both DCBQ and TriCBQ were detected after 1 hour. With increasing free chlorine concentrations, DCBQ formation increased while TriCBQ decreased (**Figure 5.4(a)**). After 24 hours, the concentrations of DCBQ increased and followed the same trend observed at 1 hour however TriCBQ was no longer detected in the reaction mixture (**Figure 5.4(b)**).

In the second set of time course experiments, both PHE and TYR produced DCBQ at pH 7.5. In this set, only DCBQ was detected as a formation product, unlike in **Figure 5.4** where TriCBQ was also detected after 1 hour of PHE chlorination but not after 24 hours. **Figure 5.5(a)** shows that over time, the formation of DCBQ from PHE was found to steadily increase and plateau at 24 hours of chlorination contact time. This is consistent with the trend of increased DCBQ from chlorination of PHE at 24 hours compared to 1 hour in **Figure 5.4**. Here, **Figure 5.5(b)** shows chlorination of TYR produced DCBQ quickly, peaking at 0.5 hours then decreased dramatically as reaction time increased. For both PHE and TYR, DCBQ formation was greater at 5:1 mM than 10:1 mM Cl:AAA reactant ratio. The overall yield of DCBQ was greater for TYR than PHE under the same reaction conditions.

Greater DCBQ formation from TYR can be rationalized due to the phenol group located on the aromatic ring. The hydroxyl can activate the ring by electron withdrawal, where electron density is polarized away from the ring towards the electronegative oxygen atom. More importantly, the lone pair of electrons from oxygen can move around the ring in resonance between alternating aromatic carbons. Previous work has found that the chlorine demand of TYR and TRP (i.e., 13 and 16 mol/mol, respectively) are much greater than PHE (i.e., 2.7 mol/mol) indicating that the activated aromatic ring is essential to their reactivity.[34] The loss of TriCBQ signal after 24 hours of PHE chlorination shown in **Figure 5.4** as well as the drop in DCBQ concentration detected from chlorination of TYR over time indicates that HBQs could be acting as intermediates in the formation of other DBPs.[35, 36]

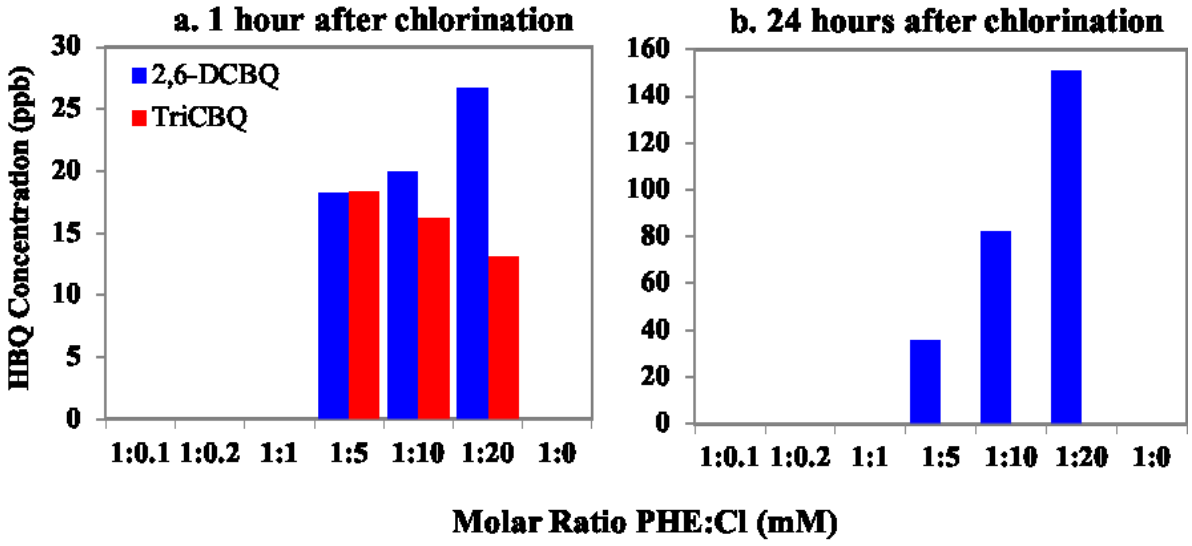


Figure 5.4 Formation of 2,6-DCBQ and TriCBQ, from phenylalanine after 1 (**a**) and 24 (**b**) hours of chlorination with free chlorine. Results reflect a single preliminary experiment with no replicates

The halogenated species of HBQs formed during drinking water treatment depends on the quality of source water. The presence of Br^- and I^- influence the formation of more toxic Br- and I-DBP congeners.[16, 17] The results in **Figures 5.4** and **5.5** have demonstrated that PHE and TYR could act as Cl-HBQ precursors. Therefore, Br-HBQs (i.e., DBBQ) were expected to form from AAAs during chlorination in high Br^- waters. As a proof of concept, PHE, TYR and TRP (1 mM) were reacted with free chlorine (NaOCl, 10 mM) in the presence of Br^- (NaBr, 0.2 mM). **Figure 5.6** shows the formation of HBQs from each AAA, 24 hours after chlorination in pH 7.5 buffered Optima water containing 0.2 mM Br^-. All AAAs formed DCBQ with the greatest yields from TRP >> PHE > TYR. After 24 hours at a 5:1, Cl:AAA molar ratio, **Figure 5.4** shows the relative formation of DCBQ was much greater from TYR (i.e., approx. 75 ppb DCBQ) compared to PHE (i.e., approx. 10 ppb DCBQ). At a molar ratio of 10:1 of Cl:AAA, the AAAs produced similar amounts of DCBQ at 24 hours, yielding approximately less than 10 ppb and 4 ppb from TYR and PHE, respectively. While DCBQ formation was in the same magnitude (i.e., both less than 20 ppb DCBQ), **Figure 5.6** shows that TYR formed less DCBQ than PHE. In the presence of Br^-, PHE and TYR both produced

greater amounts of the more cyto- and genotoxic HBQ congener, DBBQ, compared to DCBQ. This set of experiments was the first to investigate TRP as an HBQ precursor. The HBQs formed from TRP as follows DCBQ >> TriCBQ > DBBQ. The TRP reaction solution was the only AAA sample to yield detectable TriCBQ under these conditions after 24 hours. These preliminary experiments showed the interesting observations of TRP as precursors of HBQs. It warrants further investigation into the mechanisms of HBQs formation from TRP.

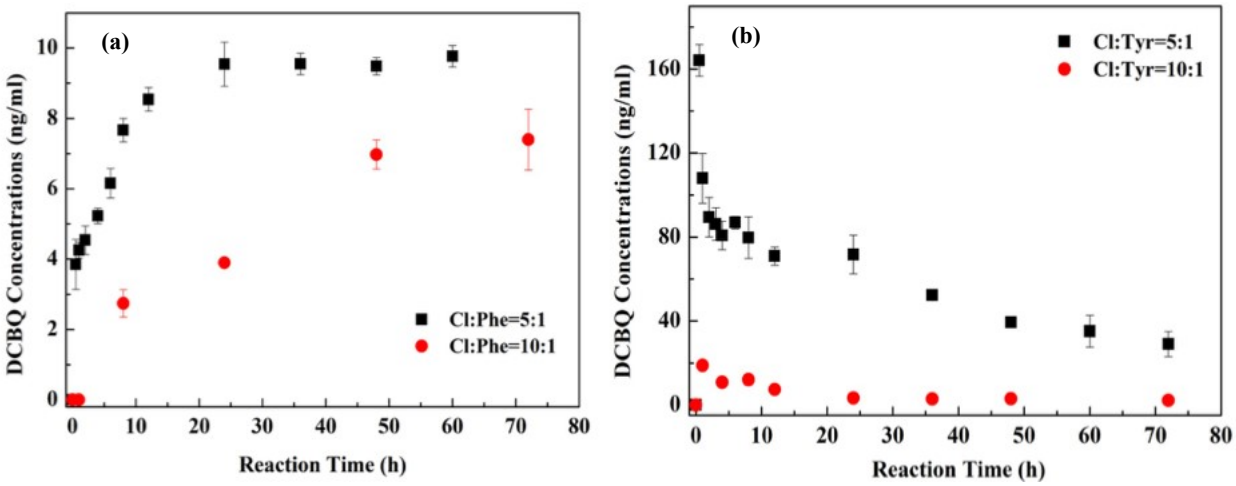


Figure 5.5 Reaction profiles of the formation of DCBQ from PHE and TYR over time at pH 7.5. Error bars represent the standard deviation of triplicate sample analysis

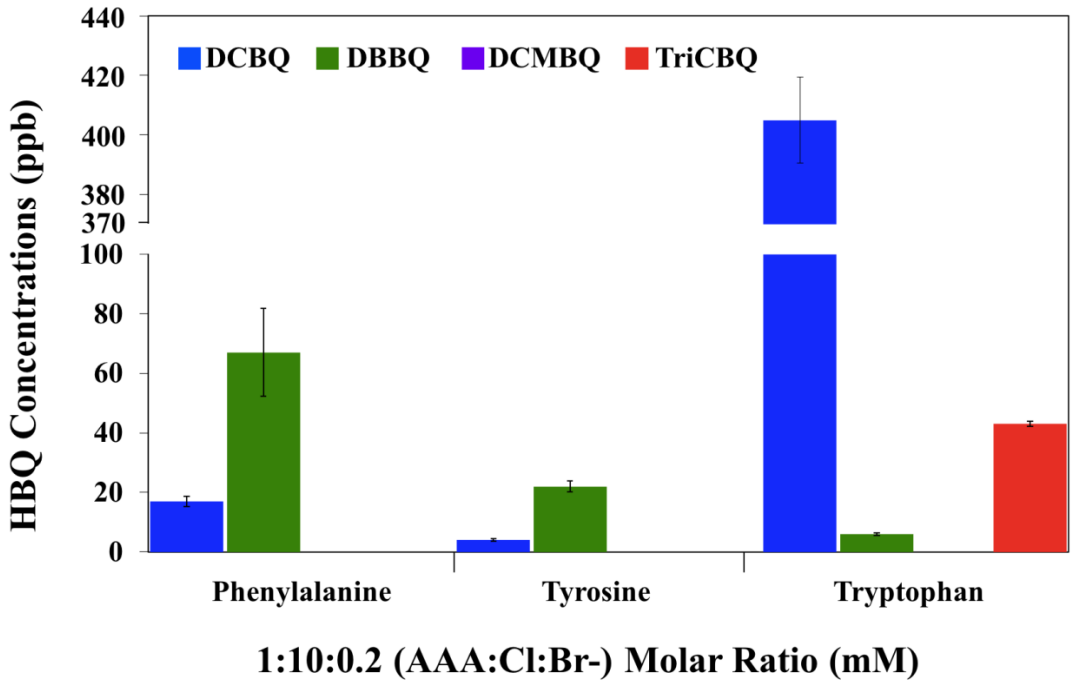


Figure 5.6 Formation of HBQs from PHE, TYR, and TRP, 24 hours after chlorination with free chlorine (NaOCl) in Optima water in the presence of Br – at pH 7.5. Error bars represent the standard deviation of triplicate sample analysis

5.3.2 Relevance of AAAs as DBP Precursors

This evidence shows AAAs are Cl-HBQ precursors under chlorination conditions and yield DBBQ in the presence of Br^-. These experiments were conducted under controlled laboratory conditions with high purity reagents. Authentic source waters contain a more complex mixture of organic content.[37, 38] Amino acids, peptides and proteins account for a majority (up to 75%) of dissolved organic nitrogen (DON) in source waters.[19] Peptides in source water can be as high as mg/L.[37, 39, 40] Based upon this idea, the Li group has developed a new strategy[41] to characterize and determine new chlorinated,[5] brominated,[7] iodinated[6] and mixed halogenated dipeptide DBPs formed after chlorination or chloramination of a variety of combinations of simple dipeptides including AAA tyrosyl-dipeptides. While unbound AAAs are not highly concentrated in environmental waters,[1] AAAs are integral in natural

systems of plants and animals,[42] therefore there is a potential that AAAs present as simple peptides could similarly serve as an additional HBQ precursor source.

Drinking water sources both in Canada[43] and internationally[44] are being increasingly impacted by seasonal algal blooms. Algal breakdown products increase the complexity and abundance of available proteins and peptides in environmental waters.[45] Recently it was reported that green algae in lakes and rivers could serve as precursors to DCBQ after 24 hours of chlorination at pH 6-9.[46] Overall, AAAs both free and bound as natural organic matter (e.g., peptides and proteins) may account for a larger source of HBQ precursors than previously understood.

5.4 Conclusion

5.4.1 AAAs as Putative HBQ Precursors

My preliminarily study has identified AAAs as HBQ DBP precursors and further research will lead to a better understanding of the mechanisms of HBQ formation from these compounds. The identification of new HBQ precursors can provide essential information to decrease their formation during water treatment disinfection processes[47] and their distribution in treated drinking water in the future. Recently, activated carbon adsorption has been proposed as a new approach to remove intermediate aromatic halogenated DBPs in chlorinated drinking water.[48] Future studies building from this foundational proof of concept work will provide information contributing to the overall understanding of DBP exposure and provide strategies to reduce exposure and subsequent potential adverse human health impacts.

5.4.2 Future Analysis

Since collecting these results, the HBQ method was adapted for more efficient future experimentation by incorporating all three AAAs as analytes. Currently, detection limits for both HBQs and AAAs are in the same order of magnitude (i.e., ppb level, **Figure 5.7**). After the conditions for HBQ formation from AAAs are validated, the impact of halide ions to precursors ratios and the effects of varying pH and reactant concentrations could be investigated over reaction time. The method could be expanded to incorporate I-HBQ congeners to repeat these experiments with a focus on the impact of iodine in the formation

of I-HBQs, found to be the most cytotoxic of all halogenated HBQ species. Finally, source water samples containing high I^- and Br^- could be used to conduct chlorination mimicking water treatment and HBQ formation could be determined in the authentic, high Br^- and I^- water sample spiked with AAAs.

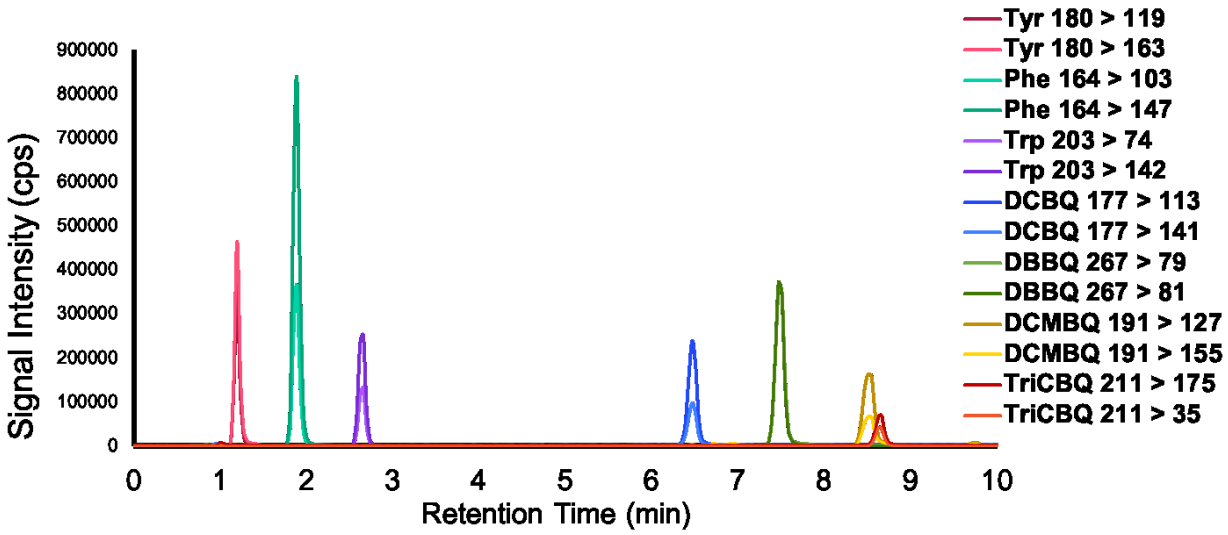


Figure 5.7 MRM Extracted ion chromatogram for method capable of future simultaneous detection of AAAs (TYR, PHE, and TRP) and HBQs (DCBQ, DBBQ, DCMBQ and TriCBQ) in negative ionization mode

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Chapter 6

Conclusions and Synthesis

"We can do anything we want to do if we stick to it long enough."

– Helen Keller (1880-1968)

6.1 Introduction

Water is a limited resource. Its quality can be impacted by waste contamination from a variety of sources (e.g., municipal, agricultural, industrial, etc.). Pathogens present in inadequately treated or untreated drinking water pose an acute risk to human health and can tragically result in waterborne-disease outbreaks.[1] Pristine water sources continue to decrease worldwide, therefore the impact of wastewater on the environment and our drinking water must be better understood to improve management strategies.[2] Wastewater treatment plants minimize the risks of discharge to receiving water bodies.[3] Some anthropogenic contaminants are incompletely removed by waste treatment processes and do not occur naturally in ground or surface waters.[4,5] Specific waste indicators can be used to trace and mitigate wastewater impact in receiving water bodies by informing treatment strategies designed to mitigate negative impacts from wastewater contamination to ensure safe, high quality drinking water.[6,7]

Disinfection is a lifesaving drinking water treatment technology that can eliminate the acute health risk of potentially fatal waterborne pathogens.[8] Unfortunately, disinfection byproducts (DBPs) are formed as an unintentional consequence from reactions between disinfectants and organic matter present in water.[9] Epidemiology studies have consistently associated long-term consumption of DBPs in chlorinated drinking water with a small, but significant, increased potential risk of developing bladder cancer.[10] Population wide consumption of domestically provided treated drinking water,[11,12] coupled with the prevalence of bladder cancer[13] have justified the ongoing investigation into the formation of, and exposure to, DBPs.[14–18]

Although trihalomethanes (THMs) and haloacetic acids (HAAs) are the dominant DBPs formed during chlorination, they are not the primary drivers of DBP toxicity.[19–21]

Research focus has shifted to identify classes or fractions of DBPs with toxicological relevance.[22,23] To decrease DBPs in drinking water, substantial efforts are employed at drinking water treatment plants to reduce the organic content in source water before disinfection.[24,25] However, DBPs formed from food and beverage preparation with chlor(am)innated water[26–29] are highlighting their relevance as an exposure source to DBPs.

The consistent themes throughout my research revolved around the assessment of wastewater indicators and the identification of toxicologically relevant DBP precursors. My thesis includes a critical assessment of the recent applications using artificial sweeteners (ASs) to assess wastewater impact in environmental waters (**Chapters 1** and **2**).[30] I developed a simple and sensitive high throughput high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for the determination of SUC and ACE in environmental waters to investigate their occurrence in previously unstudied British Columbian surface waters (**Chapter 2**). Next, I validated this method for ACE determination in a recreational water matrix. For the first time, I determined the prevalence of ACE in swimming pool and recreational waters collected from BC and Alberta. I applied the strategy of ACE as a urinary marker in two swimming pools to estimate the approximate volume of urine present (**Chapter 3**).[31] Later, I shifted from investigating non-nutritive ASs (i.e., ACE and SUC) as wastewater indicators in environmental and recreational waters to assessing a nutritional, peptide based sweetener (i.e., aspartame) and aromatic amino acids (AAAs; i.e., phenylalanine (PHE), tyrosine (TYR), and tryptophan (TRP)) as precursors of an important class of DBPs, halobenzoquinones (HBQs) under different chlor(am)ination conditions (**Chapters 4** and **5**).

6.2 Advances in Knowledge

6.2.1 Assessment of Artificial Sweeteners as Wastewater Indicators and Determination of Acesulfame and Sucralose in B.C. Surface Waters

Appropriate surface and ground water treatment measures can be employed to minimize the impact of wastewater contamination if specific indicators can be used to monitor the waste.[4,5] The occurrence of artificial sweeteners (ASs) in environmental waters has been used successfully as an indicator of wastewater impact.[30,32] Non-nutritive ASs like

acesulfame (ACE) and sucralose (SUC), were generally considered very stable compounds[33] with excellent potential as a waste indicator.[6,32,34] My review of the recent literature highlighted growing evidence suggesting ASs can undergo significant biologically mediated degradation through wastewater treatment processes.[30,35,36] Additionally, UV irradiation can result in environmental reduction in surface waters because of transformation.[37,38] Furthermore, agricultural pig feed[39] and municipal landfill leachate[40] were identified as potential unaccounted sources of AS contamination in a water body.[41] The temporal and geographical variation of ASs in wastewater reinforces the importance of accurately characterizing AS occurrence in waste sources before investigating their impact in receiving water bodies.[30]

Of the expanding reports on the occurrence of ASs in surface and ground water, relatively few investigated Canadian environmental water samples. Furthermore, no occurrence information for ASs in B.C. environmental waters was found in the existing literature. I collected surface water samples from publicly accessible surface water bodies in the Thompson Region. Using the high-performance liquid chromatography method that I developed for determination of ACE and SUC, I detected ACE in all 12 surface water samples at low parts per trillion (i.e., 1-17 ng/L) concentrations. SUC was only quantifiable in 2 lake samples. As analytical tools continue to advance, lower detection limits will enhance the understanding of the fate and behavior of trace ASs in environmental waters and their waste sources, thereby increasing the accuracy of future wastewater impact assessments.

6.2.2 Evaluation of Acesulfame as a Urinary Indicator in Recreational Waters: Occurrence in Swimming Pools and Hot Tubs and Case Study Estimation of Pool Urine Volume

ASs are pervasive in the common diet[42] and have been established as a globally occurring anthropological environmental contaminant.[30,32,33,43] ASs have several characteristics which make them ideal waste indicator compounds. They are specific to human waste, undergo incomplete removal from water treatment processes, are present in waste sources at concentrations much greater than background levels, undergo minimal transformation in the environment, and are detectable with sensitive analytical methods.[4,5,31] While ASs have been used worldwide as wastewater tracers in environmental water bodies

this strategy had never been applied to recreational waters (i.e., swimming pools and hot tubs). Many components of human bodily fluids (i.e., urine and sweat) can react with the disinfectants present in recreational waters to form disinfection byproducts (DBPs).[44] Trichloramine is a recreational water DBP with known adverse health effects including ocular and pulmonary irritation.[45,46] A primary precursor for trichloramine is urea.[47,48] While ACE and SUC are both commonly consumed and highly stable, the excretion pathway for ACE (i.e., 99% in urine) compared to SUC (i.e., 15% urine, 85% feces)[33] make it a more precise indicator of urine.

My study[31] aimed to asses ACE as a urinary indicator in recreational waters. The HPLC-MS/MS method I optimized for sensitive determination of ACE (i.e., 0.5 ng/L LOD) required no sample preconcentration or manual large volume injection. The high-throughput analysis of over 250 samples collected from 31 swimming pools and hot tubs from B.C. and Alberta detected ACE at 100% occurrence (30-7110 ng/L). These concentrations were up to 570-fold greater than ACE in the input tap water control samples. The following case study that monitored two pools for 3 weeks found the average ACE concentrations (i.e., 156 and 210 ng/L) had variation of less than 18% over the collection time. Using $C_1V_1=C_2V_2$ the approximate volume of urine required to account for average ACE was estimated to be 30 and 75 L. This publication was the first to report AS occurrence in recreational waters. My results demonstrate evidence of human waste contamination of recreational water and suggest that ACE could be used as an indicator of urine in recreational waters in similar ways as environmental water bodies.

6.2.3 Identification of Novel Precursors to Halobenzoquinone DBPs under Chlor(am)ination Conditions

Efforts continue to elucidate the link between adverse health effects (i.e., increased bladder cancer risk) and consumption of chlorinated drinking water observed consistently by epidemiologic studies.[10,49] Risk predictions using existing occurrence, toxicity, exposure and data for regulated DBPs (i.e., THMs and HAAs) could not be reconciled with the magnitude of bladder cancer specific risks estimated in epidemiological reports.[14] Therefore, focus has shifted to investigate unknown and unregulated DBP classes of potential significance in search of compounds sufficiently toxic to potentially explain the epidemiological estimates.[22]

HBQs are more cytotoxic than regulated DBPs.[20,50,51] Both *in vitro*[52] and *in vivo*[53,54] toxicity assessments have found HBQs induce oxidative stress and damage DNA. HBQs are an emerging class of DBPs with continual reports of frequent detection in disinfected drinking water across North America[50,55,56] and in Japan;[57] in Canadian[58] and Chinese[59] swimming pool water; and more recently tea prepared with chlorinated tap water.[28]

One strategy to reduce HBQ formation is to identify their precursors and design appropriate approaches to remove those precursors before reacting with common disinfectants (e.g., chlorine or chloramine). Many of the HBQ precursors identified in formation studies share a structural feature: activated aromatics and 6-membered ring structures.[28,60–62] I optimized the parameters to maximize the sensitivity of a previously described HBQ analysis method,[50,63] to investigate their formation from novel precursor candidates under both controlled laboratory and contextually relevant chlorination and chloramination conditions.

6.2.3.1 Peptide Artificial Sweetener, Aspartame in Chloraminated Water

Previous studies have identified the formation of DBPs during food and beverage preparation with disinfected water.[28,29,64] Aspartame is an AS commonly used to sweeten beverages.[65,66] No reports in the literature have evaluated DBP formation from aspartame in a beverage preparation context. It was unclear whether residual chloramine in tap water[67,68] could react with aspartame, during beverage preparation to form HBQs. Aspartame is readily available as a calorie-free tabletop alternative sweetener blend under commercially available brands like Equal® Original.[69] Here, the optimized HPLC-MS/MS analysis method[50,62] was used to determine that formed 2,6-dichloro-1,4-benzoquinone (DCBQ) concentration increased significantly from 8.0 to 11.2 ng/L just fifteen minutes after dissolving one Equal AS package into one cup of authentic chloraminated tap water. Under controlled reaction conditions mimicking tap water, DCBQ was formed at a molar yield of 0.0001% from aspartame after 24 hours. Interestingly, the presence of aspartame in Optima water solutions of DCBQ dramatically reduced the transformation of DCBQ to its relatively less cytotoxic transformation product OH-DCBQ.[50]

The fate of residual disinfectants during food and beverage preparation is most important at room temperature. Pre-treatment of tap water by boiling reduces residual

chloramine concentration,[70] and has been identified as a simple strategy to reduce volatile[71,72] and non-volatile DBPs.[73,74] Subsequently boiling can be considered to be a detoxification process for tap water as overall human exposure to DBPs is decreased.[73] In my investigation, DCBQ concentration was significantly reduced to 1.6 ng/L from to 8.0 ng/L after bringing the authentic chloraminated tap water to boiling and immediately removing from the heat source. Pre-boiling tap water decreased monochloramine, increased pH, and effectively eliminated DCBQ formation from aspartame or Equal. Overall, my work identified aspartame, as a new DCBQ precursor source in chloraminated tap water.

6.2.3.2 Aromatic Amino Acids; Phenylalanine, Tyrosine, and Tryptophan in Chlorinated Water with and without Bromide

Total amino acids (i.e., free and bound) are present in surface waters at wide ranging concentrations (i.e., 50-1000 µg/L) due to varying location and environmental conditions.[75,76] Various amino acids have been identified as precursors to DBPs[77–79] including odourous chloraldimines and chloroaldehydes.[80,81] AAAs are ideal HBQ precursor candidates because of their inherent ring structure. Under controlled chlorination conditions (i.e., 24 hours, pH 7) I found that PHE, TYR, and TRP all formed DCBQ with the greatest yields from TRP >> PHE > TYR. Natural and anthropogenic sources can alter bromide concentrations in environmental waters.[82] Source water containing high levels of bromide and iodide can produce higher proportions of the more toxic Br- and I-DBP analogs.[82–85] The halogenated species of HBQs formed during drinking water treatment depends on the raw water source characteristics. The presence of Br^- influences the formation of more toxic Br-HBQ congeners.[62] All AAAs formed 2,6-dibromo-1,4-benzoquinone (DBBQ) when they were prepared individually with free chlorine in the presence of Br^- at pH 8 for 24 hours. PHE and TYR both produced greater amounts of the more cyto- and genotoxic HBQ congener, DBBQ,[86] compared to DCBQ. Whereas TRP was found to yield DCBQ >> TriCBQ > DBBQ.

6.3 Conclusions

The application of ASs as wastewater indicators was critically assessed against guidelines for ideal tracer compounds using up-to-date literature.[31] Despite temporal and geographical variation of AS in waste, evidence of microbial degradation during wastewater treatment processes, or losses due to environmental degradation or attenuation processes, ACE and SUC were still concluded to be useful as indicators of waste impact in ground and surface waters. Additionally, CYC was highlighted as a specific indicator of untreated waste.[87] Relatively higher concentrations of ASs in waste compared to receiving water bodies in addition to continuous advancement of sensitive analytical methodologies are attributes that outweigh the challenges uncovered in recent literature.

In Canada, swimming is the third most practiced sport behind golf and hockey.[88] The formation of irritating DBPs in swimming pools can be reduced by improving swimmer hygiene practices. Many additional DBP precursors are introduced into the disinfected (e.g., chlorine and chloramine) recreational waters by swimmers who do not rinse off personal care products (i.e., shampoos, sunscreen and lotion, or dirt and sweat) in the provided showers and use the restroom before swimming.[58,89–92] The public perception of pool safety and the importance of recreational hygiene practices in key in improving swimmer compliance. The international attention generated from 'Sweetened Swimming Pools and Hot Tubs'[31] was used to disseminate a few key points for public education. In interviews I promoted awareness of proper hygiene habits.[93] Additionally, I communicated that the known benefits of swimming as regular exercise far outweigh the potential risks associated with chronic DBP exposure.

Toxicological studies continuously demonstrate the cyto- and genotoxic effects of *in vitro* and *in vivo* HBQ exposure, confirming their relevance among other important DBP classes.[94] Here, several HBQ-DBP precursors were identified. For the first time an AS, aspartame, was confirmed as a precursor DCBQ under chloramination conditions. The addition of one Equal AS package to authentic chloraminated tap water resulted in significantly greater concentration of DCBQ after 15 minutes. This warrants investigation into the formation and subsequent exposure potential of DCBQ and other DBPs via consumption of beverages prepared with aspartame or other alternative sweeteners in tap

water containing residual disinfectants. Pre-boiling tap water before beverage preparation was suggested as a simple strategy to reduce DCBQ exposure.

All AAAs were identified as viable HBQ precursors. Although environmental levels of free AAAs may be low, the potential to form HBQs from compounds containing AAA substructures may have broader implications. AAA-containing peptides and precursors are abundant in environmental waters as a humic fraction of natural organic matter.[75,95] Furthermore, AAAs are pervasive components of popular food additives like aspartame (i.e., PHE) and dietary supplements like protein powder,[96] both of which are often mixed with tap water containing residual disinfectants. The confirmation of AAAs as HBQ precursors under controlled laboratory conditions was a foundational step in understanding their significance as components of complex molecules in variable mixtures.

6.4 Future Research

In **Chapter 2**, the detection of SUC was limited by lower sensitivity compared to ACE. My research has shown that ACE is easily and sensitively detected in all the samples I collected, making it a better indicator of wastewater impact in B.C. and Alberta surface water. The simple LC-MS method I developed can be used for future studies in the Thompson Region to identify and characterize the waste sources contributing AS load into the receiving water bodies. Additionally, studies that quantitatively assess wastewater discharge into a water body using detected AS concentrations are limited in comparison to AS occurrence surveys. Comprehensive knowledge of the dynamic volume and flow rate of the water body being investigated requires future collaboration with hydrologists or environmental engineers. With careful design of experiments and controls, ASs can serve as effective tracers of wastewater impact in water bodies.

I have discussed limitations using the ACE concentration in the archived urine samples to estimate the urine volume in swimming pools in **Chapter 3.** To address this problem, we should survey a local population of swimmers for their urinary ACE levels to provide a more accurate characterization of the ‘waste source’. Conducting an investigation to precisely monitor the ACE levels in a recreational centre over time is limited by common factors. Water is lost to evaporation and splash out and fresh water is continuously added to

maintain operational levels therefore the effective volume of the swimming pool at the time of collection is highly uncertain. These inaccuracies can inflate errors in the estimate of urine volume from average ACE determined in recreational waters. Future studies could better understand these challenges by monitoring ACE over time, in hot tubs. Hot tubs are completely drained and refilled on a regular schedule.[97] Furthermore, urine input into hot tubs is diluted orders of magnitude less than swimming pools, resulting in more sensitive indication by ACE.

Many questions remain to understand daily human exposure to HBQs. The results of **Chapter 4** suggested two potentially confounding effects. The additional formation of DCBQ from residual chloramine and aspartame compared to tap water alone and enhanced stability of DCBQ (i.e., reduced transformation to OH-DCBQ) in the presence of aspartame. Future work could elucidate the impact of aspartame on DCBQ formation under chloramination conditions. It can be difficult to weigh the relevance of marginally increased DCBQ in the context of overall DBP exposure. The *in vitro* LC_{50} of DCBQ is orders of magnitude lower[20,52] than commonly regulated classes of Cl-DBPs such as THMs[98] or HAAs.[99] To define the forcing agents that generate toxicity in disinfected tap water, 'TIC-Tox' was proposed by Plewa *et al*. as a strategy to weigh DBP concentration with metrics of toxic potency.[19,22] With a more substantial data set from a variety of treated water samples, TIC-Tox could be used to estimate and compare the potential toxicological weight of increased DCBQ in authentic tap water before and after the addition of Equal. These results could contextualize whether increased HBQs from sweetened tap water play a role as a forcing agent of DBP mediated toxicity of disinfected drinking water.

Chapter 5 presented preliminary results that demonstrated the formation potential of HBQs from AAAs. To effectively monitor the minor formation of HBQs from AAAs, we should perform reactions with high molar concentrations of reactants. Several groundwater sources in Alberta are high in Br^- or I^- due to geomorphological deposits.[100,101] Future work should consider environmentally relevant concentrations of chlorine, and bromide, and expand the investigation to include iodide in the reaction mixtures and I-HBQs as potential formation products. Iodinated-DBPs include some of the most cyto- and genotoxic DBPs discovered to date[102] and HBQs follow the same trend.[86]

Advancements in analytical methodologies to quantify lower concentrations more accuracy and monitor changes in their occurrence with more precision will allow DBP researchers to continue to piece together the DBP exposome.[103] A multidisciplinary research effort is required to continue to assess novel DBPs for their toxicological impacts,[20] characterize DBP precursors and their sources, develop methods for comprehensive DBP detection,[104–106] propose strategies to minimize their formation, [21,107,108] and accurately survey their occurrence[109] to better understand total exposure to toxicologically relevant DBPs.[22]

Above all, while it is necessary to minimize the potential chemical risk associated with chronic exposure to DBPs, eliminating the acute risk of waterborne pathogens by disinfection treatment technologies remains an undisputed priority for drinking water quality.[1,110]

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"If I have seen further, it is by standing on the shoulders of Giants."

– Sir Isaac Newton (1642 – 1727)

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"Now is not the end.
It is not even the beginning of the end.
But it is, perhaps, the end of the beginning."
– Winston Churchill (1874-1965)