Influence of biofilm on disinfection byproducts formation and decay in a simulated water distribution system

Zhikang Wang

University of Toledo

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation
Wang, Zhikang, "Influence of biofilm on disinfection byproducts formation and decay in a simulated water distribution system" (2017). Theses and Dissertations. 2212.
http://utdr.utoledo.edu/theses-dissertations/2212

This Dissertation is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.
A Dissertation

Entitled

Influence of Biofilm on Disinfection Byproducts Formation and Decay in a Simulated Water Distribution System

by

Zhikang Wang

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Engineering

_________________________________________
Dr. Youngwoo Seo, Committee Chair

_________________________________________
Dr. Isabel C. Escobar, Committee Member

_________________________________________
Dr. Cyndee L. Gruden, Committee Member

_________________________________________
Dr. Leif Hanson, Committee Member

_________________________________________
Dr. Dong-Shik Kim, Committee Member

_________________________________________
Dr. Patricia R. Komuniecki, Dean

College of Graduate Studies

The University of Toledo

August 2013
An Abstract of

Influence of Biofilm on Disinfection Byproducts Formation and Decay in a Simulated Water Distribution System

by

Zhikang Wang

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Engineering

The University of Toledo

August 2013

Since biofilm has been implicated in the deterioration water quality and the increase of public health risks, various efforts have been made to minimize biofilm regrowth in drinking water distribution systems. Although traditional water treatment processes can greatly remove a large fraction of disinfection by-products (DBPs) precursors, a small portion of natural organic matter (NOM) may still enter water distribution systems. Untreated NOM can serve as nutrients for biofilm growth while also consuming maintained disinfection residuals, which can result in microbial contamination in drinking water. To suppress biofilm formation, water utilities maintain disinfectant residuals for the distribution system. However, upon disinfectant addition, toxic DBPs are inevitably produced. Biofilm and its secreted extracellular polymeric substances (EPS) produce toxic DBPs, due to the very similar chemical composition compared to traditional investigated DBP precursors.
This research investigated the role of biofilm on DBP formation and decay in simulated drinking water distribution systems with four objectives. The first objective was to investigate the influence of chemical composition and quantity of bacterial EPS on the biosorption of NOM in drinking water. Results indicated that both protein and polysaccharide based EPS adsorbed existing NOM. Biosorption capacity was mainly determined by divalent ion (Ca\(^{2+}\) and Mg\(^{2+}\)) concentrations. Mechanistically, the presence of a diffuse electrical double layer inhibited NOM biosorption by potential energy barriers, however, presence of divalent ions in the aquatic environment enhanced biosorption processes, permitting functional group interactions between EPS and NOM. In addition, hydrophobic interactions, EPS characteristics and quantity can also be used to explain biosorption results. Bridging between hydrophilic carboxyl groups on alginate EPS and NOM appeared to be the dominant form of biosorption, while hydrophobic interactions enhanced biosorption for protein-based EPS.

The second and third objectives of this study were to investigate the role of biofilm EPS on the formation of both carbonaceous DBPs (C-DBPs) and nitrogenous DBPs (N-DBPs). DBP yield (formation potential) tests of both bacterial culture and extracted EPS indicated that the chemical composition and quality of EPS played a critical role for DBP formation. In general, protein based EPS possessed higher DBP yields compared to polysaccharide based EPS, especially for N-DBPs. To further determine the relative contribution of each biomolecule in EPS to DBP formation and speciation, detailed chemical compositions of biomolecules in EPS (amino acids, polysaccharide monomers, and fatty acids) from both pure culture and mixed species biofilm isolated from a water utility were analyzed. DBP yield results from both extracted
EPS and EPS surrogates (amino acids and polysaccharide monomers) indicated that proteins in EPS have a greater impact on DBP formation, where amino acids containing unsaturated organic carbon or conjugated bonds in R-group produced higher amount of DBPs. However, DBP yields of polysaccharide monomers were lower than those of tested amino acids groups and the DBP yields were not significantly influenced by their chemical structures.

The last objective of this study was to understand the influence of biofilm on DBP formation and decay in a simulated water distribution system using lab scale annular reactors. For Cl₂ disinfection at 0.5 mg L⁻¹ Cl₂ residual concentration, no obvious DBP formation was observed. This was mainly due to the combination of low DBP formation, DBP volatilization, and biodegradation. However, when high Cl₂ residuals were maintained, the formations of both C-DBPs and N-DBPs increased dramatically beyond the DBP formation potential of the feed solution. This suggests higher Cl₂ residual not only reacted with humic acid (HA) in feed solution but also reacted with biofilm and produced extra DBPs, especially the high formation of N-DBPs (haloacetonitriles). For NH₂Cl disinfection, the DBP levels were much lower than those of Cl₂ disinfection and differences in DBP formation were not significant under different NH₂Cl residual concentrations.

Combined results suggested that biofilm can impact both C-DBP and N-DBP formation and decay in water distribution systems, where biomolecules in EPS affect DBP speciation.
This work is dedicated to all of my family members.

I appreciate that they have trusted, supported and encouraged me all this time. I am grateful for many comforts in their life they sacrificed for me. With eternal love and respect to them, the life principles they believe in have been an endless source of inspiration for me.
Acknowledgements

I would like to express sincere acknowledgements to following individuals for their valuable contributions in the completion of my dissertation:

I really appreciate my Ph.D advisor, Dr. Youngwoo Seo, for his great patience, guidance, and encouragement which turned out to this value-added scientific research.

I am also grateful to all of my committee members – Dr. Escobar, Dr. Gruden, Dr. Hanson, and Dr. Kim. They gave me so many helpful suggestions and feedback in order to finish my research dissertation.

Dr. Junsung Kim, who gave me instructions and guidance on disinfection by products (DBP) analysis, which allowed me quickly become acquainted with DBP measurement. Because of your patient guidances, I could move in the right direction.

Special thanks to Ms. Jinny Johnson, Dr. Onekyun Choi, Heng Shao, Christopher M. Hessler, and Kimberly M. Coburn. I really appreciate your generous help and suggestions during my Ph.D research. I also thank my lab colleagues Mau-yi Wu, Zongsu Wei, Xue Ding, Varun Raj Sendamangalam, Qiu Hong Jia, Emma Boff, Erin Nichols, Zheng Xue, Steven D. Cummins, Katie A. Burns, and Yahya Mohd Khan. I really enjoy the time I spent with you.

Many thanks to the Chemical and Environmental Engineering faculty for their valuable help and suggestions throughout my Ph.D study.
Table of Contents

Abstract ........................................................................................................................................ iii
Acknowledgements .................................................................................................................. vii
Table of Contents .................................................................................................................. viii
List of Tables ......................................................................................................................... xviii
List of Figures ......................................................................................................................... xx
List of Abbreviations ............................................................................................................. xxvi
List of Symbols ....................................................................................................................... xxx

1 Introduction .......................................................................................................................... 31

1.1 Disinfection By-products .............................................................................................. 31

1.2 Natural Organic Matter .............................................................................................. 33

1.3 Biofilm in Water Distribution Systems ....................................................................... 35

1.4 DBP Formation and Decay in the Water Distribution System .................................. 37

2 Literature Review ............................................................................................................. 41

2.1 Removal of Various DBP Precursors in Drinking Water ........................................... 41

2.1.1 NOM Removal ........................................................................................................ 41
2.1.2 Algal Organic Matter (AOM) Removal ........................................................... 43

2.2 Disinfectants and Disinfection Techniques ....................................................... 44

2.2.1 Chlorine ............................................................................................................ 44

2.2.2 Chloramines ..................................................................................................... 45

2.2.3 Chlorine Dioxide .............................................................................................. 46

2.2.4 Ozone ............................................................................................................... 47

2.2.5 Ultraviolet (UV) Disinfection .......................................................................... 47

2.2.6 Other Advanced Disinfection Processes ......................................................... 48

2.3 DBP Formation and Decay in Drinking Water Distribution System ............... 49

2.3.1 Type of DBPs in Water Distribution System .................................................... 49

2.3.1.1 Trihalomethanes (THMs) ........................................................................... 49

2.3.1.2 Haloacetic acids (HAAs) ............................................................................ 51

2.3.1.3 Haloketones (HKs) ..................................................................................... 53

2.3.1.4 Haloacetaldehyde (HAs) ............................................................................ 54

2.3.1.5 Haloacetonitriles (HANs) .......................................................................... 54

2.3.1.6 Halonitromethanes (HNMs) ...................................................................... 55
2.3.1.7 Haloacetamides (HAcAms) .......................................................... 56

2.3.1.8 Cyanogen Halides (CNX) .......................................................... 57

2.3.1.9 Nitrosamines ........................................................................... 57

2.3.1.10 Total Organic Halide (TOX) .................................................. 58

2.3.2 Influencing Factors on DBP Formation in Water Distribution System .... 59

2.3.2.1 Effect of Temperature on DBPs ............................................... 59

2.3.2.2 Effect of Contact Time (water age) on DBPs ............................ 60

2.3.2.3 Effect of pH on DBPs ............................................................. 62

2.3.2.4 Effect of Disinfectant Types and Concentration on DBPs .......... 63

2.3.2.5 Effect of Bromide Ion on DBPs ............................................... 65

2.3.2.6 Effect of Precursors on DBPs ................................................. 66

2.3.2.6.1 NOM .................................................................................. 66

2.3.2.6.2 Algal Organic Matter (AOM) .............................................. 68

2.3.2.6.3 Amino Acids ...................................................................... 69

2.3.2.6.4 Biofilm ............................................................................. 70

2.3.3 DBP Formation Pathway ............................................................ 71
2.3.3.1 C-DBP Formation Pathway ................................................................. 71

2.3.3.2 N-DBP formation Pathway ................................................................. 73

3 Objectives ........................................................................................................ 76

3.1 Objectives ..................................................................................................... 76

3.2 Organization of Dissertation ........................................................................ 78

4 Role of EPS on Biosorption of NOM .............................................................. 81

4.1 Abstract ........................................................................................................ 81

4.2 Introduction .................................................................................................. 82

4.3 Materials and Methods .................................................................................. 84

4.3.1 NOM Preparation ..................................................................................... 84

4.3.2 Culture Preparation and Cell Number Enumeration ............................... 85

4.3.3 Bacterial Culture and EPS Characteristics ............................................. 86

4.3.3.1 EPS Extraction and Analysis ................................................................. 86

4.3.3.2 FTIR Spectroscopy ............................................................................... 86

4.3.3.3 Hydrophobicity and Zeta-potential Measurement ............................... 87

4.3.4 Kinetic Tests ............................................................................................ 87
4.3.5   Isotherm Tests................................................................................................ 88

4.3.6 Model EPS Test ............................................................................................... 90

4.4 Results..................................................................................................................... 90

4.4.1 Culture Characteristics and EPS Analysis ....................................................... 90

4.4.1.1 EPS Analysis and Characterization ........................................................... 90

4.4.1.2 Zeta Potential and Hydrophobicity .......................................................... 93

4.4.2 Kinetic Test .................................................................................................... 94

4.4.3 Isotherm Tests .................................................................................................. 94

4.4.3.1 Four Strains Isotherm Tests ....................................................................... 94

4.4.3.2 Artificial EPS Isotherm Test ...................................................................... 98

4.4.3.3 Isotherm Model Fitting .............................................................................. 98

4.5 Discussion............................................................................................................. 101

4.6 Conclusions......................................................................................................... 104

5 Influence of Bacterial EPS on DBP formation ........................................................ 106

5.1 Abstract ................................................................................................................. 106

5.2 Introduction........................................................................................................... 107
5.3 Materials and Methods

5.3.1 Culture and Extracted EPS Preparation

5.3.2 DBP Formation and DBP Yield Tests

5.3.3 DBP Analytical Methods

5.4 Results and Discussion

5.4.1 Impact of Cl₂ Dose and pH on DBP Formation

5.4.2 Effect of Contact Time (Water Age) on DBP Formation from Biomass

5.4.3 DBP Yields of Bacterial Biomass

5.4.4 DBP Yields of Bacterial EPS

6 Relative Contribution of Biomolecules in Bacterial EPS to DBP Formation

6.1 Abstract

6.2 Introduction

6.3 Materials and Methods

6.3.1 Culture Preparation and EPS Extraction

6.3.2 Determination of the Biomolecular Compositions of EPS

6.3.3 EPS Surrogate Preparation
7.3.3 Biofilm Analysis ................................................................. 173
7.3.4 DBP Formation and DBP Yield Tests ................................. 175
7.3.5 DBP Analysis Methods ..................................................... 176
7.3.6 Fluorescent Excitation-Emission Matrix (EEM) Analysis ... 176
7.4 Results and Discussion ....................................................... 177
7.4.1 Biofilm Analysis Results ................................................... 177
7.4.2 DBP Formation Results from Reactor Effluents .................. 182
  7.4.2.1 DBP Formation upon Chlorination ...................... 182
  7.4.2.2 DBP Formation upon Chloramination ...................... 188
  7.4.2.3 Statistically Analysis of DBPs from Reactor Effluents ... 189
  7.4.2.4 DBP Speciation .......................................................... 192
7.4.3 DBP Yields of Biofilm and Biofilm Associated HA ............. 197
7.4.4 Fluorescence EEM Spectra Analysis Results ....................... 203
7.5 Conclusion .......................................................................... 211
8 Conclusion & Future Work Recommendation .......................... 212
  8.1 Conclusions ........................................................................ 212
8.2 Implication of This Research and Future Work Recommendation ....................... 215

8.2.1 Implication of This Research ................................................................. 215

8.2.2 Future Work Recommendations ......................................................... 216

References ............................................................................................................ 218

Appendix ............................................................................................................... 258

Appendix A .......................................................................................................... 259

A.1 THM/HAN/HK/TCNM/CH Analysis Methods ............................................. 259

A.1.1 Stock Solution Preparation ..................................................................... 259

A.1.2 Standard and Calibration Curve Preparation ......................................... 259

A.1.3 DBP analysis ............................................................................................ 260

A.1.4 Method Detection Limit (MDL) determination ....................................... 260

A.1.5 Spike Recovery Determination ............................................................... 261

A.2 HAA Analysis Methods .............................................................................. 261

A.2.1 Surrogate Stock Solution Preparation .................................................... 261

A.2.2 HAA Stock Solution Preparation ............................................................ 261

A.2.3 Standard and Calibration Curve Preparation ......................................... 262
**List of Tables**

4.1: Physio-chemical properties of tested strains.............................................................. 92

4.2: Freundlich and Langmuir Isotherm constants and correction coefficients ($r^2$) of tested strains and alginate beads (UVA$_{254}$ data only) ............................................................... 100

5.1: Results of one way ANOVA test for DBP yield among different bacterial cultures without bromide. ............................................................................................................. 127

5.2: Results of one-way ANOVA test of DBP yield among different bacterial cultures with bromide. .................................................................................................................. 127

5.3: t-test results of DBP yield between polysaccharide based EPS and protein based EPS without bromide. ............................................................................................................. 133

5.4: t-test results of DBP yield between polysaccharide based EPS and protein based EPS with bromide. .................................................................................................................. 133

5.5: t-test results of DBP yield (C-DBP and N-DBP) between polysaccharide based EPS and protein based EPS without bromide. ........................................................................ 134

5.6: t-test results of DBP yield (C-DBP and N-DBP) between polysaccharide based EPS and protein based EPS with bromide. .................................................................................................................. 134
6.1: Total protein and total polysaccharide contents in extracted EPS..............144

6.2: Amino acid compositions in extracted EPS.............................................. 153

6.3: Amino acid groups based on R-group properties ................................... 154

6.4: Polysaccharide monomer compositions in extracted EPS...................... 155

6.5: Polysaccharide monomer groups.............................................................. 156

6.6: DBP yields of grouped amino acids for extracted EPS............................ 159

6.7: DBP yields of polysaccharide monomers of for extracted EPS................. 160

7.1: Biofilm analysis results from three reactors a ....................................... 178

A.1: MDL of THM and HAN........................................................................... 275

A.2: MDL of HK, TCNM, and CH................................................................. 275

A.3: MDL of HAA......................................................................................... 276

C.1: chemical composition of SRFA, SRHA, and SRNOM (adapted from International Humic Substances Society’s website)........................................... 281

C.2: Contaminants in City of Toledo’s tap water (adapted from drinking water quality report from the City of Toledo in 2012)........................................ 283
List of Figures

1-1: Structures of representative C-DBPs and N-DBPs (a) chloroform, (b) TCAA, (c) DCAN, (d) 1,1-DCP, (e) TCNM, (f) NDMA. .................................................................. 32

1-2: Chemical structure of part of NOM (adapted from: http://www.acadiau.ca/~jmurimbo/Research%20Interests%202.htm) ......................... 35

1-3: Biofilm multi-cellular strategies for survival (adapted from biofilm engineering center-Montana State University) (1) waiting to grow ..................................................... 36

1-4: Biofilm growth in a simulated water distribution system. (Adapted from biofilm engineering center-Montana State University) ................................................................. 37

1-5: DBP Formation during Biofilm Disinfection. (Adapted from biofilm engineering center-Montana State University) ................................................................. 39

1-6: A Schematic Diagram of Current Research Gap ................................................................. 40

4-1: FTIR spectrum of P. aeruginosa strains and P. putida .................................................. 93

4-2: Biosorption of SRHA, SRFA and SRDOC on P. aeruginosa strains and P. putida with (Ca^{2+} =2 mM, Mg^{2+} = 1 mM) and without divalent ions at pH 7 ............................... 97
4-3: SRHA, SRFA and SRDOC biosorption on alginate beads with and without divalent ions at pH 7 (Ca$^{2+} = 2$ mM, Mg$^{2+} = 1$ mM).

5-1: The formation of chloroform and HAA$_3$ (MCAA, DCAA, TCAA) at pH=5.5 [(a) and (c)] and 7.5 [(b) and (d)].

5-2: The formation of HAN (DCAN) and HKs (1,1-DCP and 1,1,1-TCP) at pH=5.5 [(a), (c) and (e)] and 7.5 [(b), (d) and (f)].

5-3: DBP formation at different water age, Cl$_2$=5 mg L$^{-1}$ (2 hr, 4 hr, 8 hr, 24 hr, 48 hr, 96 hr) (a) THM, (b) HAA, (c) HAN (d) HK.

5-4: Detailed speciation of HAN, HK and HAA at different water age with incubation of 5 mg L$^{-1}$ Cl$_2$.

5-5: DBP yield of four tested strains. (a) DBP yield without bromide (b) DBP yield in the presence of bromide (200 $\mu$g L$^{-1}$).

5-6: DBP yield upon chlorination of bacterial cells (detailed species) (a) regulated DBPs (THMs and HAAs) without bromide.

5-7: DBP yield of extracted EPS under 20 mg L$^{-1}$ Cl$_2$. (a) DBP yield without bromide (b) DBP yield in the presence of bromide (200 $\mu$g L$^{-1}$).

5-8: Detail speciation for DBP yield by extracted EPS. (a) DBP yield without bromide (b) DBP yield in the presence of bromide (200 $\mu$g L$^{-1}$).
6-1: C-DBP and N-DBP yields of EPS and protein and polysaccharide surrogates. (a) DBP yields of extracted EPS........................................................................................................................................151

6-2: A proposed formation mechanism of DCAA and TCAA. (a) Possible formation pathway of DCAA/TCAA from β-dicarbonyl acid structure .............................................. 163

6-3: Proposed formation mechanism of DCAA and TCAA from Asp by Hong et al. [168]........................................................................................................................................................................165

7-1: The experiment setup (“P” indicates peristaltic pump).................................................. 172

7-2: CLSM images of biofilm in R1 (control reactor). (a) CLSM image at 25\textsuperscript{th} day; (b) CLSM image at 50\textsuperscript{th} day; (c) CLSM image at 75\textsuperscript{th} day ........................................................................................................... 179

7-3: CLSM images of biofilm in R2 (Cl\textsubscript{2} applied reactor). (a) CLSM image at 25\textsuperscript{th} day; (b) CLSM image at 50\textsuperscript{th} day; (c) CLSM image at 75\textsuperscript{th} day.............................................................. 180

7-4: CLSM images of biofilm in R3 (NH\textsubscript{2}Cl applied reactor). (a) CLSM image at 25\textsuperscript{th} day; (b) CLSM image at 50\textsuperscript{th} day ........................................................................................................................................ 181

7-5: Bulk phase DBP formation (a) Cl\textsubscript{2} as disinfectant; (b) NH\textsubscript{2}Cl as disinfectant. Three concentrations of disinfectant residual........................................................................................................ 184

7-6: DBP formation upon chlorination of HA. (a) at 0.5 mg L\textsuperscript{-1} Cl\textsubscript{2} dose; (b) at 2.0 mg L\textsuperscript{-1} Cl\textsubscript{2} dose; (c) at 4.0 mg L\textsuperscript{-1} Cl\textsubscript{2} dose................................................................................................. 186

7-7: The formation of THM\textsubscript{4}, HAA\textsubscript{5}, HAN\textsubscript{4}, HK\textsubscript{2}, CH, and TCNM during operation of annular reactors (100 days). (a) Cl\textsubscript{2} as disinfectant .......................................................................................... 191
7-8: Detailed THM₄ speciation from reactor effluents (a) Cl₂ as disinfectant; (b) NH₂Cl as disinfectant ................................................................................................................................. 193

7-9: Detailed HAA₉ speciation from reactor effluents (a) Cl₂ as disinfectant; (b) NH₂Cl as disinfectant ................................................................................................................................. 194

7-10: Detailed HAN₄ and TCNM speciation from reactor effluents during chlorination ....................................................................................................................................... 195

7-11: Detailed HK₂ and CH speciation from reactor effluents (a) Cl₂ as disinfectant; (b) NH₂Cl as disinfectant ....................................................................................................................................... 196

7-12: DBP yields of biofilm and biofilm associated HA in R1 (control); (a) regulated DBP yields upon chlorination (Cl₂) ................................................................................................................ 200

7-13: DBP yields of biofilm and biofilm associated HA in R2 (Cl₂ disinfected); (a) regulated DBP yields upon chlorination (Cl₂) ................................................................................................................ 201

7-14: DBP yields of biofilm and biofilm associated HA in R3 (NH₂Cl disinfected); (a) regulated DBP yields upon chlorination (Cl₂) ................................................................................................................ 202

7-15: The fluorescent EEM spectra of treated HA, isolated EPS, and regrown EPS.......... 204

7-16: The fluorescent EEM spectra of R1 (control) during the entire operation. (a) 2ₚ week; (b) 4ₚ week; (c) 6ₚ week ................................................................................................................................. 206

7-17: The fluorescent EEM spectra of R2 (Cl₂ as disinfectant) during the entire operation. ....................................................................................................................................... 208
7-18: The fluorescent EEM spectra of R3 (NH2Cl as disinfectant) during the entire operation.

A-1: Calibration curve of chloroform (CF).

A-2: Calibration curve of dichlorobromomethane (DCBM).

A-3: Calibration curve of dibromochloromethane (DBCM).

A-4: Calibration curve of bromoform.

A-5: Calibration curve of dichloroacetonitrile (DCAN).

A-6: Calibration curve of trichloroacetonitrile (TCAN).

A-7: Calibration curve of bromochloroacetonitrile (BCAN).

A-8: Calibration curve of dibromoacetonitrile (DBAN).

A-9: Calibration curve of 1,1-dichloropropane (1,1-DCP).

A-10: Calibration curve of 1,1,1-trichloropropane (1,1,1-TCP).

A-11: Calibration curve of trichloronitromethane (TCNM).

A-12: Calibration curve of chloral hydrate (CH).

A-13: Calibration curve of monochloroacetic acid (MCAA).

A-14: Calibration curve of monobromoacetic acid (MBAA).
A-15: Calibration curve of dichloroacetic acid (DCAA) ........................................... 271

A-16: Calibration curve of trichloroacetic acid (TCAA) ....................................... 271

A-17: Calibration curve of bromochloroacetic acid (BCAA) ................................. 272

A-18: Calibration curve of bromodichloroacetic acid (BDCAA) ......................... 272

A-19: Calibration curve of dibromoacetic acid (DBAA) ....................................... 273

A-20: Calibration curve of chlorodibromoacetic acid (CDBAA) ......................... 273

A-21: Calibration curve of tribromoacetic acid (TBAA) ....................................... 274

B-1: The calibration curve of total protein measurement ...................................... 279

B-2: The calibration curve of total polysaccharide measurement ......................... 280
List of Abbreviations

AOC ····················································································· Assimilable Organic Carbon
AOM ······················································································ Algal Organic Matter
AOP ····················································································· Advanced Oxidation Processes
ALA ····················································································· Alanine
ARG ····················································································· Arginine
ASN ····················································································· Asparagine
ASP ····················································································· Aspartic Acid
ASX ····················································································· Asparagine/Aspartic Acid
AWWA ····················································································· American Water Works Association

BCAA ····················································································· Bromochloroacetic Acid
BCAN ····················································································· Bromochloroacetonitrile
BDCAA ····················································································· Bromodichloroacetic Acid
BDCM ····················································································· Bromodichloromethane
BDOC ····················································································· Biodegradable Dissolved Organic Carbon
BSA ····················································································· Bovine Serum Albumin

CDBAA ····················································································· Chlorodibromoacetic Acid
C-DBPs ····················································································· Carbonaceous DBPs
CF ····················································································· Chloroform
CFU ····················································································· Colony Forming Unit
CH ····················································································· Chloral Hydrate
CLSM ····················································································· Confocal Laser Scanning Microscopy
CNCl ····················································································· Cyanogen Chloride
CNX ····················································································· Cyanogen Halide
CYS ····················································································· Cysteine

DAcAms ····················································································· Dichloroacetamide
DAPI ····················································································· 6-Diamidino-2-Phenylindole
DBAA ····················································································· Dibromoacetic Acid
DBAN ····················································································· Dibromoacetonitrile
DBCM ····················································································· Dibromochloromethane
DBPs ····················································································· Disinfection By-products
D/DBPR ····················································································· Disinfectants/Disinfection By-products rule
DCA ····················································································· Dihaloacetaldehydes
DCAA ····················································································· Dichloroacetic Acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCAN</td>
<td>Dichloroacetonitrile</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved Organic Matter</td>
</tr>
<tr>
<td>DXP</td>
<td>Dihalopropanones</td>
</tr>
<tr>
<td>EDTA</td>
<td>Tetrasodium Salt of Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EEM</td>
<td>Excitation Emission Matrices</td>
</tr>
<tr>
<td>EFM</td>
<td>Epifluorescence Microscopy</td>
</tr>
<tr>
<td>EOM</td>
<td>Extracellular Organic Matter</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular Polymeric Substances</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>GLN</td>
<td>Glutamine</td>
</tr>
<tr>
<td>GLU</td>
<td>Glutamic Acid</td>
</tr>
<tr>
<td>GLX</td>
<td>Glutamine/Glutamic Acid</td>
</tr>
<tr>
<td>GLY</td>
<td>Glycine</td>
</tr>
<tr>
<td>HA</td>
<td>Humic Acid</td>
</tr>
<tr>
<td>HAAs</td>
<td>Haloacetic Acids</td>
</tr>
<tr>
<td>HAA₅</td>
<td>the Sum of Five Haloacetic Acids</td>
</tr>
<tr>
<td>HAA₉</td>
<td>the Sum of Nine Haloacetic Acids</td>
</tr>
<tr>
<td>HAcAms</td>
<td>Haloacetamides</td>
</tr>
<tr>
<td>HANs</td>
<td>Haloacetonitriles</td>
</tr>
<tr>
<td>HAN₄</td>
<td>the Sum of Four Haloacetonitriles</td>
</tr>
<tr>
<td>HIS</td>
<td>Histidine</td>
</tr>
<tr>
<td>HKs</td>
<td>Haloketones</td>
</tr>
<tr>
<td>HK₂</td>
<td>the Sum of Two Haloketones</td>
</tr>
<tr>
<td>HPC</td>
<td>Heterotrophic Plate Counting</td>
</tr>
<tr>
<td>HMW</td>
<td>High Molecular Weight</td>
</tr>
<tr>
<td>HNMs</td>
<td>Halonitromethanes</td>
</tr>
<tr>
<td>HPSEC</td>
<td>High-pressure Size Exclusion Chromatography</td>
</tr>
<tr>
<td>HS</td>
<td>Humic Substances</td>
</tr>
<tr>
<td>ICR</td>
<td>Information Collection Rule</td>
</tr>
<tr>
<td>IOM</td>
<td>Intracellular Organic Matter</td>
</tr>
<tr>
<td>ILE</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>IS</td>
<td>Internal Standard</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LEU</td>
<td>Leucine</td>
</tr>
<tr>
<td>LMW</td>
<td>Low Molecular Weight</td>
</tr>
<tr>
<td>LYS</td>
<td>Lysine</td>
</tr>
<tr>
<td>MBAA</td>
<td>Monobromoacetic Acid</td>
</tr>
<tr>
<td>MCAA</td>
<td>Monochloroacetic Acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>MCL</td>
<td>Maximum Contamination Level</td>
</tr>
<tr>
<td>MDL</td>
<td>Minimum Detection Limit</td>
</tr>
<tr>
<td>MET</td>
<td>Methionine</td>
</tr>
<tr>
<td>MMW</td>
<td>Middle Molecular Weight</td>
</tr>
<tr>
<td>MTBE</td>
<td>Methyl tert-butyl Ether</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cutoff</td>
</tr>
<tr>
<td>N-DBPs</td>
<td>Nitrogenous DBPs</td>
</tr>
<tr>
<td>NDMA</td>
<td>N-nitrosodimethylamine</td>
</tr>
<tr>
<td>NOM</td>
<td>Natural Organic Matter</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OD$_{600}$</td>
<td>Optical Density Absorbance at 600 nm</td>
</tr>
<tr>
<td>Org-N</td>
<td>Organic Nitrogen</td>
</tr>
<tr>
<td>PHE</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>PRO</td>
<td>Proline</td>
</tr>
<tr>
<td>QA/QC</td>
<td>Quality Assurance and Quality Control</td>
</tr>
<tr>
<td>SER</td>
<td>Serine</td>
</tr>
<tr>
<td>SMP</td>
<td>Soluble Microbial Products</td>
</tr>
<tr>
<td>SUVA</td>
<td>Specific Ultraviolet Absorbance</td>
</tr>
<tr>
<td>SRDOC</td>
<td>Suwannee River Dissolved Organic Carbon</td>
</tr>
<tr>
<td>SRFA</td>
<td>Suwannee River Fulvic Acid</td>
</tr>
<tr>
<td>SRHA</td>
<td>Suwannee River Humic Acid</td>
</tr>
<tr>
<td>SRNOM</td>
<td>Suwannee River Natural Organic Matter</td>
</tr>
<tr>
<td>SS</td>
<td>Sample Size</td>
</tr>
<tr>
<td>TBAA</td>
<td>Tribromoacetic Acid</td>
</tr>
<tr>
<td>TCAA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TCAN</td>
<td>Bromochloroacetonitrile</td>
</tr>
<tr>
<td>TCNM</td>
<td>Trihalonitromethane</td>
</tr>
<tr>
<td>TCP</td>
<td>Trichloropropanones</td>
</tr>
<tr>
<td>THMs</td>
<td>Trihalomethanes</td>
</tr>
<tr>
<td>THR</td>
<td>Threonine</td>
</tr>
<tr>
<td>TYR</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>TOC</td>
<td>Total Organic Carbon</td>
</tr>
<tr>
<td>TOX</td>
<td>Total Organic Halide</td>
</tr>
<tr>
<td>TOCl</td>
<td>Total Organic Chloride</td>
</tr>
<tr>
<td>TOBr</td>
<td>Total Organic Bromide</td>
</tr>
<tr>
<td>TOI</td>
<td>Total Organic Iodide</td>
</tr>
<tr>
<td>TRP</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>TXP</td>
<td>Trihalopropanones</td>
</tr>
<tr>
<td>US EPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
</tbody>
</table>
UV$_{254}$ \hspace{1cm} \textit{Ultraviolet Absorbance at 254 nm}

UV/H$_2$O$_2$ \hspace{1cm} \textit{Ultraviolet/Hydrogen Peroxide}

VAL \hspace{1cm} \textit{Valine}

XAA \hspace{1cm} \textit{One Halogen Acetic Acid}

X$_2$AA \hspace{1cm} \textit{Two Halogen Acetic Acid}

X$_3$AA \hspace{1cm} \textit{Three Halogen Acetic Acid}

1,1-DCP \hspace{1cm} \textit{1,1-dichloro-2-propanone}

1,1,1-TCP \hspace{1cm} \textit{1,1,1-trichloropropanone}
List of Symbols

- Da: \( \text{Dalton} \)
- Ce: the aqueous phase concentration of absorbate at equilibrium
- \( C_0 \): the initial absorbate concentration
- \( q_e \): equilibrium adsorbent-phase concentration of absorbate
- V: volume of aqueous-phase absorbate solution
- M: the quantity of adsorbent (cell numbers)
- \( K_f \): the Freundlich constant indicative of adsorptive capacity
- \( n_f \): the Freundlich constant related to adsorption intensity
- \( K_L \): the Langmuir isotherm constant related to the equilibrium constant or binding energy
- \( S_m \): the amount of sorption corresponding to complete surface coverage
Chapter 1

Introduction

This chapter includes the brief introduction of disinfection by-products, natural organic matter, biofilm, and their interactions in drinking water distribution systems.

1.1 Disinfection By-products

Disinfection by-products (DBPs) were first discovered in drinking water utilities in the early 1970s by Rook et al. [1, 2]. Since then, extensive investigations have been and are still being conducted on the formation of DBPs in drinking water systems [3-7]. Over 600 indentified DBPs have been reported in drinking water or simulated laboratory disinfection tests using chlorine and other disinfectants [8]. Humans and animals may be directly exposed to DBPs by ingestion, inhalation, or through skin contact [9]. Exposure to DBPs may cause cancer and birth defects because of the genotoxicities and cytotoxicities of DBPs [10, 11].

For most DBP formation studies, specific emphasis has been placed on the formation of trihalomethanes (THMs) and haloacetic acids (HAAs), the two most prevalent classes of DBPs resulting from chlorination in drinking water treatment [6, 12-
Due to major health risks, multiple regulations have been put in place to control DBP formation in drinking water. The disinfection/disinfection by-products rules (D/DBPR) from the US Environmental Protection Agency (EPA) regulated that the total concentration of the four THMs \([\text{THM}_4, \text{chloroform}, \text{bromodichloromethane (BDCM)}, \text{dibromochloromethane (DBCM)}\text{ and bromoform})\] should be lower than 80 μg L\(^{-1}\) and that the total concentration the of five main HAAs \([\text{HAA}_5, \text{monochloroacetic acid (MCAA)}, \text{dichloroacetic acid (DCAA)}, \text{trichloroacetic acid (TCAA)}, \text{monobromoacetic acid (MBAA)}\text{ and dibromoacetic acid (DBAA)})\] should be lower than 60 μg L\(^{-1}\) \[\text{[16]}\].

The presence of nitrogenous DBPs (N-DBPs), which include haloacetonitriles (HANs), haloacetamides (HAcAms), halonitromethanes (HNMs), cyanogen chloride (CNX) and N-nitrosodimethylamine (NDMA), have also been continuously detected in drinking water (Fig. 1-1) \[\text{[17, 18]}\].

**Figure 1-1:** Structures of representative C-DBPs and N-DBPs (a) chloroform, (b) TCAA, (c) DCAN, (d) 1,1-DCP, (e) TCNM, (f) NDMA.
In recent years, interest in the formation of N-DBPs has increased because toxicological research has indicated more genotoxicity, cytotoxicity, and carcinogenicity of those N-DBPs than many of the previously investigated carbonaceous DBPs (C-DBPs) [19]. The levels of emerging DBPs in drinking water are not yet regulated by the US EPA. The switch from chlorination to chloramination disinfection is an option for water utilities to reduce the formation of regulated DBPs such as THMs and HAAs. However, it has been shown that chloramination can also increase certain N-DBPs formation [20]. Due to the presence of DBPs in drinking water systems, water utilities are required to identify locations with high DBP concentration in the distribution system [21].

1.2 Natural Organic Matter

NOM, primarily comprised of humic substances (HS), is a complex mixture of organic compounds present in all fresh water, particularly in surface waters [22-24]. NOM is composed of a range of different organic compounds, from largely aliphatic to highly colored aromatics [25]. Some of this organic matter is negatively charged, consisting of a wide variety of chemical compositions and molecular sizes [26]. NOM present in water is comprised of both hydrophobic NOM, high molecular weight (HMW) organic matter rich in aromatic carbon and nitrogenous compounds (e.g. carbohydrates, proteins, sugars and amino acids) [24], and hydrophilic NOM, which consists mainly LMW (low molecular weight) organic matter with hydrophilic functional groups [27].
To control NOM in drinking water, a number of approaches focus on NOM removal technologies [22, 25, 28, 29]. Among various available technologies, the most common and economically feasible approaches applied in most drinking water treatment plants are coagulation and flocculation followed by sedimentation, flotation, and filtration [30, 31]. While most of the NOM can be removed by the coagulation method, the hydrophilic low molecular weight (LMW) fractions of NOM are apparently removed less efficiently than the hydrophobic high molecular weight (HMW) compounds [22]. However, treatment of the NOM is limited by its complicated structure, variable solubility, and a broad range of molecular weights [22, 32]. Thus, water utilities have difficulties in removing all fractions of NOM through conventional water treatment processes [22]. One well-established vector for DBP formation in distribution systems is the presence of NOM. Due to the limitations of the treatment capability of conventional water treatment plants, untreated NOM enters the water distribution system and reacts with disinfectants generating DBPs in water distribution systems [6, 22].
1.3 Biofilm in Water Distribution Systems

Biofilm is a collection of organic and inorganic, living and dead material collected on a surface [33]. Formation of microbial biofilms on pipe walls have serious implications in water distribution systems, which can be responsible for loss of distribution system disinfectant residuals, increased bacterial levels, reduction of dissolved oxygen, taste and odor changes, red or black water problems due to iron or sulfate-reducing bacteria, microbially influenced corrosion, hydraulic roughness, and reduced materials life [34, 35].

To remove biofilm in water distribution systems, various disinfection approaches have been applied [36-39]. However, maintaining disinfection residual alone in drinking water does not result in the inactivation of all microorganisms; the regrowth of bacteria and other microorganisms in distribution system has been continuously reported (Fig. 1-
3) [40]. It is well accepted that biofilm extracellular polymeric substances (EPS) play a significant role in the reduced efficacy of disinfectants by providing a protective barrier to EPS embedded bacteria [41, 42].

Figure 1-3: Biofilm multi-cellular strategies for survival (adapted from biofilm engineering center-Montana State University) (1) waiting to grow, (2) initial attachment, (3) building communities, (4) meeting antimicrobial challenges, (5) gradients and niches, (6) nutrient transport, (7) detachment, (8)signaling, and (9) differentiation.

Within the distribution systems, untreated NOM has also been linked to enhanced biological instability, providing a nutrient source, and contributing to microbial regrowth even in the presence of residual disinfectants [43]. A key factor enhancing NOM utilization and retention by microorganisms or biofilm is the presence of EPS, which have been shown to provide binding sites for nutrients to permit the adsorption of metal ions and organic compounds [33, 44]. Extracted EPS from biofilm and activated sludge have been shown to contain high concentration of HS [45, 46], suggesting an interaction between EPS and NOM.
1.4 DBP Formation and Decay in the Water Distribution System

Water supply industries have commonly applied higher doses of disinfectants to maintain a high disinfectant residual concentration as operational responses to the biofilm outbreak [38]. Since water distribution systems are continuously fed with unremoved organic materials, high concentrations of organic DBP precursors may accumulate on biofilm surfaces as well as in its internal structures [6, 33], providing a reservoir of organic material for subsequent DBP formations. A key feature in this process is the EPS secreted by biofilm attached to pipe walls. Therefore, biofilm may contribute the highest concentration of DBP precursors and biofilm control practices may significantly enhance DBP formation in water distribution systems (Fig. 1-4 and 1-5).

![The Distribution System as Reactor](image)

**Figure 1-4**: Biofilm growth in a simulated water distribution system. (Adapted from biofilm engineering center-Montana State University)

Recent studies reported DBP formation by biomolecules from algae entering water treatment processes [47, 48]. Biomolecules derived from aquatic organisms may
pose a greater risk for DBP formation than traditionally NOM due to their higher contribution to toxic N-DBP formation [49]. Production of DBPs from algae suggests biofilm could produce similar DBPs because comparable biopolymers are presented in biofilm or planktonic cells. In microbial biofilms, more than 90% of the biomass is contributed by EPS, which consists of reactive organic composition and chemical structure as aquatic DBP precursors [50]. However, biofilm contributions to DBP formation in distribution systems have been largely overlooked, even though previous studies reported increased DBP formation in the distribution system assuming that organic deposits on pipe walls were the culprit [6]. Despite these findings, current research for preventing DBP formation and investigating DBP behavior in water distribution systems focuses on: (1) removing allochthonous DBP precursors as well as autochthonous precursors during water treatment processes [30, 51], (2) evaluating modulations in DBP formation and decay in water distribution systems as result of seasonal changes, pH, disinfectant type, etc. [6, 14], and (3) identifying the formation of emerging and unregulated DBPs. DBPs research and biofilm control research have been unfortunately separated into two areas of study, although significant interaction likely occurs between them.
While both the DBP formation and their potential health risks have undergone considerable scrutiny, the contribution of biofilm to DBP formation is still not clear. Chlorine is reported to have high reactivity with biofilm, but non-selective reactions limit its penetration [52]. Thus, transport limitation of Cl₂ prevents identification and quantification of DBP formation from specific biofilm components. Beyond limited chlorine transport, the structural heterogeneity of biofilm (voids, channels, cell distribution) does not permit differentiation of bacterial vs. EPS contributions to DBP formation upon exposure to chlorine. Certain DBPs, such as HAAs, were reported to be biodegradable by heterotrophic bacteria in distribution systems [53]. Their concentration may be spatially and temporally affected as a result of biofilm growth. However, very few studies have investigated the role of biofilm on NOM accumulation and simultaneous DBP formation under the presence of disinfectants. Biofilm disinfection and DBPs
formation in water distribution system have been treated as separate research topics, even though there are significant interactions between them. Specifically, while researchers are focusing on the disinfection of persistent biofilm, they are lacking robust and quantitative information about the role of biofilm on the formation of DBPs [14, 54]. Currently, there is a significant knowledge gap for water utilities to access and optimize disinfectant dosage to control biofilm growth while reducing DBPs in water distribution systems (Fig. 1-6).

Figure 1-6: A Schematic Diagram of Current Research Gap.
Chapter 2

Literature Review

In this chapter, previous studies related to the removal of DBP precursors, DBP species detected in drinking water distribution systems, and factors that affect DBP formation and decay were summarized.

2.1 Removal of Various DBP Precursors in Drinking Water

2.1.1 NOM Removal

The abundance of NOM in natural waters significantly affects drinking water purification [22, 25]. NOM has been widely regarded as an important DBP precursor, which can enhance the risk of drinking water [24, 55, 56]. In addition, the presence of NOM in drinking water deteriorates water quality and taste [28, 57]. Accordingly, NOM removal before entering water distribution systems is necessary.

From the previous studies, various NOM constituents were evaluated in terms of physical and chemical characteristics [10, 22, 24, 25, 27, 29]. NOM is usually divided into the following fractions: (1) colloidal and noncolloidal NOM, separated by a dialysis membrane with a molecular weight cutoff of 3500 Da and (2) hydrophobic, transphilic,
and hydrophilic NOM constituents, each portion divided by XAD-8/4 resins [55]. The characteristics of NOM are listed below:

- The major functional groups are carboxyl and hydroxyl groups. The hydrogen ions in those groups have easier ionizing potential, which result in NOM having electronegativity (deprotonation) property [58].
- NOM usually possesses the larger surface area and surface energy [59].
- The major chemical elements in NOM are C, H, O, N and minor S, P.
- Molecular weight of NOM is mostly from 100 Da to 1000 kDa [22].

Molecular weight is a significant factor in determining the removal of NOM. HMW organic matter was clearly easier to remove by coagulation and clarification than LMW organic matter [22, 60]. MW range can be usually determined by high pressure size exclusive chromatography (HPSEC) [22, 29].

In traditional water treatment processes, coagulation and flocculation are usually used to remove HMW organic matter. Aluminum doses and pH significantly affect the removal efficiency of the coagulation and flocculation processes [22]. Furthermore, in the continued purification practices, depending on the regeneration of activated carbon filters, activated carbon filtration, and sand filtration, the process was effective to remove NOM from HMW organic compounds to middle molecular weight (MMW) organic compounds, but most of the LMW compounds cannot be completely eliminated [27]. Due to the presence of LMW organic matter entering the water distribution system, the carbon source from LMW organic matter contributes to biofilm growth [57]. In addition to traditional treatment processes, the advanced oxidation processes, such as
ultraviolet/hydrogen peroxide (UV/H₂O₂) disinfection is currently applied in advanced drinking water applications for the removal of various organic pollutants [61]. It has been demonstrated that the UV/H₂O₂ oxidation process for NOM removal: (1) reduce the aromaticity portion in NOM; (2) shift from HMW to LMW organic carbon; (3) form readily biodegradable compounds; (4) and decrease the NOM hydrophobicity. Therefore, both traditional processes and advanced oxidation processes can greatly reduce HMW of NOM, but most of LMW organic matter stays in drinking water.

2.1.2 Algal Organic Matter (AOM) Removal

In addition to NOM, which have been known to cause DBP formation in drinking water, in recent years, due to the eutrophication, algal blooms in drinking water reservoirs or lakes can also cause increased DBP formation issues [47, 62-64]. One major problem of algae species contributing DBP formation in drinking water is the interaction between disinfectants and untreated algal organic matter (AOM) [30, 65, 66]. Water resources commonly contain high concentrations of AOM and these components were usually separated as algal extracellular organic matter (EOM) and intracellular organic matter (IOM) [62, 67]. Neither EOM nor IOM is readily removed by traditional water treatment processes [30, 63, 64]. For the chemical components of algal EOM and IOM, polysaccharides, proteins and nucleic acids/lipids are the major components, the content of those biomolecules varies based on algal species [47, 62]. Cellular organic matter from algae EOM and IOM was reported to have analogous DBP yields compared to traditionally investigated DBP precursors [47, 62-64, 68].
2.2 Disinfectants and Disinfection Techniques

2.2.1 Chlorine

Chlorine is widely applied as the common disinfectant for drinking water distribution systems and disinfection steps because of low cost, high disinfection efficiency, easy handling, and easy transport, etc. [69]. Disinfection efficiency of chlorine is higher than chloramines but lower than chlorine dioxide and ozone [70]. The U.S. Environmental Protection Agency (EPA) determined that 0.5 to 2 mg L\(^{-1}\) of chlorine residual is necessary to maintain biological stability because of microbial regrowth in water distribution systems [38, 71]. However, it has been reported that higher disinfectant doses are reached to control biofilm growth or microbial activity in actual practice. Nagy et al. [72] reported that maintenance of a residual of 3 to 5 mg of chlorine per liter was necessary to reduce bacterial biofilms by more than 99.9%.

In past decades, various studies have been conducted to evaluate the factors affecting chlorine disinfection. For instance, Tsai et al. [73] conducted the chlorine consumption experiments in drinking water. They found added chlorine was rapidly consumed by the components in water at the beginning, but the consumption rate dramatically decreased to 0.1% of the initial rate after about 50 minutes. Le Dantec et al. [74] investigated the chlorine resistance of various mycobacteria isolated from a water distribution system. Their results indicated that the better inactivation was observed at higher temperature and lower pH. For the pH, HOCl was proved to be more than 50-fold more effective than OCl\(^{-}\) as a disinfectant [75]. At pH =6.0, HOCl serves as the major component (~98%) of free chlorine, whereas at pH=10.0, OCl\(^{-}\) accounts for over 99%.
The equilibrium of the transition between HOCl and OCl\(^-\) is temperature dependent, with lower temperatures resulting in forming slightly higher proportions of HOCl. The limitation of using chlorine is the result of the fact that it could generate high levels of both regulated DBPs and emerging DBPs.

### 2.2.2 Chloramines

Chloramines have been used to control taste and odor in drinking water from the beginning of the twenty-first century [76]. Chloramines are amines which contain at least one chloride atom, which is directly bonded to nitrogen atoms. Inorganic chloramines are formed when dissolved chlorine and ammonia react. During this reaction three different inorganic chloramines are formed: monochloramine, dichloramine, and trichloramine [76].

Replacing free chlorine with chloramines in drinking water treatment plants could further improve DBP control and have good efficiency of controlling bacterial regrowth. The advantage of chloramines is the gradual production of a more stable residual than free chlorine and thus providing continuous protection against bacteria regrowth [76, 77]. LeChevallier et al. have demonstrated that chloramines were more effective for inactivation of attached biofilm in lab scale experiments [38]. Although chloramines are considered to be a more stable disinfectant than chlorine, depletion can occur in the distribution system through reactions involving both the corroded surface of pipes and natural organic matter in bulk water [11]. Moreover, poor operation of chloraminated systems may cause nitrification which also promotes the dissipation of chloramines [78]. The subsequent growth and release of bacteria from pipe surfaces in such situations may
not be controlled because chloramines are well-known to be a less effective disinfectant than chlorine.

Chloramines would not affect DBP precursor’s removal as they are not effective enough to be used for oxidizing or decomposing DBP precursors such as NOM. In addition, chloramines do not produce appreciable amounts of US EPA regulated DBPs (THMs and HAAs) compared with chlorine disinfection, but some of toxic N-DBP formation (cyanogen chloride and NDMA) were discovered at higher concentration during chloramine disinfection practices. Although, most N-DBPs were not regulated by US EPA and with very low concentration in drinking water at the level from several ng/L to μg/L, the relatively higher formation of some very toxic N-DBPs like NDMA limited the application of chloramines. In addition, chloramines are a poor primary disinfectant and oxidant and not effective for taste and odor control [79].

2.2.3 Chlorine Dioxide

In recent years, a promising and powerful disinfectant—chlorine dioxide, has been used in drinking water disinfection. During disinfection practices, chlorine dioxide does not provide high levels of THMs and HAAs compared to traditional chlorine disinfection [69, 80]. The advantages of chlorine dioxide include: (1) effective control of odor and taste, (2) high disinfection efficiency, (3) effective over a wide pH range and five times more soluble in water than chlorine, and (4) generation of a much lower TOX than chlorination [20]. In addition, chlorine dioxide has been listed by the US EPA in a subsequent amendment as a suitable alternative treatment method. However, the
inorganic DBP, chlorite, was discovered in drinking water disinfected by chlorine dioxide. Typically, about 70% of the applied chlorine dioxide reduced to chlorite [81].

2.2.4 Ozone

Ozone is a potent disinfectant used in drinking water mainly because of high disinfection efficiency and very low DBP formation [82]. However, due to the instability of ozone molecules, ozone cannot produce a persistent disinfectant residual in distribution systems [83]. Ozone, similar to chlorine dioxide, is also a powerful disinfectant and oxidant and has the similar advantages compared to chlorine dioxide. In addition, because of the strong oxidation properties of ozone, oxidation of NOM could produce categories of oxidation by-products similar to chlorine dioxide, which consist of LWM and hydrophilic organic matter. Those NOM are difficult to remove in traditional water treatment processes. Moreover, those LMW organic by-products mainly consist of assimilable organic matter (AOC) and biodegradable organic matter (BDOC), which could easily be utilized by microorganisms and lead to enhanced biological instability in the water distribution system [43].

2.2.5 Ultraviolet (UV) Disinfection

UV water treatment offers many advantages over other forms of water treatment for microbiological contaminants. Most importantly, it does not introduce any chemicals to the water, and it does not alter the taste, pH, or other properties of the water [84]. Commonly, UV radiation can be used as a pretreatment or polishing step to sterilize and disinfect water. A majority of studies indicated that UV could kill microorganism
efficiently in the guarantees of UV intensity and exposure time [85]. In addition, UV is simple to install and requires little maintenance and space. For DBP formation control, utilizing UV does not produce any DBPs. However, using UV for drinking water disinfection has limitation because UV cannot control the microbial regrowth in drinking water distribution systems.

### 2.2.6 Other Advanced Disinfection Processes

Peracetic acid is used mainly in the food industry, where it is applied as a cleanser and as a disinfectant and can be applied for the deactivation of a large variety of pathogenic microorganisms. It also deactivates viruses and spores. Recently, peracetic acid has been utilized in drinking water disinfection to eradicate biofilm. Peracetic acid can be applied for the deactivation of a large variety of pathogenic microorganisms, viruses, and spores [86]. Similar to ozone and chlorine dioxide, the high oxidation properties make peracetic disinfection very efficient. In addition, peracetic acid does not produce any halogen DBPs or total organic halide (TOX).

Peracetic acid activity is hardly influenced by organic compounds that are present in the water. However, pH and temperature do influence peracetic acid activity. Peracetic acid is more effective when the pH value is 7 than at a pH range between 8 and 9. At a temperature of 15 °C and a pH value of 7, five times more peracetic acid is required to effectively deactivate pathogens than at a pH value of 7 and a temperature of 35 °C [87]. Besides, its instability limits its usage in the drinking water industry [88].
2.3 DBP Formation and Decay in Drinking Water Distribution System

2.3.1 Type of DBPs in Water Distribution System

In recent years, new DBPs have been continuously identified in drinking water distribution systems alongside traditional investigated DBPs [8, 21, 89, 90]. In addition to carbonaceous emerging DBPs, such as haloketones (HKs) and haloacetaldehyde (HAs), more N-DBPs have been discovered in drinking water utilities, such as HANs, HNMs, CNX, haloacetamides (HAcAms), and nitrosamines, etc. [8, 90-92]. The concentrations of those DBPs were much lower than regulated DBPs in drinking water distribution systems. Although the number of known DBPs is always increasing, there are still many unknown DBPs in drinking water distribution system.

2.3.1.1 Trihalomethanes (THMs)

Trichloromethane, or chloroform, was the first DBP identified in drinking water and was also linked to reactions between chlorine and natural organic matter (NOM) [93]. In addition to chloroform, the chloride atoms on molecules are easily substituted by bromide and iodide atoms due to the presence of bromide and iodide ions in drinking water [90]. Since that discovery, the THM levels in the drinking water distribution system were regulated at 80 μg L$^{-1}$ in the USA because of the cancer risk. In drinking water, chloroform (CF) is the major THM species detected, usually accounting for approximately 80% of total THMs, followed by brominated THMs, iodinated THMs and CF, respectively [8]. The speciation of THMs was determined by the concentrations of halogen (bromide and iodide) ions in drinking water.
Several studies indicated that higher THM concentrations were measured in pilot scale distribution systems as compared to actual treatment plants, which was mainly attributed to the presence of higher organic material content [2, 54, 94, 95]. Rossman et al. [6] conducted the DBP formation experiments in a lab scaled drinking water distribution system, which aimed to compare the production rate of THMs in a simulated pilot environment compared to batch THM formation experiments upon chlorination. Their results showed that the rate of chlorine consumption in the simulated distribution system was much greater than those in bottles (batch experiment). THMs levels were elevated by an average of 15% compared to batch experimental results. Separate tests confirmed that this increase was most likely due to a reservoir of organic precursor material associated with deposits on the surface of pipe wall.

Generally, increased THM levels were reported in actual water distribution systems [8, 96-98]. For a case study in Newport (NJ), the THM4 levels ranged from 70 to 145 μg L⁻¹ (average=90 μg L⁻¹) when chlorine was used as the disinfectant. After the period disinfection by chlorine, chloramine was used as the secondary disinfectant in the distribution system. Under chloramination, THM formation decreased to the range of 45 to 108 μg L⁻¹ (average=90 μg L⁻¹). The relatively high levels of THMs under chloramine disinfection might be attributed to the long retention time in the water distribution system after immediately switching the disinfectants from Cl₂ to NH₂Cl [98]. Singer et al. [99] studied the THMs behavior in a variety of waters from different geographically regions and distribution system characteristics; the study found around 56-89 μg L⁻¹ of THM₄ were formed in the midpoint location in drinking water distribution system. However, almost double levels (67-159 μg L⁻¹ of THM₄) were detected in the remote point of
drinking water distribution system. Booth et al. [96] investigated the THM formation in distribution systems with water of relatively high bromide concentration. In addition to any treatment methods to remove bromide, the THM₄ levels reached approximately 200 μg L⁻¹. However, after applying various pretreatment methods to eliminate bromide, THM₄ levels decreased to the range of 55 to 125 μg L⁻¹. Vikesland et al. [100] studied the THM₄ behavior in actual water distribution systems under both chlorine and chloramine disinfection where THM₄ levels were in the range of 37 to 160 μg L⁻¹. Overall, when the disinfection method switched from chloramine to chlorine, significant THM₄ formation was observed; in contrast, when chloramine was applied, THM₄ levels dropped relatively slowly. Johnson et al. [101] investigated the impact of treatment methods on THM₄ formation and reformation in drinking water distribution systems. They found both air stripping and granular activated carbon (GAC) treatments methods could remove 75-80% of THMs precursors. In addition, the reformation of THM₄ was around 50 μg L⁻¹ at the far point of water distribution system due to the re-chlorination to maintain the Cl₂ residual based on EPA regulations. Valentine et al. [97] tried to correlate the role of disinfectant loss and DBP formation with the deposits on distribution system pipelines. They found the deposits could significantly consume the disinfectant residuals and contribute to higher DBP reformation, a similar conclusion was also found by Nuckols et al. [102]

2.3.1.2 Haloacetic acids (HAAs)

HAAs are another major group of DBPs discovered in drinking water. The levels of HAAs were analogue to those of THMs in the water distribution system [36, 89].
Similar to brominated THMs, brominated HAAs can be produced in drinking water due to the presence of bromide. By replacing the hydrogen atoms with halogen atoms, partially or completely, a total of nine HAAs were observed, which were grouped as monohaloacetic acid (XAA), dihaloacetic acid (X₂AA), and trihaloacetic acid (X₃AA) [90].

There were a great number of studies focused on HAA formation in drinking water distribution system [8, 36, 103-106]. Singer et al. [99] reported that the presence of chlorine residual and increasing water age could contribute higher HAA formation in water distribution system. In comparison to THM₄ levels, other studies also indicated that HAA₅ levels were higher than THM₄ in batch experiments; whereas, opposite trend were observed in water distribution system, which may be attributed to biodegradation of HAAs [14, 53, 107]. Tung and Xie [53] investigated HAA degradation in a full scale water distribution system and found that increases in HPC were strongly correlated to the decay of HAAs. Bayless and Andrew [108] investigated biodegradation of six HAAs in drinking water. The study reported that XAA (MCAA and MBAA) was the most easily degraded HAA species followed by X₂AA (DCAA, BCAA, and DBAA) and TCAA, respectively.

For case studies of HAA formation in water distribution systems, the levels of HAAs ranged from several to several hundred μg L⁻¹. Rodriguez et al. [14] reported that HAA levels were from 10 to 70 μg L⁻¹ in a full scale water distribution system where the formation in summer was higher than formation in winter. Singer et al. [99] studied the HAA behaviors in drinking water distribution system over 12 months and discovered
higher pH contributes to higher HAA₅ levels compared to THM₄. In addition, the hydrophobic portion of NOM tends to produce more HAA₅. Rossman et al. [6] investigated the effect of deposited organic material on simulated drinking water distribution system pipe wall on HAA formation and speciation. Over a 24 hour period, there was no statistically significant increase of HAAs from simulated drinking water distribution system than those from bottle batch experiments. However, there was a tendency for the pipe to produce more dichloro- and less trichloroacetic acid than the bottle as reaction time progressed. Johnson et al. [101] reported the HAA₅ levels in drinking water distribution system with the impact of GAC carbon treatment, the HAA₅ formation were monitored around 40% decrease.

2.3.1.3 Haloketones (HKs)

HKs or halopropanones are groups of DBPs detected in drinking water usually at low levels [89, 109]. Propanone can be replaced by two or three halogen atoms to form dihalopropanones (DXP) or trihalopropanones (TXP), respectively. Usually, chlorinated HKs were monitored in drinking water, which include in 1,1-dichloro-2-propanones (1,1-DCP) and 1,1,1-trichloropropanone (1,1,1-TCP). Due to the lack of commercial standards and even lower concentrations of brominated HKs, DXP and TXP formation were usually not reported [8]. Previous studies reported that the concentration ranges were usually lower than 10 μg L⁻¹ in drinking water distribution system and with the increase of water age, HK levels were observed to decrease [110]. Nikolaou et al. [109] investigated the behavior of three HK species (1,1-DCP, 1,1,1-TCP, and 1,3-DCP) in drinking water samples with the impact of two temperatures. They found higher
temperature led to faster decomposition of studied HKs, formation of chloroform and chloral hydrate were observed from decomposition of HKs.

2.3.1.4 Haloacetaldehyde (HAs)

HAs are commonly detected in drinking water when chlorine was applied as the disinfectant. Since TCA is also called chlortal and very easily forms TCA hydrate in water, the name chlortal hydrate (CH) is commonly used [90]. Among HAs, dihaloacetaldehydes (DCA) and trichloroacetaldehyde (TCA) were the main species monitored [8, 21]. In addition to DCA and TCA, monohaloacetaldehydes can also be produced in chlorinated drinking water, but their occurrence and dominance in chlorinated water is very limited since they can be subsequently oxidized into TCA [21, 90]. The concentrations of HAs in drinking water distribution system were much lower than THMs and HAAs [111]. According to Krasner et al. [8], the maximum DCA concentration reached 16 μg L⁻¹ observed in one water distribution system was under chloramine-ozone disinfection. In addition, ozonation without biological filtration followed by chlorination/chloramination was reported to increase HAs formation [112, 113]. McKnight and Reckhow [114] found that acetaldehyde (an organic by product from ozonation) can react with chlorine to form DCA initially; then react with excess amount of chlorine residual to form CH.

2.3.1.5 Haloacetonitriles (HANs)

Since N-DBPs were continuously detected in drinking water, the formation of HANs, the most prevalent N-DBP, has been studied in recent years [21, 89]. Previous
studies have indicated that the toxicity of HANs was higher than C-DBPs due to the presence of the nitrile group in molecules of HANs [21]. For the formation of HAN, the hydrogen atoms on α-carbon could be replaced by halogen atoms to form HANs through the reaction between disinfectants and precursors. Currently, four species of HANs [dichloroacetonitrile (DCAN), trichloroactetonitrile (TCAN), bromochloroacetonitrile (BCAN), and dibromoacetonitrile (DBAN)] have been monitored in drinking water.

In the US survey for 2000-2002, HANs were observed with median and maximum levels of 3 and 14 μg L⁻¹ in drinking water distribution system, respectively, where dichloroacetonitrile (DCAN) was the dominate species [8]. The formation of HANs in water distribution systems was usually attributed to DBP precursors containing organic nitrogen (org-N) such as biofilm and untreated algal organic matter (AOM) [13, 47, 62, 89, 115]. Previously, the higher content of org-N in precursors, more HANs formation was reported [49]. Oliver reported that the formation of HAN in drinking water was mainly due to the reaction of amino acids and algal organic matter with chlorine based disinfectants [70]. Fang et al. compared the HAN formation of NOM and algae and the results indicated that HAN formation from algae was two times higher than that of NOM [47].

### 2.3.1.6 Halonitromethanes (HNMs)

Although low levels of HNMs were reported in finished water at some water utilities, rigorous studies has been conducted for HNM due to their high toxicity (most cyto- and genotoxic classes among emerging DBPs) [116]. HNM molecules are composed of a nitro group and hydrogen atoms on α-carbon which can be partially or
fully replaced by halogen atoms. There are nine species of chlorine and bromine substituted HNMs and trichloronitromethane (TCNM) is the main species which has been investigated in the early of 1980s [8]. For the sum of HNMs, median and maximum levels of 1 and 10 μg L\(^{-1}\) was observed in drinking water distribution system in the early US EPA survey [117]. However, the formation of TCNM was observed as higher levels during a study conducted in 2006-2007 with median and maximum values of 0.5 and 7.6 μg L\(^{-1}\), respectively [8]. The higher formation may be attributed to wastewater impacted source water and algae bloom in natural water, since algal organic matter (AOM) is important precursor for HNMs [118]. In addition, the disinfection method could also affect TCNM formation [119, 120]. For example, TCNM levels in drinking a water distribution system were reported to increase from 2 to 6 μg L\(^{-1}\) after preozonation processes [121]. Hu et al. [116] reported the effect of various disinfection methods on the formation of HNMs and they found ozonation coupled with chlorination produced the highest HNM yields followed by chlorination, ozonation-chloramination, and chloramination.

### 2.3.1.7 Haloacetamides (HAcAms)

HAcAms formation in drinking water distribution system was first reported during the 2000-2002 US survey [117]. After that, increased number of studies began to investigate the formation of HAcAms for both laboratory and actual drinking water distribution systems [21, 89, 122]. For HAcAm species, dichloroacetamide (DAcAms) has been identified as the dominant species formed in drinking water with the median concentration of 1.3 μg L\(^{-1}\) [117]. Krasner et al. reported the highest level (14 μg L\(^{-1}\)) of
total HAcAms detected in actual drinking water distribution system by chlorine-chloramines disinfection [8]. Previous studies indicated that higher org-N in precursors and using chloramine as disinfectant could enhance HAcAm formation in drinking water [13, 123, 124]. In addition, HAcAms can also be formed by hydrolysis of HANs [123, 124]. It is possible that the high level of HAcAm formation of was affected by the hydrolysis of HANs.

2.3.1.8 Cyanogen Halides (CNX)

CNX, a very toxic class of DBPs, was discovered in drinking water in the 90s, [91]. Similar to HAN, CNX molecules also contain a nitrile group where the hydrogen atom connected to the nitrile group could be substituted by halogen. Researchers suggested that using chloramine as disinfectant would enhance the CNX formation in the drinking water distribution system [13]. Based on the survey conducted for CNX formation in the US water utilities, median values of cyanogen chloride (CNCI) in drinking water with free chlorine and chloramine were 0.4 μg L⁻¹ and 2.2 μg L⁻¹, respectively [8].

2.3.1.9 Nitrosamines

N-nitrosodimethylamine (NDMA) is a very toxic and semi-volatile organic compound that has been detected in drinking water with low concentration, typically observed in the range of ng L⁻¹. However, the information for the formation and fate of NDMA in full-scale distribution systems is very limited. A survey conducted by Valentine et al. indicated that NDMA levels in water distribution system were higher than
those from plants effluents at 24 water utilities in US and Canada, which used chlorine or chloramines for disinfection [125, 126]. Studies also reported that NDMA levels would increase when monochloramine was applied as the primary disinfectant in water distribution systems [97, 126]. Field study results indicated that 50% of NDMA levels were below 2 ng L\(^{-1}\) in drinking water distribution systems when monochloramine was utilized as a disinfectant. On the other hand, the distribution system applied chlorine as a disinfectant showed below 1 ng L\(^{-1}\) of NDMA levels [126].

### 2.3.1.10 Total Organic Halide (TOX)

In addition to identified DBPs, there are still large fractions of unknown DBPs in drinking water. To estimate the fraction of unknown DBPs in drinking water, total organic halide/halogen (TOX, sum of TOCl, TOBr, and TOI) has been used. However, there are few studies focused on TOX formation in the drinking water distribution system. Krasner et al. measured the TOX from water treatment plant effluents [8] and reported that the median level of TOCl was 178 μg L\(^{-1}\), which was higher than the average median value (122 μg L\(^{-1}\)) from Information Collection Rule (ICR). In addition, THM\(_4\), HAAs and HA accounted for 14, 12, and 2% of the TOX, respectively [8]. Baribeau et al. [127] observed that TOX concentrations normally increased with increased contact time in both cold and warm water. Singer and Chang [128] reported TOX formation of 12 full-scale treatment plants and tried to correlate the TOX and THM formation; their results indicated that the TOX to THM ratio was approximately 3.4:1.
2.3.2 Influencing Factors on DBP Formation in Water Distribution System

Both laboratory and field studies indicated that DBP formation in the water distribution systems is significantly affected by environmental conditions. Several factors were commonly investigated to evaluate DBP levels and speciation in water distribution systems, which include temperature (seasonal changes), residence time (water age), pH, DBP precursor properties, disinfectant residuals, bromide levels, and microbial activity [13, 15, 79, 89, 91].

2.3.2.1 Effect of Temperature on DBPs

A large number of studies have focused on understanding the impact of temperature on the changes of DBP level in drinking water distribution systems [36, 54, 105, 109]. Considering the influence of temperature on DBP reaction kinetics, lower temperature (winter) would inhibit the reaction between DBP precursors and disinfectants. Temperature variations not only affect the formation of DBPs but also influence the DBP speciation [79, 129]. Chlorinated THMs and HAAs showed higher formation rates compared to brominated species with the increase of temperature [14, 54, 130]. Greater THM formation in drinking water distribution systems was observed with higher temperature (above 25 °C), whereas other DBPs were not as sensitive compared to THMs [14, 36].

To investigate the influence of temperature on regulated DBP formation in the drinking water distribution system, Rodriguez et al. [14] monitored the seasonal
variations of chlorinated DBPs in a drinking water distribution system located in a region where very significant seasonal variations of temperature existed. They found that THM levels in summer and fall were about five times higher than those in winter on average, whereas average HAAs in spring were about four times higher than in winter. Lebel et al.[105] studied the DBP behavior in distribution systems during one year period. Both THMs and HAAs were found at higher levels during summer than winter. Dion-Fortier et al. also investigated the effect of temperature on the formation of THMs and HAAs in selected water distribution systems [131]. THMs increased significantly (faster than HAAs) when water remains in a hot water tank. In hot water, the median levels of total THMs were approximately 2 to 5 times higher than those in cold water; whereas median levels of HAAs in hot water only showed 1.5 times higher than those in cold water. However, not all DBP formation is increased at higher temperature. For HKs, higher formation was detected during winter than summer, which is mainly attributed to lower decomposition rate of HKs at higher temperature [104, 109, 132]. Nikolaou et al. [109] reported HK decomposition rates increased at higher temperatures (30 °C), where the main decomposition products included chloroform and CH.

2.3.2.2 Effect of Contact Time (water age) on DBPs

Previous studies also indicate that residence time is another important factor that could affect DBP levels in the water distribution system [106]. Due to the complexity in the water distribution system, water age in water distributions system and storage tanks is easily affected by retention in customer demand (peak and off-peak hours) [133]. Those fluctuations contributes to considerably variations in DBP concentration [36].
Commonly, increased THM levels were observed with increased distance between water treatment plants and consumer tap; in contrast, levels of HAAs were not consistently found increased with longer distance from the water distribution system [54, 95, 106]. Case studies reported that THM levels were increased and stable in the extremities (end) of the distribution system; whereas HAA levels were increased at the beginning, and then decreased continuously (mainly due to a reduction of DCAA) [53, 106]. Their results indicated that HAAs may be biodegraded by biofilm in drinking water distribution systems [53, 106]. Baribeau et al. [36] investigated the THM concentration and speciation changes with the water age change in a distribution system (utility A) using chlorine as a primary disinfectant. The results demonstrated that increased water age facilitated THM formation. From treated water just entering into the distribution system (0 hour) to a location with a high water age (323 hours), the THM levels increased from 44.3 to 179.2 μg L⁻¹. For HAAs, in the same distribution system, a similar trend was found similar to previous studies [53, 106]. Specifically, HAA₉ levels was observed around 26 μg L⁻¹ at the point of entry of the distribution system, then increased to around 40 μg L⁻¹ from 3.5 to 18 hours (water age), then decreased to around 30 μg L⁻¹ at the furthest distance in the water distribution system (323 hours).

For some emerging DBPs, DCAN and 1,1,1-TCP levels were also found to be decreased with the increased distance in the water distribution system [109, 134]. Reckhow et al. [134] reported that DCAN concentrations decreased with large water age, which suggests the decrease is mainly attributed to DCAN hydrolysis. Nikolaou et al. [109] reported that 1,1,1-TCP was hydrolyzed as water age increased, decreasing 90% in 10 hours. In our previous study, we evaluated the effect of contact time on formation of
HANs, HKs, and TCNM of selected bacterial cultures upon chlorination. With the increase of contact time, a decrease of both HANs and HKs was detected [135].

### 2.3.2.3 Effect of pH on DBPs

A pH change in drinking water distribution system can affect the DBP formation and speciation [13, 15, 79, 136, 137]. Although mild pH fluctuation occurs in water distribution systems (usually ranged in 6.5-8.5), obvious impacts on DBP formation and speciation were noticed [130].

Many studies reported that higher THM formations are related to pH increases [2, 36, 90, 95]. According to Liang and Singer [15], increased THM formation was observed at pH=8 compared to pH=6, where THM formation was more sensitive than that of HAA with pH changes. From a study by Hooper et al. [138], a 50 to 60% of THM$_4$ concentration increase corresponded with a 2-unit increase of pH (6.5 to 8.7) with CHCl$_3$ being the main species influenced by pH variation compared to other brominated THMs. Increased THMs were also found at higher pH by Stevens et al. [136] when they measured the THM$_4$ formation under the chlorination of raw and filtered water samples from a water treatment plants (pH from 5 to 7 to 9.4).

For the influence of pH variation on HAA formation, opposite trends were found compared to that of THMs [15, 99]. Previous studies reported HAA formation decreased at higher pH level [13, 15]. Laboratory studies performed by Hooper et al. [138] indicated that pH (6.5 to 8.7) may not greatly impact HAA$_6$ formation, although, a slight decrease in concentration with increased pH (mainly TCAA) was observed above pH 8.2.
Similar results were also reported by the study of Liang and Singer [15] and no significant HAA increase were detected from pH 8 to 6. Besides the influence of pH on HAA formation, pH change was also reported to affect HAA speciation. TCAA and DCAA formation decreased with increased pH; whereas, the decrease in DCAA levels was much less significant than those of TCAA [132].

pH variation also influence the formation of some emerging DBPs [13, 47, 135]. Fang et al. [47] studied the effect of pH changes (pH 4-9) on formation of HANs, HK, TCNM and CH [13]. For HANs, DCAN levels firstly increased and then decrease (with the increase pH from 4 to 9), where the highest DCAN concentration was found at pH=6. Compared to change in formation of the DCAN, TCAN levels were not sensitive with the pH variation. For HKs, 1,1-DCP and 1,1,1-TCP levels decreased with the increase of pH. CH levels increased from pH 4 to 8, but a decrease was observed at pH=9. For TCNM, the pH did not affect the formation significantly.

2.3.2.4 Effect of Disinfectant Types and Concentration on DBPs

To maintain biological stability in drinking water, disinfectant residuals are usually applied to the water distribution system [139, 140]. Previous investigations confirmed that higher disinfectants residuals usually contributed to higher DBP levels [36, 47, 91]. For most water treatment plants and distribution systems, chlorine has been used as the primary disinfectant due to the low cost and high disinfection efficiency. However, chlorine usually contributes to higher DBP production in drinking water [89, 90]. In addition to chlorination, there has been an increased interest in applying chloramines as primary or secondary disinfectant due to their lower DBP formation in
water distribution systems [13, 141]. Monochloramine is commonly acknowledged to produce much lower levels of THM and HAA compared to chlorine. However, some toxic N-DBP formation, such as CNX, HAcAms, and NDMA, are of concern in using monochloramine as primary disinfectant [49]. For other commonly reported N-DBPs such as HANs and TCNM, the formation was lower using the monochloramine as disinfectant compared to Cl₂ [13, 62, 141, 142].

Increasing chlorine dosage not only promotes DBP formation but also affects changes in DBP speciation [36, 91], since higher chlorine dose can change the reaction pathway. Johnson and Jensen indicated that in the presence of higher chlorine dose, oxidation and cleavage reactions dominate while substitution reactions dominate at lower chlorine dose [143]. For example, higher chlorine dose or residuals were reported to favor the formation of HAAs over THMs [91]. Besides, higher chlorine doses result in a greater proportion of TCAA than DCAA or MCAA [92], which could be attributed to more reactions involving HOCl than the substitute of chlorine atoms on precursors. In a chlorinated distribution system, Oblensky and Frey observed that the change in DBP concentrations were strongly associated with total chlorine residuals [144]. For distribution systems using chloramine disinfections, higher residuals did not appear to be associated with changes in THM levels but appeared to suppress DCAA and TCAA decay in water distribution systems [144].

Enhanced emerging DBPs formation was also reported with the increased monochloramine doses [13, 47]. Yang et al. [13] showed increased formation of CF, 1,1-DCP, DCAN and CNCl with the increase of monochloramine doses. DBP formation per
unit dose of monochloramine decreased as follows: CNCl, 1,1-DCP, DCAN, CF. Fang et al. studied the influence of different Cl₂ doses on formation of HANs, HKs, CH, and TCNM [47]. Their results indicated that the formation of CH and 1,1,1-TCP increased with the rise of Cl₂ dose. In contrast, the formation of TCAN and 1,1-DCP were independent to the Cl₂ dose. Due to the oxidation properties of Cl₂, 1,1-DCP can be continually oxidized to 1,1,1-TCP, which would explain the steady level of 1,1-DCP formation. For DCAN, the highest formation was observed at the Cl₂ dose of 10.2 mg L⁻¹ and the decrease of DCAN may be explained by the faster decomposition rate of oxidation or hydrolyzation.

### 2.3.2.5 Effect of Bromide Ion on DBPs

In drinking water distribution systems, bromide is a very important ion affecting chlorination chemistry [90, 129]. From a survey conducted during 2000-2002, many selected water treatment plants had high bromide levels and the median concentration was reported as 120 μg L⁻¹ [8]. Until now, a great number of studies have focused on the influence of bromide on DBP speciation, where bromide could react with HOCl to produce HOBr [13, 79, 129, 145]. Several researchers indicated that HOBr is a more effective oxidant and it reacts with DBP precursors faster than HOCl [91, 129]. Since HOBr is involved in producing brominated DBPs, it is expected that the presence of bromide ions increases the overall mass concentration of DBPs formed.

Cowman and Singer [79] investigated the influence of bromide ion on HAA₉ speciation while chlorinating NOM solution. Larger fractions of brominated HAAs were found compared to chlorinated HAAs under high bromide concentrations [129, 145, 146].
At the higher levels of bromide, results indicated that three halogen acetic acid (X₃AA) species constituted the greatest mole fraction of the total HAA₉ (61 to 67%); the two halogen acetic acid (X₂AA) species accounted for 30 to 36%, and one halogen acetic acid (XAA species) comprised around 3 to 5% of total HAA₉ [79]. In addition, with increased bromide ion (0 to 25 μM), some HAA species (DCBAA and BCAA) increased first and then decreased, which further suggests more bromide ions were incorporated into chlorinated HAA species and formed brominated HAA species [79]. In addition to HAAs, higher bromide ions contributed to the formation of other brominated DBPs such as brominated THMs, HANs, and HNMs [129, 145, 147].

2.3.2.6 Effect of Precursors on DBPs

2.3.2.6.1 NOM

Previous studies indicated that the hydrophobic fraction of NOM is the most important precursor for DBP formation with a higher DBP yield compared to other factions [148, 149]. The hydrophobic fraction of NOM contains higher portions of aromatic/phenolic content and conjugated double bonds, which were reported to contribute to the formation of higher THMs during disinfection processes [150].

Kitis et al. [151] investigated the reactivity of DOM isolated by XAD-8 resin to chlorine and found that the hydrophobic fraction of DOM was the most reactive component. Reckhow et al. have shown that halogenated DBP formation was increased with the “activated (defined as electron-rich) aromatic” content of NOM [152]. Many efforts have been made to correlate various fundamental characteristics of NOM (e.g.
size, structure, functionality) to DBP yield. Specific ultraviolet absorbance (SUVA) has been demonstrated to be a good predictor of the aromatic carbon content of the NOM and DBP yield [153-155]. For example, Ates et al. [153] reported that UV$_{254}$ (UV absorption band of aromatic structure) has a good correlation with DBP yield, which further proved that hydrophobic groups of NOM could dominantly produce DBPs.

In water treatment plants, the hydrophobic portion (phenolic fraction) is more easily removed compared to the hydrophilic portion (carboxylic fraction) by conventional water treatment processes [15, 156]. Although previous studies reported the hydrophobic fraction in NOM produced higher DBPs than hydrophilic fraction, hydrophilic NOM could also contribute appreciably to DBP yields [149]. Hua and Reckhow [149] reported hydrophilic and low MW (<0.5 kDa) NOM fractions produced the highest X$_2$AA yields compared to those of hydrophobic and high MW (>0.5 kDa) fractions of NOM. Liang and Singer [15] reported the hydrophilic fraction of NOM also produces relatively high DBPs and is more reactive to bromide compared to hydrophobic fractions. Zhao et al. [155] studied DBP formation of different fractions of DOM isolated from the Pearl River in China and found that hydrophilic LMW fraction of DOM contributed to higher THM formation.

In addition to investigating properties of NOM, previous studies also indicated that spatial variations of DBPs in water distribution systems are related to the long reaction time of chlorine and NOM [53]. Higher THM concentrations are often found in the water distribution systems compared to water treatment plant effluents, especially at maximum residence time locations in the distribution system [6, 14]. Understanding the
role of dissolved organic matter (DOM) characteristics on DBP formation may provide insights to develop more effective solutions for DBP control via drinking water treatment operations [157].

2.3.2.6.2 Algal Organic Matter (AOM)

Many studies have investigated the influence of algae species and disinfection techniques on DBP formation [48, 63, 68, 158]. Huang et al. conducted the THM and HAA formation experiments for two different algae species (*Anabaena flos-aquae* and *Microcystis aeruginosa*). They found both algal cells and EOM of these two species exhibited a high potential for DBP formation. Hong et al. investigated DBP yields of surrogates for biomolecules in algae to predict DBP yield upon chlorination of algal cells [48]. The polysaccharide surrogate (starch) showed the lowest overall DBP yields. The results also revealed that the surrogate protein (bovine serum albumin) possessed the highest HAA yields, while the lipid surrogate (fish oil) had the highest THM yields. Widrig et al. [159] reported that coagulation poorly removed the org-N fragments. The high organic nitrogen content of AOM affects the disinfection efficiency and DBP formation during chlorination and chloramination in drinking water distribution systems. Bond et al. [160] also reported that identified N-DBP precursors tend to be LMW and low electrostatic charge organic matter, which suggested org-N fragments may be difficult to be removed by traditional water treatment processes.
2.3.2.6.3 Amino Acids

In addition to DBP formation from NOM and AOM, other DBP precursors are also present in water distribution system, such as free and combined amino acids. The contents and concentrations is are also related to the algae activity in surface water [161]. Due to the LMW characteristics of amino acids, it is difficult to remove amino acids by conventional water treatment unit processes. In addition to amino acids, other DBP precursors such as amines, carboxylic acids and phenols were also found in water distribution systems [162]. The presence of those DBP precursors in drinking water distribution systems may be attributed to the advanced oxidization process in water treatment trains such as ozonation [82, 163]. Kasiske et al. [164] reported that seven amino acids (aspartic acid, serine, glutamic acid, glycine, alanine, leucine, and phenylalanine) were present in the range from 0.33 to 1.05 μg L⁻¹ in treated drinking water.

Several studies conducted DBP yield tests with selected amino acids to evaluate DBP formation and pathways of different amino acids [165-169]. Hong et al. studied THM and HAA yield of twenty amino acids; high chlorine demands of all amino acids were observed (3.4-10 mg Cl₂ mg⁻¹C). For DBP yield, low THM yields were observed for most amino acids, except for tryptophan (Trp) and tyrosine (Tyr) [168]. For HAAs, large variations of yields occurred in tested amino acids; aspartic acid and asparagines were found as the important HAA precursors. For emerging DBPs, Yang et al. [169] reported DCAN and HNM formation from selected amino acids. Tryptophan and alanine generated the greatest amount of TCNM upon chlorination and asparagine and tyrosine
yielded the highest levels of TCNM upon chloramination. In addition, Trp, Tyr, asparagine, and alanine produced higher DCAN upon both chlorination and chloramination than other amino acids.

### 2.3.2.6.4 Biofilm

Biofilm and its secreted extracellular polymeric substances (EPS) possess very similar chemical characteristics to NOM and AOM [5, 41, 42], which are comprised of organic carbon (org-C) and org-N rich compounds, such as polysaccharides, protein, combined amino acids/peptides, lipids, and trace amount of humic substances [170].

Although biofilm associated problems in drinking water distribution systems have been widely reported and studied [35, 37, 38], the role of biofilm on DBP formation has been largely overlooked. Baribeau et al. [103] conducted the lab-scale annular reactor experiments to evaluate the stability of THMs and HAAs. They reported that X₂AA species were continuously formed in the presence of free or combined Cl₂ residual, where other species in THMs and HAAs were observed either slightly increased or decreased depending on experimental conditions. The results obtained from this experiment could be attributed to biofilm affected disinfectant demands and DBP formation. In our previous study, DBP yields from both protein and polysaccharide based EPS were investigated upon chlorination [137]. Our results indicated that protein based bacterial EPS possesses higher DBP yields compared to polysaccharide based EPS, especially for nitrogenous DBP (N-DBP). Wang et al. [171] also conducted lab scaled annular reactor experiments to evaluate the role of biofilm on formation and decay of DBPs in simulated water distribution systems. Results indicated that at lower Cl₂ residual (0.5 mg L⁻¹)
contributed to lesser DBP formation, at the same time, HAAs may be biodegraded by biofilm. At higher Cl₂ residual, biofilm increased DBP formation beyond the DBP yield of NOM in bulk solution.

2.3.3 DBP Formation Pathway

Given the application of various disinfectants in water distribution system, both individually and in combination, an increase in DBPs levels was inevitable [8, 21, 36, 89, 117, 127, 160]. Surprisingly, more information is known for the formation of C-DBPs and N-DBPs in drinking water distribution system; whereas, less is known the precursor characteristics and identities of the chemical structures that contribute to form DBPs [160]. To further elucidate the chemical reaction mechanisms for DBP formation on certain reaction sites, DBP yields from selected organic surrogates were examined [17, 48, 168, 172].

2.3.3.1 C-DBP Formation Pathway

A great number of studies investigated the formation pathway of regulated DBPs (THMs and HAAs) [161, 165, 166, 168, 172, 173]. For THM formation pathways, most studies indicated that phenolic and conjugated structures in precursors produce higher THM formation compared to aliphatic structures. To find the representative intermediate chemicals to elucidate the reaction and DBP formation pathways, amino acids and other typical organic compounds (such as tannic acid and resorcinol) were selected and tested for DBP yield experiments [165, 168]. Joll et al. [172] investigated THM yields from selected terpenoids (beta-carotene, retinol, beta-ionone, geranyl acetate) and
polyhydroxyphenol model compounds (4',5,7-trihydroxyflavanone, and ellagic acid). DBP yield results of terpenoid compounds indicated that reactions occur on methyl ketone groups and produce THMs by forming many intermediates. For polyhydroxyphenol compounds, extremely high THM formation was observed, which could be attributed to the presence of phenol structures. For amino acids, Tyr and Trp also showed higher THM yields due to the presence of activated aromatic structures [167, 168]. Hong et al. [168] reported that Tyr and Trp showed 147 μg mgC\(^{-1}\) and 45.8 μg mgC\(^{-1}\) CF yields, respectively. Other amino acids showed lower CF yields upon chlorination. Bond et al. [165, 166] studied several representative organic model compounds, which include five amino acids, two sugars, tannic acid, and resorcinol. Their results showed resorcinol possessed the highest THM yields because of the di-phenol structure.

Previous studies indicated Asp is the main precursor to produce high levels of HAAs [165, 166, 168]. Hong et al. [168] reported that Asp possessed the highest HAA yields compared to other amino acids and similar results were also reported by Bond et al. [166, 174]. DCAA was the main species produced by chlorination of Asp with yields as high as around 600 μg mgC\(^{-1}\). Based on the earlier research [175, 176], cyanoacetic acid (HOOC-CH\(_2\)-CN) was reported as a critical intermediate formed during the initial reactions of Asp upon chlorination. This reaction includes several steps. (1): The hydrogen atoms on -CH\(_2\)- could be quickly replaced by chlorine atoms due to the electron-withdrawing effect of nitrile group, forming dichlorocyanoacetic acid (HOOC-CCl\(_2\)-CN). (2): Since the carboxyl group is not stable in the intermediate, decarboxylation occurred on HOOC-CCl\(_2\)-CN which forms HCCl\(_2\)-CN. (3): The addition of HOCI to the nitrile group on HCCl\(_2\)-CN leads to the formation of N-chloroamides (HCCl\(_2\)-CO-NHCl),
which can be rapidly hydrolyzed to DCAA (main product) and TCAA (minor product) [168].

While most studies have focused on understanding regulated C-DBPs, it appears that a few studies have been focusing on exploring emerging C-DBPs (HKs and CH) formation pathways. This may be attributed to lower formation of emerging C-DBPs (HKs and CH in drinking water distribution system usually at the several μg L⁻¹) compared to regulated DBPs [8, 20, 21, 89]

2.3.3.2 N-DBP formation Pathway

In the water distribution system, the formation of N-DBPs was attributed to org-N in DBP precursors entering or present in distribution systems. Although untreated NOM contains the org-N, the biofilm grown in the water distribution systems has been recently identified as a potential N-DBP precursor since it possess higher org-N content than that of NOM (amino acids are the fundamental units for protein in biofilm). Thus org-N may also be from the biofilm grown in the water distribution system.

To understand N-DBP pathways, most studies have focused on the HAN formation pathway of amino acids. One of the important steps for HAN formation is nitrile formation by chlorination of the terminal amine groups, which is governed by lower pH [177]. For HAN formation from amino acids, previous studies reported that Asn, Asp, Tyr and His could produce higher HAN levels upon chlorination [123, 124, 167, 169, 176, 178, 179]. The proposed mechanism indicated that dichlorocyanoacetic acid (HOOC-CCl₂-CN) could be formed after chlorination and then this intermediate
could be further hydrolyzed to DCAN [123]. For Tyr, Chu et al. reported that benzyl cyanide was first produced after chlorination. Then it could be further substituted by Cl on the α-carbon and the dephenylation reaction occurred to form DCAN or TCAN [167]. Li and Blatchley [179] reported the formation pathway of DCAN during chlorination of His. The formation pathway includes: (1) the amine group forms a nitrile group by decarboxylation and dechlorination reactions, (2) Due to the electrical withdrawing properties of nitrile group and heterocycle, the hydrogen atom on the α-carbon can be replaced by a chlorine atom. (3) Then DCAN will be formed by the cleavage of the bond of the α-carbon and heterocycle.

Besides HANs, the formation pathways of other N-DBPs (HAcAms, CNX, HNMs, and NDMAs) were also reported [123, 180, 181]. Chu et al. [180] investigated the formation pathway of DCAcAms and TCAcAms from Asp. Their results suggested that both DCAcAms and TCAcAms could be hydrolyzed from DCAN and TCAN. In addition, Chu et al. also conducted the fluorescence excitation and emission matrix (EEM) spectra to target the precursors of HAcAms, which revealed that a mass of protein-like substances in hydrophilic acid DOM, made up of amino acids, were likely the DCAcAm precursors [181].

For CNX, formation pathways were also reported using amino acids and other organic molecules [124, 182, 183]. Na and Olson [182] reported a formation mechanism of CNCl from Gly. In detail, the hydrogen atoms of an amine group on Gly are replaced by chlorine atoms. Then decarboxylation occurs to form an intermediate (CH$_2$NCl), which is not stable, to form hydrogen cyanide (HCN) and further react with hypochlorous
acid (HOCl) to form cyanogen chloride. In addition, Pedersen et al. [183] studied the formation mechanism of CNCl from formaldehyde and proposed that formaldehyde reacts with NH₂Cl to oxidize the aldehyde group, then dehydration reaction occurs to form the same intermediate, which is not stable, and then forms HCN and further forms cyanogen chloride.

For HNMs, previous studies reported that the disinfection methods (with/without preozonation) are more critical for TCNM formation than the chemical structure of amino acids contained in the treated water [120, 184]. Hu et al. [116] reported the formation pathway of TCNM from Asp. This pathway includes (1) the continuous oxidation of Asp by HOCl, (2) the β-elimination of carboxyl groups, (3) oxidation of the amino group, and (4) the substitution of chloride atoms on α-carbon.

The formation mechanisms for NDMA were reported by several approaches [185-187]. To understand formation pathways of NDMA upon chloramination, Choi and Valentine used a ¹⁵N isotope labeled NH₂Cl to react with dimethylamine (DMA) [186]. They reported that DMA firstly reacted with NH₂Cl to produce (CH₃)₂NNH₂ (UDMH), then the intermediate could be further oxidized by NH₂Cl to produce NDMA. Chen and Young [185] studied NDMA formation from N′-(3,4-dichlorophenyl)-N, N-dimethylurea (Diuron) by reacting with NH₂Cl. DMA was one of the intermediates produced by Diuron, followed by the same mechanism proposed by Choi and Valentine [186]. Mitch and Sedlak [187] reported NDMA formation upon chlorination. The intermediate (1,1-dimethylhydrazine) was formed by the reaction between NH₂Cl and DMA, then rapidly oxidized to NDMA and other products (dimethylecyanamide and dimethylformamide).
Chapter 3

Objectives

This chapter outlines the research objectives in my PhD study and the organization of this dissertation.

3.1 Objectives

The objective of this study was to elucidate the role of biofilm and biofilm associated organic matter on the formation and decay of DBPs in simulated drinking water distribution systems. Emphasis was placed on how composition and quantity of biofilm growth in simulated distribution systems contributed to the formation and degradation of both regulated and emerging DBPs.

Below are the specific objectives:

(1) Determine the role of EPS on biosorption of NOM

This objective would give the fundamental information on how selected NOM interacts with biofilm/EPS in water distribution systems. Two bacterial species producing different compositions of EPS were examined in this study. *Pseudomonas putida*, which excretes a protein based EPS, was compared to *Pseudomonas aeruginosa*, whose primary
EPS component is polysaccharides. In addition, the effect of EPS quantity was evaluated using a wild type and two mutant strains of *P. aeruginosa*, which secrete different amounts of alginate (polysaccharide) based EPS. Sodium alginate beads, the artificial EPS, were also tested to exclusively observe EPS biosorption of NOM.

(2) Elucidate the role of biofilm EPS on DBP formation in drinking water distribution system conditions

The EPS quantity and composition on DBP formation kinetics and DBP yields (formation potential) were examined. Bacterial cultures that secreted protein and polysaccharide based EPS were also selected. In addition, the effect of EPS quantity on DBP formation was evaluated using a wild type and two mutant strains of *P. aeruginosa*. Both bacterial species have been found in various drinking water distribution systems [33, 41, 42, 188]. Formation of C-DBPs and N-DBPs were examined with consideration to conditions typically found in water distribution systems (pH, chlorine dose, and contact time). Furthermore, DBP yield experiments were conducted to elucidate DBP contribution from biomass and EPS.

(3) Investigate the relative composition of biomolecules in EPS on DBP formation

The main chemical compositions in EPS include proteins/амino acids, polysaccharides, and trace amount of lipids, DNA, and NOM, etc. [33, 189]. Proteins and polysaccharides are acknowledged as the most significant constituents in biofilm EPS [33, 170, 190]. Specifically, amino acids (derivatized from protein) and polysaccharide monomers (broken down from polysaccharides) of four different extracted EPS from
both single bacteria species and multi-species biofilm were analyzed. For DBP analysis, the influence of detailed chemical compositions in biofilm EPS on DBP yields were conducted by EPS protein and polysaccharides surrogates. Subsequently, the DBP formation pathways were examined.

(4) Monitor both regulated and emerging DBP formation and decay in a simulated water distribution system.

The formation and decay of DBPs in a simulated water distribution system was monitored by lab scale annular reactors. Considering factors in water distribution systems can affect DBP formation, such as precursors, disinfectant type, temperature, presence of bromide, and pH, this objective focused on the influence of disinfectants (types and residuals) and on DBP formation and decay. Multi-species biofilm obtained from a water utility were grown in annular reactors at selected hydraulic retention times and using selected NOM as the sole carbon source. DBP levels of reactor effluents were continuously monitored and DBP yield of biofilm and associated NOM from reactor coupons were analyzed every two weeks. In addition, biofilm behavior was monitored to provide fundamental understanding of DBP formation and decay.

### 3.2 Organization of Dissertation

To elucidate the role of biofilm and associated NOM on DBP formation and decay in the drinking water distribution system, this dissertation was separated into several chapters.
Chapter One briefly introduces the background of this study.

Chapter Two provides a literature review of DBP formation and decay in water distribution system. First, general information of disinfectants, disinfection techniques, and DBP precursors were summarized in detail. Second, DBP formation and speciation in both lab scale and full scale drinking water distribution systems were reviewed. Third, DBP formation pathways and DBP degradation mechanism were discussed.

Chapter Three discusses research objectives of this study and dissertation organization.

Chapter Four describes the role of EPS on biosorption of selected NOM with the influence of divalent ions. In water distribution system, untreated NOM may be adsorbed by biofilm/EPS and subsequently utilized by biofilm as nutrient for growth. In addition, harbored NOM may increase DBP formation by reacting with disinfectant residuals. Two bacterial cultures which secrete different types of EPS and three different NOM were selected in this study.

Chapter Five reports the influence of bacterial EPS on C-DBP and N-DBP formation considering the conditions in water distribution systems (pH, chlorine doses, and contact time).

Chapter Six further discusses the influence of major biomolecules (proteins, polysaccharides, and lipids) in bacterial EPS on DBP formation and speciation. EPS from single bacterial cultures and mixed species biofilm isolated from water utilities were characterized and their relative contributions to DBP formation and speciation were determined.

Chapter Seven focuses on monitoring the impact of biofilm development on DBP
formation and decay in a simulated water distribution system using lab scaled annular reactors.

Chapter Eight discusses the overall conclusions of this study and provides recommendations for further work.
Chapter 4

Role of EPS on Biosorption of NOM


4.1 Abstract

In this study, the influence of extracellular polymeric substances (EPS) composition and quantity was explored for biosorption of natural organic matter (NOM), using variants of Pseudomonas aeruginosa and Pseudomonas putida. Model EPS (sodium alginate beads) were tested and sorption capacity for NOM was also elucidated. In the absence of divalent ions, minimal NOM biosorption was observed and differences among strains were negligible. Under presence of divalent ions, biosorption of NOM was proportional to the amount of EPS secreted by P. aeruginosa variants. For sorption tests with model EPS, divalent ions also promoted biosorption of tested NOM, and total biosorption was also proportional to alginate quantity. Carboxyl group content in both alginate EPS and NOM appeared to be linked to increased biosorption via bridging with divalent ions. The alginate overproducing strain possessed more potential NOM biosorption sites, while the wild-type and alginate deficient strains possessed fewer
potential binding sites. In comparison, *P. putida*, secreting protein-based EPS, behaved differently for NOM biosorption, due to its hydrophobicity and the structural characteristics of proteins. Hydrophobic interactions appeared to enhance the biosorption of more hydrophobic Suwannee River humic acid (SRHA) by *P. putida*, whose biosorption of more hydrophilic NOM variants was similar to the alginate deficient strain. Mechanistically, the presence of a diffuse electrical double layer will present potential energy barriers limiting biosorption; however, divalent ion concentrations in the aquatic environment will promote biosorption processes, permitting functional group interactions between EPS and NOM. Bridging between hydrophilic carboxyl groups on alginate EPS and NOM appeared to be the dominant form of biosorption, while hydrophobic interactions enhanced biosorption for protein based EPS.

4.2 Introduction

Natural organic matter (NOM), greatly comprised of humic substances (HS), is a persistent problem in engineered water systems. Since NOM has been identified as a precursor to the formation of harmful DBPs [191, 192], significant effort has been made to remove NOM prior to disinfection practices, as stipulated by stringent regulation. However, treatment of the NOM is limited by its complicated structure, variable solubility, and a broad range of molecular weights [22, 32]. Thus, water utilities have difficulties removing all fractions of NOM through conventional water treatment processes [22]. Subsequently, untreated NOM enters the water distribution system,
where it is shown to generate DBPs in excess of measurements taken at the chlorination point during treatment, due to accumulation of precursor materials on the pipe wall [6].

Within the distribution systems, untreated NOM has also been linked to enhanced biological instability, providing a nutrient source and contributing microbial regrowth even in the presence of residual disinfectants [193, 194]. A key factor enhancing NOM utilization and retention by microorganisms is the presence of EPS [194], where most bacterial strains produce EPS as both suspended cultures and microbial biofilms [170]. EPS have been shown to provide binding sites for nutrients from the environment and to permit the absorption of metal ions and organic compounds [33, 44]. However, previous NOM biosorption studies primarily focused on the removal of NOM by aerobic and anaerobic biomass without considering biomass composition, although sludge and biofilm are mostly comprised of EPS [195-197]. Extracted EPS from biofilm and activated sludge have been shown to contain high concentration of humic substances [45, 46], suggesting an interaction between EPS and NOM. However, evaluating the interaction between NOM and EPS is complicated by void spaces, channels, and highly heterogeneous structure typical of both sludge and biofilm [42, 195, 198], where these attribute may influence the biosorption process.

Accordingly, the contribution of EPS quantity and composition on NOM biosorption is not well understood. Different factors may influence the biosorption of NOM on EPS. Intrinsically, the chemical composition of EPS includes a variety of biomolecules, such as polysaccharides, proteins, and nucleic acids, which contain different prominent functional groups that may affect the extent of NOM biosorption
Furthermore, extrinsic variation in solution chemistry, such as ionic strength, may also affect EPS interaction with NOM. Therefore, examining the presence and composition of capsular EPS on planktonic bacterial culture may simplify the variables influencing the biosorption of NOM and provide more fundamental understanding of EPS interactivity with NOM.

The primary objective of this study is to elucidate the role of EPS on biosorption of NOM. Two bacterial species producing different compositions of EPS were examined in this study. *P. putida*, which excretes a protein based EPS, was compared to *P. aeruginosa*, whose primary EPS component is polysaccharides. In addition, the effect of EPS quantity was evaluated using a wild type and two mutant strains of *P. aeruginosa*, which secrete different amounts of alginate based EPS. Sodium alginate beads, the artificial EPS, were also tested to exclusively observe EPS biosorption of NOM.

4.3 Materials and Methods

4.3.1 NOM Preparation

Suwannee River NOM (SRNOM), Suwannee River humic acid (SRHA) and Suwannee River fulvic acid (SRFA) were purchased from International Humic Substances Society (IHSS). NOM stock solutions were prepared by adding 100 mg of SRNOM, SRFA or SRHA, respectively to 1L deionized (DI) water (18.2 MΩ). From SRNOM stock solution, a Suwannee River dissolved organic carbon (SRDOC) stock
solution was prepared by filtering the SRNOM stock solution through a 0.45 μm nitrocellulose membrane (Millipore, USA).

4.3.2 Culture Preparation and Cell Number Enumeration

Three *P. aeruginosa* strains (mucA, PAO1, algT), which secrete different amounts of alginate based EPS (Provided by Dr. Daniel Hassett, University of Cincinnati), as well as *P. putida* (ATCC# 12633), whose primary EPS component is protein [190], were used. Cultures were grown in 1/10 LB broth (Becton, Dickinson and Company) at 30°C for *P. putida* and 37 °C for *P. aeruginosa*, respectively. Cultures were harvested at late exponential phase and centrifuged at 2700 rpm for 20 minutes to minimize stripping of EPS, and then washed in pH=7 phosphate buffer (0.54 g Na₂HPO₄•H₂O and 0.88 g KH₂PO₄ for 1 L solution) to remove excess media. Optical density (OD) of the washed culture suspension was measured and adjusted at 600 nm to obtain approximately 10⁹ cells/mL for isotherm experiments.

All culture suspensions were 4-log inactivated through exposure to a UV-C (254 nm, 8 W, Spectroline) lamp for 4 hours. UV-C disinfection inhibits cell replication without causing cell lysis [199]. Heterotrophic plate counting (HPC) with R2A agar media [200] was used before and after UV-C inactivation to enumerate cells and to confirm bacterial inactivation. Cell numbers were also confirmed by epifluorescence microscopy (EFM). For EFM enumeration, bacteria suspension was stained with 5 μg/L DAPI (6-Diamidino-2-Phenylindole) and incubated in the dark for 30 minutes, then filtered onto 0.2 μm hydrophilic black polycarbonate membrane [201]. Membranes were viewed on an epifluorescence microscope (IX51, Olympus, USA) and images were
captured with a Qimaging Systems camera (QImaging, Canada). Thirty microscopic fields were randomly selected for bacterial enumeration and counted using Image J software.

4.3.3 Bacterial Culture and EPS Characteristics

4.3.3.1 EPS Extraction and Analysis

All extraction procedures were performed on bacterial suspensions prepared from culture harvested at the exponential phase. The modified EDTA extraction method combined with high speed centrifugation method was employed [202]. In this method, 2% tetrasodium salt of ethylenediaminetetraacetic acid (EDTA) was added to each culture suspension in 1:1 ratio, and then shaken for 3 h at 4°C. The solutions were vortexed, then centrifuged at 14,000 x g for 20 min at 4°C. The supernatant was collected and analyzed to quantify EPS composition.

Total protein and polysaccharide were measured using colorimetric techniques on extracted EPS suspension. Protein concentrations were determined using the modified Lowry Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin (BSA) as standard. Polysaccharide concentration was measured using the phenol-sulfuric acid method using glucose as standard [46].

4.3.3.2 FTIR Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) was employed to probe both the chemical composition of the capsulated EPS. Cultures were grown and prepared as
described in Section 4.2.2. 50 µL of suspension was added onto stainless steel substrate, and then permitted to dry before analysis. A FTS-4000 Varian Excalibur Series FTIR spectrometer (Varian, Palo Alto, CA) was used for collecting the infrared spectra. Each spectrum was an average of 256 scans from 4000 to 400 cm⁻¹ was collected with a resolution of 2 cm⁻¹, and the ordinate was expressed as absorbance.

4.3.3.3 Hydrophobicity and Zeta-potential Measurement

Hydrophobicity was measured using a modified bacterial adhesion to hydrocarbons (BATH) method [203]. The hydrophobicity index was calculated in percentage by the following equation.

\[
\text{Hydrophobicity}% = \left(1 - \frac{\text{OD}_{\text{final}} - \text{OD}_{\text{initial}}}{\text{OD}_{\text{initial}}} \right) \times 100\%
\]  

(1)

Zeta-potential of the four cultures and three NOM were measured on a Malvern zetasizer Nanoseries (Malvern Instruments, UK) in both the absence and presence of divalent ions (2mM Ca²⁺ and 1mM Mg²⁺). Cultures were prepared as described in Section 4.2.2. All values were expressed as the mean of triplicate analysis.

4.3.4 Kinetic Tests

Kinetic tests were conducted at neutral pH (7±0.3) to determine the equilibrium time of the NOM biosorption. To monitor the influence of divalent ions present in water distribution system, CaCl₂ and MgCl₂·6H₂O were added to a final concentration of 2 mM Ca²⁺ and 1 mM Mg²⁺ for biosorption experiments [204]. For tests without divalent ions,
NaCl solution was added to match the ionic strength (IS) of the samples containing divalent ions. 20 mL of NOM stock solution was added to each 40 mL vial followed by 2 mL of culture suspension, then followed by 0.5 mL ion solution for a total volume of 22.5 mL.

Sample vials were placed in a rotary tumbler at 12 rpm. Samples were harvested at 15 min, 1 h, 2 h, 4 h, 8 h, and 20 h and filtered through 0.45 μm nitrocellulose membranes. The liquid phase concentrations of the tested NOM were monitored by measuring filtrate solution total organic carbon (TOC; TOC-V<sub>SCH</sub>, Shimadzu, Japan) and ultraviolet light absorbance at 254 nm (UVA<sub>254</sub>) on a UV spectrophotometer (UV-1800, Shimadzu, Japan). The calibration curve between UVA<sub>254</sub> and TOC of SRDOC, SRHA and SRFA was created to correlate absorbance to TOC.

Blank samples without cultures were prepared to assess changes in NOM and solution chemistry. Blank samples with culture suspension alone were also prepared as biological control to monitor any increases in TOC and UVA<sub>254</sub> due to the release of soluble EPS from the culture solution. The final TOC and UVA<sub>254</sub> of each sample were corrected by subtracting the average concentration of blank samples from experimental samples.

### 4.3.5 Isotherm Tests

Isotherm tests were conducted with the four tested cultures to determine the role of EPS on the biosorption of NOM. Seven concentrations of absorbate solutions were prepared from NOM stock solutions. Triplicate samples were prepared at each sampling
point and the isotherm equilibrium time was based on the results from kinetic experiments. Other experimental procedures were followed as outlined in section 4.2.4.

The final equilibrium concentrations, Ce of SRDOC, SRHA and SRFA were measured and corrected by subtracting organic matter content (soluble EPS) from culture blank samples (C_{culture blank}) as follows [197]:

\[ C_{e} = C_{sample} - C_{culture \ blank} \]  \hspace{1cm} (2)

Equilibrium absorbent (biomass) phase concentration was calculated based on using the mass balance expression:

\[ q_{e} = \frac{V}{M} (C_{o} - C_{e}) \]  \hspace{1cm} (3)

where \( q_{e} \) is equilibrium adsorbent-phase concentration of absorbate, \( \mu g \ (10^{10} \text{cells})^{-1} \); \( C_{e} \) is the aqueous phase concentration of absorbate at equilibrium, \( mg \ L^{-1} \); \( C_{o} \) is the initial absorbate concentration, \( mg \ L^{-1} \); \( V \) is volume of aqueous-phase absorbate solution, \( L \); \( M \) is the quantity of adsorbent (cell numbers), \( 10^{10} \) cells.

In order to quantify the biosorption capacity of the tested cultures, obtained experimental data were fitted according to the Freundlich and Langmuir Isotherm equations.

**Freundlich isotherm:**

\[ q_{e} = K_{f} C_{e}^{1/n} \]  \hspace{1cm} (4)

**Langmuir isotherm:**

\[ q_{e} = \frac{S_{m} K_{L} C_{e}}{1 + K_{L} C_{e}} \]  \hspace{1cm} (5)
where $K_f$ is the Freundlich constant indicative of adsorptive capacity, $[\mu g (10^{10} \text{cells}^{-1} (\text{mg L}^{-1})^{n_f})]$; $n_f$ is the Freundlich constant related to adsorption intensity; $K_L (\text{mg}^{-1})$ is the Langmuir isotherm constant related to the equilibrium constant or binding energy; $S_m [\mu g (10^{10} \text{cells}^{-1})]$ is the amount of sorption corresponding to complete surface coverage.

4.3.6 Model EPS Test

Sodium alginate beads were tested as an EPS surrogate to quantify the NOM sorption capacity of EPS. The alginate beads were formed by pumping autoclaved sodium alginate solution ($20 \text{ g L}^{-1}$) through a 0.25 mm syringe needle, where droplets solidified into beads upon contact with in 0.1 M CaCl$_2$ solution [205]. Beads were stabilized in CaCl$_2$ solution and washed daily with DI water until minimal TOC was measured in rinse water. 0.5 g alginate beads (wet weight) were used for both kinetic and isotherm experiments, following the procedure outlined in sections 4.2.4 and 4.2.5.

4.4 Results

4.4.1 Culture Characteristics and EPS Analysis

4.4.1.1 EPS Analysis and Characterization

Protein and polysaccharide concentration of four cultures are summarized in Table 4.1. For $P. aeruginosa$ EPS, the mucA strain has the highest polysaccharide and
protein content, followed by PAO1 then algT. *P. putida* EPS contained more protein than all *P. aeruginosa* strains. Upon visual inspection of cultures, clear differences were also observed for the variants. The mucA variant produced excessive biomass attributed to EPS, as compared to other strains and culture.

FTIR spectrums of four strains are shown in Fig. 4-1. For *P. aeruginosa* strains, strong absorbance was observed in the range from 700 – 1150 cm\(^{-1}\), which has been identified as the polysaccharide region [206]. Absorbance in this region is enhanced for the mucA strain in comparison to observed spectral absorbance at 1537 and 1650 cm\(^{-1}\), classified as the amide I and amide II bands. For PAO1 and algT strains, the difference between these spectral regions is less pronounced, thus suggesting the preeminence of polysaccharide content for the alginate overproducing strain. In addition, strong absorbance was observed at 3300 cm\(^{-1}\) for all strains, signifying NH\(_2\) stretching vibration of proteins. For *P. putida*, absorbance was also observed in the 700-1650 cm\(^{-1}\) and 3000-3600 cm\(^{-1}\) region. Absorbance in the polysaccharide region is present and reflects polysaccharide contributions from the cell and cell membrane. Absorbance at 1650 cm\(^{-1}\), 1537 cm\(^{-1}\), and 1240 cm\(^{-1}\) belong to the amide I, amide II, and amide III bands, respectively, indicating cell protein content. Differences in absorbance intensity for polysaccharide and protein regions in *P. putida* are not pronounced, although this may suggest increased contribution by proteins for this strain.
**Table 4.1:** Physio-chemical properties of tested strains

<table>
<thead>
<tr>
<th>Cultures</th>
<th>EPS characteristics</th>
<th>Hydrophobicity (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Zeta-potential (mV)</th>
<th>EPS concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>without divalent ions</td>
<td>with divalent ions</td>
</tr>
<tr>
<td>algT</td>
<td>alginate deficient</td>
<td>14.9 ± 3.2</td>
<td>-18.0 ± 0.8</td>
<td>-7.1 ± 1.8</td>
</tr>
<tr>
<td>PAO1</td>
<td>wild type (normal)</td>
<td>11.2 ± 1.7</td>
<td>-25.4 ± 3.0</td>
<td>-16.5 ± 0.3</td>
</tr>
<tr>
<td>mucA</td>
<td>alginate over-produce</td>
<td>7.2 ± 0.8</td>
<td>-28.7 ± 1.4</td>
<td>-18.9 ± 1.1</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>protein EPS</td>
<td>46.2 ± 6.6</td>
<td>-19.5 ± 2.2</td>
<td>-6.7 ± 0.4</td>
</tr>
</tbody>
</table>

<sup>1</sup>Percent transferred into hexadecane phase
4.4.1.2 Zeta Potential and Hydrophobicity

Table 4.1 indicates the zeta potential of four strains with and without divalent ions. For both conditions, algT and *P. putida* possessed lower zeta potential compared to the PAO1 and mucA. In the presence of divalent ions, the zeta potential value was reduced significantly for all cultures. In addition, zeta potential of three NOM solutions was measured, and revealed a strong charge reduction for all NOM with divalent ion addition.

Table 4.1 also outlines hydrophobicity results for four cultures. *P. putida* is the most hydrophobic among the four strains, based on organic phase concentration. For *P. aeruginosa* strains, algT (alginate deficient strain) was observed as the most hydrophobic,
followed by PAO1 and mucA, suggesting that EPS functional groups may contribute to hydrophobicity.

### 4.4.2 Kinetic Test

Kinetic tests showed that the average equilibrium time for all tested conditions was less than 15 minutes (data were not shown). Fast equilibrium may be attributed to occurrence of biosorption at the surface of cells and alginate beads. Several researchers reported an approximately 600 daltons molecular weight cutoff (MWCO) for the Gram-negative outer cell membrane, which constitutes a permeability barrier for hydrophilic substances [207, 208]. The MW range of NOM has been reported as several hundred Da to ten thousands Da [22, 32], thus NOM biosorption would occur on the surface of cultures rather than permeating into cell. Similarly, the biosorption on alginate beads would also occur at the surface due to small pore size [205]. Accordingly, an extended equilibrium time was selected for all isotherm tests to guarantee equilibrium.

### 4.4.3 Isotherm Tests

#### 4.4.3.1 Four Strains Isotherm Tests

Conventionally, absorbent quantity is presented with the mass of absorbent to calculate the equilibrium phase concentration of absorbate [195-197]. In this study, however, absorbent quantity was expressed as the cell numbers, $10^{10}$ cells, for calculating equilibrium adsorbent-phase concentration of absorbate ($q_e$). This elucidates the role of capsular EPS, because biomass contributions from the microorganism and EPS cannot be
differentiated using total absorbent mass in biosorption.

Both TOC and UVA$_{254}$ analysis were conducted to measure the NOM biosorption on the tested strains. However, TOC data resulted in a larger standard deviation (SD) compared to UVA$_{254}$ results, which might be related to dissolution of soluble EPS during isotherm tests. UVA$_{254}$ indicates the presence of aromatic carbon-carbon double bonds found in NOM. Therefore, UVA$_{254}$ may provide more accurate measurement for NOM biosorption to biomass [197]. Thus, only UV$_{254}$ data was presented in this study.

Fig. 4-2 (a) and (b) show the biosorption of SRHA at pH 7 both with and without divalent ions. The results indicated that mucA had the highest $q_e$ [77 $\mu$g (10$^{10}$ cells)$^{-1}$] followed by $P$. putida [60 $\mu$g (10$^{10}$ cells)$^{-1}$], PAO1 [35 $\mu$g (10$^{10}$ cells)$^{-1}$] and algT [32 $\mu$g (10$^{10}$ cells)$^{-1}$]. However, without divalent ions, biosorption capacity of tested strains decreased and differences among the four tested strains were not significant.

Fig. 4-2 (c) and (d) show biosorption of SRFA on tested strains. Compared to the SRHA biosorption data, mucA showed slightly lower $q_e$ [49 $\mu$g (10$^{10}$ cells)$^{-1}$] for SRFA. SRFA biosorption on PAO1 showed the similar $q_e$ [35 $\mu$g (10$^{10}$ cells)$^{-1}$] to that of SRHA. For the algT, $q_e$ [23 $\mu$g (10$^{10}$ cells)$^{-1}$] was lower than that of SRHA. For $P$. putida, $q_e$ [19 $\mu$g (10$^{10}$ cells)$^{-1}$] was much lower than determined for SRHA. Without divalent ions, biosorption of SRFA on strains decreased similar to results found for SRHA biosorption test.

Fig. 4-2 (e) and (f) showed biosorption of SRDOC on four tested strains. SRDOC showed higher affinity than SRFA in the presence of divalent ions. The results showed
that mucA had the highest qe [75 μg (10^{10} cells)^{-1}] followed by PAO1 [54 μg (10^{10} cells)^{-1}], whereas algT [32 μg (10^{10} cells)^{-1}] and *P. putida* [36 μg (10^{10} cells)^{-1}] showed similar biosorption capacity. In addition, SRDOC biosorption on strains without divalent ions was also decreased; however the affinity was slightly higher than results for SRHA and SRFA.
Figure 4-2: Biosorption of SRHA, SRFA and SRDOC on *P. aeruginosa* strains and *P. putida* with (Ca$^{2+} = 2$ mM, Mg$^{2+} = 1$ mM) and without divalent ions at pH 7. (a) SRHA with divalent ions. (b) SRHA without divalent ions. (c) SRFA with divalent ions. (d) SRFA without divalent ions. (e) SRDOC with divalent ions. (f) SRDOC without divalent ions. Error bars represent standard deviation (SD) of triplicate samples. (●—mucA, ○—PAO1, ▼—algT, Δ—*P. putida*)
4.4.3.2 Artificial EPS Isotherm Test

Fig. 4-3 showed three NOM absorbed on sodium alginate, used as a model EPS component. With divalent ions, SRHA (50 μg/g) showed the highest biosorption, followed by SRDOC (32 μg/g), then SRFA (16 μg/g). Without divalent ions, SRDOC and SRFA were not absorbed by sodium alginate beads; however, SRHA was minimally adsorbed on alginate beads (10 μg/g), suggesting higher hydrophobicity of SRHA may contribute to increased biosorption [209].

4.4.3.3 Isotherm Model Fitting

Freundlich and Langmuir isotherm models were used to evaluate biosorption of NOM on cultures and alginate beads. Isotherm constants were determined and summarized in Table 4.2. In the presence of divalent ions, both isotherm models fit the data well ($r^2 > 0.9$). However, isotherm models did not fit biosorption data without divalent ions, which may be attributed to low biosorption.
Figure 4-3: SRHA, SRFA and SRDOC biosorption on alginate beads with and without divalent ions at pH 7 (Ca$^{2+}$ = 2 mM, Mg$^{2+}$ = 1 mM). SRDOC and SRFA had negligible biosorption without divalent ions. Error bars represent standard deviation (SD) of triplicate samples.
<table>
<thead>
<tr>
<th>Cultures/model EPS</th>
<th>algT</th>
<th>PAO1</th>
<th>mucA</th>
<th>P. putida</th>
<th>alginate beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRHA(*)</td>
<td>0.32</td>
<td>1.58</td>
<td>0.59</td>
<td>0.25</td>
<td>1.54</td>
</tr>
<tr>
<td>SRDOC(*)</td>
<td>0.65</td>
<td>1.33</td>
<td>0.90</td>
<td>0.13</td>
<td>2.09</td>
</tr>
<tr>
<td>SRFA(*)</td>
<td>1.15</td>
<td>1.17</td>
<td>0.66</td>
<td>0.40</td>
<td>1.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRHA( **)</td>
<td>7.81</td>
<td>0.79</td>
<td>0.97</td>
<td>13.78</td>
<td>0.49</td>
</tr>
<tr>
<td>SRDOC( **)</td>
<td>8.41</td>
<td>0.68</td>
<td>0.96</td>
<td>13.22</td>
<td>0.74</td>
</tr>
<tr>
<td>SRFA (**)</td>
<td>6.97</td>
<td>0.57</td>
<td>0.93</td>
<td>7.89</td>
<td>0.74</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cultures/model EPS</th>
<th>algT</th>
<th>PAO1</th>
<th>mucA</th>
<th>P. putida</th>
<th>alginate beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRHA(*)</td>
<td>-1.13</td>
<td>0.18</td>
<td>0.73</td>
<td>-20.96</td>
<td>-0.02</td>
</tr>
<tr>
<td>SRDOC (*)</td>
<td>-8.80</td>
<td>-0.07</td>
<td>0.88</td>
<td>-2.83</td>
<td>-0.10</td>
</tr>
<tr>
<td>SRFA (*)</td>
<td>-60.98</td>
<td>-0.02</td>
<td>0.77</td>
<td>-8.22</td>
<td>-0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRHA( ** )</td>
<td>106.38</td>
<td>0.07</td>
<td>0.97</td>
<td>48.08</td>
<td>0.33</td>
</tr>
<tr>
<td>SRDOC( **)</td>
<td>69.44</td>
<td>0.11</td>
<td>0.96</td>
<td>119.05</td>
<td>0.12</td>
</tr>
<tr>
<td>SRFA( ** )</td>
<td>32.90</td>
<td>0.23</td>
<td>0.91</td>
<td>98.04</td>
<td>0.07</td>
</tr>
</tbody>
</table>

1 Isotherm constants are not available (NA)
4.5 Discussion

In the absence of divalent ions, minimal biosorption of the different NOM was observed for all tested strains. Furthermore, discernable differences in biosorption between strains for the tested NOM solutions were not evident. Zeta potential measurements of both the individual cultures and NOM solutions suggested that the potential energy barrier limited biosorption by minimizing steric interaction between culture and NOM. Zeta potential reduction and greatly increased biosorption of three NOM was observed in the presence of divalent ions, which have previously been reported [197, 210]. The results indicated that the addition of divalent ions promotes biosorption by compression of the electrical double layer and reduction of potential energy, which is explained by Derjaugin, Landau, Verwey, and Overbeek (DLVO) theory [211]. Moreover, divalent ions are more efficient in this process than the single charged ions (Na⁺, K⁺) according to Schulze-Hardy rule [212].

Upon meting this energy threshold, physical interaction between functional moieties in both NOM and bacterial cultures was permissible, as facilitated by the quantity and composition of EPS. P. aeruginosa EPS is comprised primarily of polysaccharides, while P. putida EPS is protein based. As illustrated in Table 4.1, the polysaccharide content of the alginate over-producing P. aeruginosa strain was considerably higher than wild-type and alginate deficient strains, while the protein content in P. putida EPS greatly exceeds all P. aeruginosa strains confirming results of other study [190]. The presence of these biomolecules has a pronounced effect on the functional moieties accessible at the fluid interface. The acidic polysaccharide alginate is the predominant component in P. aeruginosa EPS, thus more negatively charged
hydrophilic carboxyl groups are present [60]. However, our data suggested that negatively charged functional groups in the protein-based EPS of *P. putida* do not influence the surface interactions.

These EPS functional groups are significant in the biosorption of NOM due to the excessive presence of hydrophilic carboxyl groups on the NOM. The addition of divalent ions in solution has been shown to bind negatively charged function groups, promoting biosorption between absorbent and absorbate by creating a bridge between negatively charged functional groups. Several studies have reported that the “bridging effect” occurred between carboxyl groups on both NOM and membrane surfaces in the presence of divalent ions, where this effect was described as the mechanism of membrane surface fouling [213-215]. This bridging effect was also observed between the polysaccharide components in EPS and reverse osmosis membranes, whereas the increased protein binding in the presence of divalent ions was not observed [216]. In consideration of the biosorption of NOM by the tested strains, both the composition and quantity of the EPS had a profound impact on biosorption in the presence of divalent ions. The alginate EPS over-producing strain (mucA) was a more efficient biosorbent for all tested NOM, as compared to the alginate deficient strain (algT) and the *P. putida* with protein-based EPS. The increased amounts of alginate EPS corresponded to a greatly increased presence of carboxyl groups for bridging with the carboxyl groups on the NOM, while the wild-type and alginate deficient strains possess fewer potential binding sites.

However, functional group bridging does not provide the sole explanation for the biosorption process [Fig. 4-2 (a)]. Differences in hydrophobicity for both the different NOM and bacterial cultures had a distinct effect on biosorption. SRHA, SRFA and
SRDOC showed different biosorption trends for tested strains and model EPS, where SRHA had higher biosorption than SRFA in the presence of divalent ions. Esparza-Soto and Westerhoff examined NOM biosorption with activated sludge and found that SRHA had higher sorption onto the sludge compared to SRFA [197]. This trend was attributed to the greater hydrophobicity of SRHA due to its high aromatic carbon content [209]. With the highest biosorption of all tested NOM, the hydrophobicity of SRHA facilitated its partitioning from the bulk fluid for subsequent biosorption by the tested strains and model EPS, as compared to the more hydrophilic SRFA and SRDOC [217]. Furthermore, hydrophobic interactions were significant for the biosorption of SRHA on *P. putida*. As determined by the BATH method, *P. putida* is more hydrophobic than all *P. aeruginosa* strains, due to the hydrophobic surface properties of its protein-based EPS. Therefore, hydrophobic interactions appear to enhance the biosorption of SRHA by *P. putida*, whose surface charge and biosorption of more hydrophilic NOM variants was similar to algT (alginate deficient strain).

In consideration of these prevalent biosorption effects, a more robust explanation of the dominant biosorption mechanism can be suggested. Although hydrophobic interactions influenced biosorption, divalent ion facilitated bridging between negatively charged functional groups on EPS and NOM appeared to be the prevalent biosorption mechanism. Polysaccharide-based EPS is highly hydrophilic and contributes carboxyl groups accessible to NOM. Examination of the alginate EPS producing variants confirms that an increased amount of carboxyl groups are present with greater quantities of alginate EPS. The presence of these polar and hydrophilic carboxyl groups facilitates bridging with NOM in the presence of divalent ions. Conversely, the microbial strain
secreting protein-based EPS behave differently for NOM biosorption in aqueous systems, due to the structural characteristics of proteins. The primary amine structure possesses many charged functional groups but does not appear to influence the surface interactions between EPS and NOM. Instead, these functional groups are known to have far greater impact on the secondary and tertiary structure of proteins [218]. This level of molecular organization determines the surface characteristics of protein in the EPS, implying a reduced availability of binding sites. Indicated by lower surface charge and greater hydrophobicity, the functional groups at the fluid interface are less polar and do not appear to facilitate biosorption through divalent ion bridging, although they enhance the biosorption of hydrophobic NOM.

4.6 Conclusions

The combined results of this study provided a more exhaustive evaluation of the mechanisms responsible for NOM biosorption by EPS. The presence of a diffuse electrical double layer appears to limit the biosorption regardless of EPS type and quantity. In natural and engineered aquatic systems, however, conditions will ultimately favor biosorption due to the concentration of divalent ions present, thus potential energy barriers will be limited by compaction of the electrical double layer and functional group interactions will be favored. In this sense, the composition and quantity of EPS becomes more critical for NOM biosorption under environmental conditions as binding sites become accessible for biosorption. Hydrophobic interactions are observed to have an impact on the biosorption of hydrophobic NOM by hydrophobic EPS. Nevertheless, the
impact of these occurrences is less critical compared to the dominant effect from divalent ion bridging between negatively charged functional groups on the NOM and EPS, where increasing the quantity of these sites enhance biosorption regardless of hydrophobicity.

The hydrophobicity of different NOM may facilitate its removal from the aquatic environment, but does not individually contribute to the biosorption process. Hydrophobic interactions may enhance biosorption by some EPS components, but these occurrences appear to be dominated by bridging effect. A comparison of these mechanisms in the biosorption of NOM in the presence of divalent ions reveals the overall importance of functional group interaction on the biosorption process.
Chapter 5

Influence of Bacterial EPS on DBP formation


5.1 Abstract

Considering the regulatory presence of residual chlorine in distribution systems, untreated organic matter may not be the sole contributor to DBP formation, given the presence of microbial biofilm with extracellular polymeric substances (EPS). This study investigated the influence of bacterial EPS on the formation of carbonaceous DBPs (C-DBPs) and nitrogenous DBPs (N-DBPs), reacting chlorine with *Pseudomonas* strains that produce different quantities and composition of EPS. When biomass is reacted in excess to chlorine, both C-DBPs and N-DBPs were produced without preference for speciation. However, under an excess of chlorine compared to biomass, increased EPS content led to enhanced formation of DBPs. The DBP yield of haloacetic acids (HAAs) was higher than those of trihalomethanes where dichloroacetic acid was dominant in HAA species. Additionally, chemical composition of EPS influenced the yields of DBPs. The N-DBP yield from *P. putida* EPS was two times higher than that of *P. aeruginosa* EPS, which
suggested that higher organic nitrogen content in EPS contributes to higher N-DBP yield. Moreover, time based experiments revealed that DBP formation from biomass occurs rapidly, reaching a maximum in less than four hours. Combined results suggest that bacterial EPS have significant roles in both the formation and fate of DBPs.

5.2 Introduction

Maintaining the biological stability in water distribution systems is a great challenge due to ubiquitous presence of microbial biofilm [37, 38]. Biofilm consists of aggregated microbial cells embedded with a matrix of extracellular polymeric substances (EPS) [41, 42], comprised of various biomolecules such as nucleic acids, proteins, polysaccharides, and lipids [33]. Biofilm EPS protects the embedded bacteria and promotes enhanced biological instability in distribution systems by reducing the disinfectant efficacy. Biofilm EPS has been shown to limit the transport of chlorine through the biofilm structure while simultaneously consuming residual chlorine [52]. Given these limitations, biofilm proliferation in distribution systems reduces disinfectant efficacy. Currently, common biofilm control practices employed by water utilities include applying high doses of residual chlorine to maintain the biostability in water distribution systems [37, 38]. However, biofilm control using high disinfectant doses may deteriorate water safety by increasing disinfection by-product (DBP) formation through reaction with attached biofilm. Additionally, given rapid reaction between chlorine and biomass, continuous maintenance of disinfectant residuals may contribute to DBP formation
exceeding the limits of US Environmental Protection Agency (EPA) DBPs regulations [52, 219].

Recent studies reported DBP formation by biomolecules from algae entering water treatment processes [47, 48]. Biomolecules derived from aquatic organisms may pose a greater risk for DBP formation than traditionally investigated natural organic matter (NOM) due to their higher contribution to toxic nitrogenous DBP (N-DBP) formation [19, 21, 49]. Many environmental microorganisms produce various biopolymers as both biofilm and planktonic cells [170]. In microbial biofilms, more than 90% of biomass is contributed by EPS, which constitute similar organic composition and chemical structure as aquatic DBP precursors [50, 166, 220]. However, biological contributions to DBP formation in distribution systems have been largely overlooked, even though previous studies reported increased DBP formation in the distribution system assuming organic deposits on pipe walls were culprit [6, 8, 221]. Despite these findings, current research for preventing DBP formation and investigating DBP behavior in water distribution systems focuses on: (1) removing allochthonous DBP precursors as well as autochthonous precursors during water treatment processes [30, 51]; (2) evaluating modulations in DBP formation and decay in water distribution systems as result of seasonal changes, pH, disinfectant type, etc. [6, 14]; and (3) identifying the formation of emerging and unregulated DBPs from different disinfectants [8, 21]. DBP research and biofilm control research have been unfortunately separated into two areas of study, although significant interaction likely occurs between them.
While both the DBP formation and their potential health risks have undergone considerable scrutiny, the contribution of biofilm to DBP formation is still not clear. Chlorine is reported to have high reactivity with biofilm, but non-selective reactions limit its penetration through biofilm [52]. Thus, transport limitation of Cl\textsubscript{2} prevents identification and quantification of DBP formation from specific biofilm components. Beyond limited chlorine transport, the structural heterogeneity of biofilm (voids, channels, and cell distribution) does not permit differentiation of bacterial vs. EPS contributions to DBP formation upon exposure to chlorine. Relative chemical compositions of biofilm EPS have been reported slightly different from those of capsular EPS of planktonic cells [222]. Different growth conditions such as pH, nutrient conditions, divalent ions, and even material of biofilm substratum affect EPS production [170, 222]. However, previous studies also reported that major chemical species of biofilm EPS are mostly analogous to capsular EPS [222]. The EPS amount significantly increases for biofilm compared to those of planktonic cells under same bacteria cell numbers [222]. Therefore, evaluating DBP formation by capsular EPS on planktonic bacterial cells and extracted EPS may provide a more fundamental understanding toward the impact of EPS on DBP formation, thus minimizing variability of biofilm samples due to its heterogeneous characteristics.

The primary objective of this chapter was to elucidate the role of EPS on DBP formation. Two representative bacterial species producing different compositions and quantity of EPS were examined in this study. \textit{P. putida}, which excretes protein based EPS, was qualitatively compared to \textit{P. aeruginosa}, whose primary EPS components are polysaccharides (alginate). In addition, the effect of EPS quantity on DBP formation was also evaluated using a wild type and two isogenic mutant strains of \textit{P. aeruginosa}, which
secrete different amounts of polysaccharide based EPS. Both species produce the most commonly found chemical components of bacterial EPS (protein and polysaccharides) and have been found in various drinking water distribution systems [33, 41, 42, 188]. Formation of carbonaceous DBPs (C-DBPs) and N-DBPs was examined considering conditions typically found in water distribution systems (pH, chlorine dose, bromide, and contact time). Furthermore, DBP yield experiments were also conducted considering total biomass and EPS contribution to determine the impact of the composition and quantity of EPS on DBP yield.

5.3 Materials and Methods

5.3.1 Culture and Extracted EPS Preparation

Three *P. aeruginosa* variants (mucA, PAO1, and algT), which secrete different quantities of polysaccharide based EPS, as well as *P. putida* (ATCC# 12633), which secretes protein based EPS, were used in this study. Detailed preparation method for bacterial cultures and measured EPS quantity and composition are described elsewhere [135]. For DBP formation experiments, $10^8$ CFU mL$^{-1}$ cell density was selected to ensure Cl$_2$ limiting condition based on preliminary tests at 5 mg L$^{-1}$ Cl$_2$ dose. For DBP yield experiments, $10^6$ CFU mL$^{-1}$ was selected to make biomass limiting under a high Cl$_2$ dose (20 mg L$^{-1}$). Cell density of this order of magnitude ($\sim10^6$ CFU mL$^{-1}$) has been found for water distribution system biofilm in the presence of residual Cl$_2$ [35, 223].
To quantify EPS contribution to DBP formation, bacterial EPS were extracted using cation exchange resin (CER) method [224]. To minimize the influence of CER on DBP formation, resins were carefully washed. Blank extraction tests confirmed that there was no DBP formation contribution by resins [225]. Total organic carbon (TOC) content of both strains and extracted EPS were determined using a TOC analyzer (Shimadzu, Japan, TOC-V<sub>SCS</sub>).

### 5.3.2 DBP Formation and DBP Yield Tests

Both C-DBPs [trihalomethanes (THMs), haloacetic acids (HAAs), and haloketones (HKs)] and N-DBPs [haloacetonitriles (HANs) and trichloronitromethane (TCNM)] were measured. For both DBP formation and DBP yield tests, batch experiments were conducted in 40 mL amber glass vials. For DBP formation tests, five chlorine concentrations (0.5, 1, 2, 3, and 5 mg L<sup>-1</sup>) were selected to simulate doses commonly observed in water distribution systems as well as a higher dose for biofilm control [38]. All experimental solutions were prepared using deionized water (18.2 MΩ) and buffered with phosphate solution. Free chlorine concentration was measured by DPD method [226]. Considering the impact of pH on DBP formation, all tests were conducted at drinking water pH range (pH=5.5 and 7.5) and samples were incubated in the dark for 4 days.

To elucidate the water age impact on DBP formation, various contact times (2, 4, 8, 24, 48, and 96 hours) representing water ages reported in distribution systems were tested using two representative strains (PAO1 and *P. putida*) at a fixed initial Cl<sub>2</sub> dose (5 mg L<sup>-1</sup>) [227].
To quantify and compare EPS contribution to DBP formation, DBP yield experiments were conducted with and without 200 μg L⁻¹ bromide following the standard method 5710B. In accordance to the method, excessive Cl₂ was applied to fixed cell or extracted EPS quantities and incubated for 7 days at 25 °C (pH=7) [226]. Bromide solution was prepared and the final concentration was verified with an ion chromatograph (Dionex, USA, ICS-1000). Final chlorine residual concentration was also measured to confirm complete biomass reaction with chlorine.

For DBP yield tests with total biomass (cells and EPS), DBP yield was expressed as both μg mgC⁻¹ and μg (10¹⁰ cells)⁻¹. DBP yield results from planktonic cells experiments were presented as the cell numbers, 10¹⁰ cells, to elucidate the contribution of capsular EPS on DBP yield as the cells from individual strains possess different amounts of EPS.

5.3.3 DBP Analytical Methods

Analysis of THM₄ [chloroform, bromodichloromethane (BDCM), dibromochloromethane (DBCM) and bromoform], HAA₉ [monochloroacetic acid (MCAA), monobromoacetic acid (MBAA), dichloroacetic acid (DCAA), bromochloroacetic acid (BCAA), trichloroacetic acid (TCAA), bromodichloroacetic acid (BDCAA), dibromoacetic acid (DBAA), chlorodibromoacetic acid (CDBAA), and tribromoacetic acid (TBAA)], HAN₄ [trichloroactetonitrile (TCAN), dichloroacetonitrile (DCAN), bromochloroacetonitrile (BCAN), and dibromoacetonitrile (DBAN)], HK₂ [1,1-dichloro-2-propanone (1,1-DCP) and 1,1,1-trichloropropanone (1,1,1-TCP)], and trichloronitromethane (TCNM) were carried out using a gas chromatograph (GC).
(Shimadzu, Japan, GC-2010 Plus) with dual electron-capture detectors (ECD) coupled with DB-1 (30 m, 0.25 mm, 1.00 μm) and DB-5 (30 m, 0.25 mm, 0.25 μm) capillary columns (Agilent, USA). THM₄/HAN₄/HK₂/TCNM extraction followed EPA method 551.1 [228], where DBPs were extracted from water samples by methyl tert-butyl ether (MTBE) and organic phase was analyzed. HAA₉ were recovered by liquid/liquid extraction with MTBE, followed by methylation with acidic methanol based on EPA method 552.2 with small modifications [229, 230]. The oven temperature for THM₄/HAN₄/HK₂/TCNM analysis was programmed as follows: an initial temperature of 35 °C was held for 22 mins, ramped to 145 °C at 5 °C/min (held 2 mins) and ramped to 225 °C at 25 °C/min (held 2 mins). For HAA₉ analysis: 37 °C for 11 mins, ramped to 136 °C at 5 °C/min (held 3 mins) and ramped to 236 °C at 20 °C/min (held 6 mins). 1,2-dibromopropane served as internal standard for all DBPs and 2-bromobutanoic acid was used as a surrogate for HAA₉ to monitor methylation efficiency. Spike recovery was calculated to verify the validity of entire analysis in this study. Both average spike recoveries and minimum detection limits (MDL) for all tested DBPs were determined. For regulated DBP (THMs and HAAs) yields, relative percent difference (RPD) were below 20% in duplicate measurements. For emerging DBP (HANs, HKs and TCNM) yields, the RPD was slight higher than those of regulated DBP yields between duplicate samples. One way ANOVA tests and t-tests were conducted to determine statistical significance of DBP yield data.
5.4 Results and Discussion

5.4.1 Impact of Cl₂ Dose and pH on DBP Formation

In DBP formation tests using excessive biomass, strains with different EPS quantity and composition did not significantly influence DBP formation and speciation due to limited amount of Cl₂ for preferential reactions with biomass. Free chlorine concentrations at the end of experiments were negligible in all tested samples, which suggested complete reaction of chlorine with biomass. Fig. 5-1 shows formation of chloroform and chlorinated HAA₃ (sum of MCAA, DCAA, TCAA) at pH=5.5 and 7.5 under various chlorine doses with 4-day incubation. At pH=5.5, low chloroform formation was observed (1-5 μg L⁻¹) from all tested strains, where Cl₂ dose had minimal impact on chloroform formation. In contrast, chloroform formation increased significantly at pH=7.5 and exhibited Cl₂ dose dependency. Same trend has been previously observed by chlorination of NOM [13]. At 5 mg L⁻¹ Cl₂, chloroform formation reached approximately 25 μg L⁻¹ for all tested strains [Fig. 5-1 (b)].

The effect of Cl₂ dose on chlorinated HAA₃ formation was also investigated [Fig. 5-1 (c) and (d)]. At pH=5.5, the formation of chlorinated HAA₃ was slightly higher than at pH=7.5. A similar study on HAA formation upon chlorination of NOM indicated that HAA formation decreased at higher pH [15]. However, the influence of pH on chlorinated HAA₃ formation was less significant compared to that of chloroform, congruent with a previous report by Liang and Singer [15]. Furthermore, increased chlorine dose contributed minimal increase to chlorinated HAA₃ formation. In the presence of 0 to 2 mg L⁻¹ Cl₂ doses, MCAA and DCAA were the dominant species.
However, DCAA and TCAA were the dominant species upon application of 3 to 5 mg L\(^{-1}\) Cl\(_2\). While increased HAA\(_3\) formation has been reported at higher chlorine dose [13, 47], the total chlorinated HAA\(_3\) formation did not increase proportionally with chlorine dose in this study. Among three detected chlorinated HAA species, MCAA concentration was similar (1-2 μg L\(^{-1}\)) at all tested chlorine doses, which may be explained by fast biodegradation of MCAA [12, 53, 107], given the presence of viable cells from heterotrophic plate count (HPC) results between 8 and 24 hrs.

Previous studies reported increased DBP formation in water distribution systems. Based on our experiment results, the highest THM and HAA formation by biomass and EPS can be up to one third of regulated DBP concentrations. However, there are many variables (pH, precursor, disinfectant residual, temperature, pipe materials, water age, etc.) which affect both biofilm and DBP formation in the distribution system. The role of biofilm on DBP formation in water distribution system is still elusive and further studies are needed [6, 14, 36, 53].

The formation of HANs and HKs is presented in Fig. 5-2. For N-DBPs, TCNM formation was negligible. As shown in Fig. 5-2 (a, b), DCAN was the only HAN species detected (in the absence of bromide) and exhibited slight dependency with the increased Cl\(_2\) dose. The increased chlorine dose showed minimal impact on DCAN formation, which may be attributed to decomposition of DCAN by hydrolysis [13, 47]. Overall, Fig. 5-2 (c-f) shows 1,1-DCP and 1,1,1-TCP formation at pH=5.5 were slightly higher than formations at pH=7.5. Previous studies reported lower DCAN, 1,1-DCP, and 1,1,1-TCP formations upon chlorination of NOM at pH=7.5 compared to those at low pH [13, 231].
Among four tested strains, slightly higher HK formation (around 1-2 μg L⁻¹) was observed for *P. aeruginosa* strains compared to the *P. putida* strain. However, an application of 5 mg/L Cl₂ resulted in a decrease of HK formation for *P. aeruginosa*, which might be related to 1,1,1-TCP decomposition into chloroform and other species [109].

**Figure 5-1**: The formation of chloroform and HAA₃ (MCAA, DCAA, TCAA) at pH=5.5 [(a) and (c)] and 7.5 [(b) and (d)], at the chlorine doses (0.5, 1, 2, 3, and 5 mg L⁻¹); ●–algT, ○–PAO1, ▼–mucA, Δ–*P. putida*. 
Figure 5-2: The formation of HAN (DCAN) and HKs (1,1-DCP and 1,1,1-TCP) at pH=5.5 [(a), (c) and (e)] and 7.5 [(b), (d) and (f)], at the chlorine doses (0.5, 1, 2, 3, 5 mg L⁻¹) (●–algT, ○–PAO1, ▼–mucA, Δ–P. putida).
5.4.2 Effect of Contact Time (Water Age) on DBP Formation from Biomass

Fig. 5-3 shows the impact of contact time on the DBP formation at fixed Cl₂ concentration (5 mg L⁻¹). To investigate this occurrence, *P. aeruginosa* and *P. putida* with distinctive EPS composition were tested [Fig. 5-4 (a)-(h) for detailed DBP species affected by water age]. For chloroform, formation occurred and increased rapidly during the first 4 hrs then continued at a slower rate from 8 to 96 hrs [Fig. 5-3 (a)]. Chlorinated HAA₃ formation also occurred quickly during the first 4 hr incubation. Both *P. putida* and PA01 produced similar amounts of MCAA and TCAA, while PA01 produced 15-20% greater amounts of DCAA than *P. putida*. However, after 4 hr incubation, a sharp HAA₃ concentration decrease was observed between 8 and 24 hrs for all tested strains. After 24 hrs, the concentration of HAA₃ stabilized for the remaining 72 hrs. The concentration decrease of HAA₃ came in the form of decreased MCAA and DCAA, which may be attributed to the biodegradation of these two species [12, 107]. Contact time evaluation also revealed rapid formation of HANs. Among HAN species, DCAN was the dominant species over TCAN (less than 20% of HAN) [Fig. 5-4 (a) and (b)]. There was a rapid formation of HAN after 4 hr incubation, but HAN formation stabilized and began to slightly decrease with prolonged contact time (after 48 hrs). The hydrolysis of DCAN may explain these results [134]. In addition, the formation of total HKs at different contact times is shown in Fig. 5-3 (d). Similar to HAA and HAN formation, rapid HK formation was observed after 4 hrs. However, similar to HAA₃ decay, HK concentration decreased rapidly as contact time increased. Detailed speciation of HK revealed that 1,1-DCP and 1,1,1-TCP were the only species present, where 1,1,1-TCP
was the dominant HK species [see Fig. 5-4 (c) and (d)]. Compared to initial formation (at 4 hr), reduction of 1,1,1-TCP was observed around 75% and 87.5% at pH=5.5 and 7.5, respectively, while concentration of 1,1-DCP was not significantly changed during 96 hr contact time. This might be attributed to the decomposition of 1,1,1-TCP by hydrolysis [47, 232].

**Figure 5-3**: DBP formation at different water age, Cl₂=5 mg L⁻¹ (2 hr, 4 hr, 8 hr, 24 hr, 48 hr, 96 hr) (a) THM, (b) HAA, (c) HAN (d) HK (●–PAO1, pH=5.5, ▲–P. putida, pH=5.5, ○–PAO1, pH=7.5, △–P. putida, pH=7.5).
Figure 5-4: Detailed speciation of HAN, HK and HAA at different water age with incubation of 5 mg L\(^{-1}\) Cl\(_2\). ●–PAO1 at pH=5.5; ▲–P. putida at pH=5.5; ○–PAO1 at pH=7.5; ∆–P. putida at pH=7.5.
Rapid reactions between Cl₂ and bacteria strains, as well as rapid production of DBPs were reported in previous studies, which observed DBP formation with different organic precursors under chlorine/chloramines [13, 15, 47]. Yang et al. [13] investigated time dependent formation of DBPs from the chlorination of NOM, where rapid chloroform formation was observed within 24 hrs followed by a plateau. In their study, rapid DCAN formation was observed within 4 hrs, followed by a decrease in formation. Similar trends were also reported in the study of *Microcystis aeruginosa* chlorination with different contact times [47]. For HAA formation, the decrease in MCAA and DCAA concentration may be attributed to biodegradation, where several studies have shown bacterial biodegradation of HAAs. Tung and Xie [53] investigated HAA degradation in a full scale water distribution system; they found that increases in HPC were strongly correlated to decay of HAAs. Bayless and Andrew [108] investigated biodegradation of six HAAs in drinking water. The study reported that XAA (MCAA and MBAA) was the most easily degraded HAA species followed by X₂AA (DCAA, BCAA, and DBAA) and TCAA.

5.4.3 DBP Yields of Bacterial Biomass

Fig. 5-5 shows DBP yields of tested strains reported per cell numbers [µg (10¹⁰ cells⁻¹)] with and without bromide (200 µg L⁻¹) [(Fig. 5-6 (a)-(d)) for detailed DBP yield for each species]. DBP yields without bromide addition are shown in Fig. 5-5 (a). *P. aeruginosa* strains possessed DBP yields proportional to the capsular EPS quantity. To compare different DBP yields among four bacterial strains, one-way ANOVA test was applied. Our one-way ANOVA test analysis results indicated that there was a significant
statistical difference among four bacterial strains (see Table 5.1 and 5.2 for details). The mucA (EPS overproducing strain) had the highest DBP yield among the tested *P. aeruginosa* strains (P<0.05). In comparison, *P. putida* exhibited higher chloroform and HAA yields compared to the *P. aeruginosa* strains (P<0.001); however, similar HAN and HK yields were observed for all tested strains. Additionally, TCNM yields of all tested strains were low [~1 μg (10^{10} cells)^{-1}] in the absence of bromide. In the presence of bromide, TCNM yields decreased further and became lower than the MDL (0.1 μg L^{-1}). The decrease of TCNM yields suggest that there were brominated HNMs formed in the presence of bromide. However, the yields of other brominated HNMs were not investigated in this study because of low TCNM yield and even lower expected brominated HNM yields at tested bromide concentration (200 μg L^{-1}).

In consideration of unregulated DBP formation, DCAN was the major species (~80%) in total HAN formation for all tested strains without bromide addition. Examination of HK formation revealed that 1,1,1-TCP yields for all tested strains were similar to those of 1,1-DCP. For HAA\textsubscript{3}, DCAA showed the highest yield compared to those of MCAA and TCAA. Total HAA formation and yields with bromide addition were higher than those without bromide, due to bromide incorporation [79]. Considering THM occurrence, DCBM yield was similar to chloroform yield in all tested strains with the addition of bromide. Small bromoform yields were also observed compared to chloroform and DCBM formation, but no DBCM was detected.

Varied DBP yields from strains can be attributed to differences in EPS quantity and composition. It has been reported that *P. aeruginosa* produces EPS with the
polysaccharide alginate as its major component [33]. In addition to polysaccharides, proteins, lipids, and nucleic acids have been reported in the EPS at much lower orders of magnitude [33, 42]. Compared to the *P. aeruginosa* EPS, the primary biomolecules present in *P. putida* EPS are proteins [190], where the differences in EPS composition may explain DBP speciation. For *P. putida*, both THM and HAA yields were higher compared to those for *P. aeruginosa* (P<0.001). THM precursors are reported to consist mainly of aromatic structures [15, 148]. Thus, the polysaccharide based EPS of *P. aeruginosa* likely contains much less aromatic content compared to protein based EPS of *P. putida*, which may explain the lower THM yield. In addition, proteins or amino acids were reported as important HAA formation precursors when compared to polysaccharides [48, 161, 168]. Therefore, the higher HAA yield for *P. putida* can be attributed to higher protein content in the biomass compared to *P. aeruginosa* strains.

Besides the contribution of capsular EPS to DBP yield, different characteristics and composition of bio-macromolecules in bacterial cells may also affect DBP yield and speciation. Observable N-DBPs produced by chlorination of algT (EPS deficient strain) indicate that intracellular materials of bacterial cells could serve as important DBP precursors as reported previously [62].
Figure 5-5: DBP yield of four tested strains. (a) DBP yield without bromide (b) DBP yield in the presence of bromide (200 μg L⁻¹) (error bar represents the standard deviation of duplicate samples)
DBP yield (µg/10¹⁰ cells)

(a) CHCl₃, MCAA, DCAA, TCAA

(b) TCAN, DCAN, 1,1-DCP, 1,1,1-TCP, TCNM

algT, PAO1, mucA, P.putida
Figure 5-6: DBP yield upon chlorination of bacterial cells (detailed species) (a) regulated DBPs (THMs and HAAs) without bromide; (b) emerging DBPs (HANs, HKs and TCNM) without bromide; (c) regulated DBPs with bromide; (d) emerging DBPs with bromide.
Table 5.1: Results of one way ANOVA test for DBP yield among different bacterial cultures without bromide.

<table>
<thead>
<tr>
<th>Bacterial cultures</th>
<th>algT Ave¹</th>
<th>PAO1 Ave</th>
<th>mucA Ave</th>
<th>P.putida Ave</th>
<th>DBP yield (μg/10¹⁰cells)</th>
<th>SD²</th>
<th>SD</th>
<th>SD</th>
<th>SD</th>
<th>P³</th>
</tr>
</thead>
<tbody>
<tr>
<td>THM</td>
<td>10.9ᵃᵇ</td>
<td>15.1ᵃ</td>
<td>19.3ᵃ</td>
<td>29.3ᶜ</td>
<td>12.9ᵃ</td>
<td>0.7</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HAA</td>
<td>19.4ᵃ</td>
<td>25.7ᵇ</td>
<td>35.9ᶜ</td>
<td>95.8ᵈ</td>
<td>28.5ᵃ</td>
<td>2.9</td>
<td>0.2</td>
<td>4.4</td>
<td>6.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HAN</td>
<td>1.1ᵇ</td>
<td>1.8ᵃ</td>
<td>2.1ᵇ</td>
<td>2.1ᶜ</td>
<td>0.9ᵃ</td>
<td>0.6</td>
<td>0.2</td>
<td>0.6</td>
<td>0.6</td>
<td>0.006</td>
</tr>
<tr>
<td>HK</td>
<td>0.8ᵃ</td>
<td>1.1ᵇ</td>
<td>1.6ᵇ</td>
<td>0.9ᵃ</td>
<td>1.2ᵃ</td>
<td>0.1</td>
<td>0.1</td>
<td>1.2ᵃ</td>
<td>0.1</td>
<td>0.011</td>
</tr>
<tr>
<td>TCNM</td>
<td>0.2ᵃ</td>
<td>0.2ᵇ</td>
<td>0.3ᵃ</td>
<td>0.3ᵃ/c</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Table 5.2: Results of one-way ANOVA test of DBP yield among different bacterial cultures with bromide.

<table>
<thead>
<tr>
<th>Bacterial culture</th>
<th>algT Ave¹</th>
<th>PAO1 Ave</th>
<th>mucA Ave</th>
<th>P.putida Ave</th>
<th>DBP yield (μg/10¹⁰cells)</th>
<th>SD²</th>
<th>SD</th>
<th>SD</th>
<th>SD</th>
<th>P³</th>
</tr>
</thead>
<tbody>
<tr>
<td>THM</td>
<td>12.9ᵃ</td>
<td>17.1ᵇ</td>
<td>21.4ᵃ</td>
<td>28.2ᵃ</td>
<td>12.9ᵃ</td>
<td>0.7</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HAA</td>
<td>28.5ᵃ</td>
<td>33.4ᵃ</td>
<td>43.4ᵇ</td>
<td>121.2ᵃ</td>
<td>28.5ᵃ</td>
<td>2.9</td>
<td>0.2</td>
<td>4.4</td>
<td>6.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HAN</td>
<td>0.9ᵃ</td>
<td>1.6ᵇ</td>
<td>1.8ᵇ</td>
<td>2.3ᵇ</td>
<td>0.9ᵃ</td>
<td>0.1</td>
<td>0.0</td>
<td>0.3</td>
<td>0.0</td>
<td>0.005</td>
</tr>
<tr>
<td>HK</td>
<td>0.6ᵃ</td>
<td>0.6ᵇ</td>
<td>1.0ᵇ</td>
<td>1.2ᵃ</td>
<td>0.6ᵃ/b</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.011</td>
</tr>
<tr>
<td>TCNM</td>
<td>0.1</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.086</td>
</tr>
</tbody>
</table>

1: Average category of DBP yield from duplicate sample

2: Standard Deviation

3: P-value of one way-ANOVA test. P<0.05 indicates there is significant statistical difference in certain DBP yield among four tested strains.

Values were marked by a common letter (a,b,c). Different letter shows certain DBP yield is statistically different from individual bacteria strain (P < 0.05, Tukey test).
5.4.4 DBP Yields of Bacterial EPS

To further elucidate the EPS contribution to DBP yield, EPS were extracted from both *P. aeruginosa* and *P. putida* bacterial cells. Fig. 5-7 and 5-8 show the DBP yield for extracted EPS with DBP categories and detailed speciation, respectively. t-test was used to verify the statistical difference of DBP yields between *P. aeruginosa* EPS and *P. putida* EPS (Table 5.3 to 5.6). In all tested conditions, *P. putida* EPS produced higher DBP yields for both C-DBPs and N-DBPs compared to *P. aeruginosa* EPS (P=0.02 for C-DBPs). In Fig. 5-7, the chloroform, HAA, and HK yields of extracted *P. aeruginosa* EPS were 46.9, 52.7, and 1.1 μg mgC⁻¹, respectively, while *P. putida* EPS yielded 70.7 μg mgC⁻¹ for chloroform, 75.1 μg mgC⁻¹ for HAA₃, and 1.8 μg mgC⁻¹ for HKs. For N-DBPs, *P. putida* EPS also showed a higher DBP yield compared to *P. aeruginosa* EPS. Our t-test analysis indicated that there were statistical differences in regulated DBP yields between two types of EPS tested. However, for some emerging DBPs, the difference was not significant enough according to the t-test, which might be due to small sample size (SS=2). Considering brominated DBP production, Fig. 5-7 (b) shows DBP yield for EPS with the addition of 200 μg L⁻¹ bromide ion. Again, *P. putida* EPS had a higher DBP yield compared to *P. aeruginosa* with added bromide (P=0.04 for C-DBPs). THM and HAA yields increased compared to tests in the absence of bromide ion, due to the formation of brominated DBP species. However, similar DBP yields were observed for HAN and HK formation compared to tests without bromide addition, consistent with a previous study [13]. Furthermore, TCNM yield decreased in the presence of bromide for both types of extracted EPS due to the formation of brominated halonitromethane species.
Figure 5-7: DBP yield of extracted EPS under 20 mg L\(^{-1}\) Cl\(_2\). (a) DBP yield without bromide (b) DBP yield in the presence of bromide (200 μg L\(^{-1}\)) (error bar represents the standard deviation of duplicate samples).

Fig. 5-8 shows the detailed speciation of observed DBP yields. Without bromide addition, chloroform yield was the highest among all DBPs. In the presence of bromide, nearly equal amounts of DCBM and chloroform were formed, while a small bromoform
yield was also observed. DCAA yield was the highest for HAA species as compared to TCAA and MCAA during formation by both types of extracted EPS. In comparison to algal DBP studies, higher formation of DCAA was observed compared to TCAA (DCAA to TCAA ratio was approximate 2.5:1), which may be attributed to bio-macromolecules containing more DCAA precursors [64, 158, 168]. For HAA₉, higher total formation was observed due to the presence of brominated DBPs [129, 145].

Similar to results observed in whole cell biomass experiments, the EPS composition appears to influence DBP yields. However, compared to whole cell DBP yield experiments, the compositions of extracted EPS strongly influence not only DBP yields but also speciation, where protein content of *P. putida* EPS enhanced DBPs yield. Hong et al. [48] investigated DBP yields of surrogate biomolecules [a polysaccharide (starch), a lipid (fish oil) and a protein surrogate (bovine serum albumin)] to predict DBP yield upon chlorination of algal cells. The results indicated that the surrogate protein produced the highest HAA yields, while the lipid surrogate had the highest THM yields. The polysaccharide surrogate showed the lowest overall DBP formation. Thus, the increased protein content in *P. putida* EPS contributed to higher DBP yields when compared to *P. aeruginosa*. In addition, although extracted *P. aeruginosa* EPS primarily consist of alginate, proteins and other organic biomolecules were also detected in its EPS [189, 233], which likely contributed to N-DBP formation. However, detailed information regarding the chemical components and corresponding composition of biomolecules in EPS require further investigations to elucidate DBP formation pathway.
Several studies reported a wide range of DBP yields upon the chlorination of algal extracellular organic matter (EOM), which is composed of amino acids, aliphatic amines,

Figure 5-8: Detail speciation for DBP yield by extracted EPS. (a) DBP yield without bromide (b) DBP yield in the presence of bromide (200 μg L⁻¹)
and nitrogen-heterocyclic aromatics [47, 62]. THM yields were reported in the range of 0.23 to 3.2 chloroform μmol mgC\(^{-1}\), but the yields were dependent upon the chlorine contact time, algal species and growth phases [64]. In our study, the yields of chloroform from EPS were similar to those of algal DBP studies, which suggests algal EOM and extracted bacteria EPS may have similar chemical compositions. For both extracted polysaccharide and protein based EPS, slightly higher HAA yields were observed compared to THM yields (the HAA: THM ratio was approximately 1.1-1.3), and the trend was consistent with studies for algal EOM [63, 64]. For N-DBPs, \(P. \) putida EPS resulted in a higher HAN yield (1.82 μg mgC\(^{-1}\)) compared to that of \(P. \) aeruginosa EPS (0.98 μg mgC\(^{-1}\)). Similarly, TCNM yield of \(P. \) putida EPS (0.99 μg mgC\(^{-1}\)) was also considerably higher than that of \(P. \) aeruginosa EPS (0.53 μg mgC\(^{-1}\)), which indicates compounds containing higher organic nitrogen content in EPS may produce more toxic N-DBPs [62]. Overall, results obtained from this study provide observations on DBP formation by bacteria EPS, suggesting that chemical composition of bacteria EPS plays a significant role in DBP yield and speciation. Based on the findings of this study, the application of high chlorine dose as a biofilm control strategy needs to be applied with caution to prevent unwanted DBP formation during biofilm disinfection.
Table 5.3: t-test results of DBP yield between polysaccharide based EPS and protein based EPS without bromide.

| Extracted EPS | P. aeruginosa EPS | P. putida EPS | P  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave.</td>
<td>SD</td>
<td>Ave.</td>
</tr>
<tr>
<td>DBP yield (μg mgC⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| THM         | 46.9  | 0.5  | 70.7  | 4.8  | 0.010  
| HAA         | 52.7  | 8.7  | 75.0  | 4.7  | 0.043  
| HAN         | 1.0   | 0.0  | 1.8   | 0.5  | 0.076  
| HK          | 1.1   | 0.0  | 1.8   | 0.4  | 0.049  
| TCNM        | 0.5   | 0.1  | 1.0   | 0.3  | 0.102  

1: One-tailed P-value of t-test. P<0.05 indicates that there is significant statistical difference between two extracted EPS

Table 5.4: t-test results of DBP yield between polysaccharide based EPS and protein based EPS with bromide.

| Extracted EPS | P. aeruginosa EPS | P. putida EPS | P  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave.</td>
<td>SD</td>
<td>Ave.</td>
</tr>
<tr>
<td>DBP yield (μg mgC⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| THM         | 51.2  | 3.3  | 62.6  | 3.4  | 0.038  
| HAA         | 82.5  | 11.2 | 102.8 | 7.6  | 0.083  
| HAN         | 1.0   | 0.1  | 1.7   | 0.5  | 0.101  
| HK          | 0.7   | 0.1  | 1.2   | 0.1  | 0.023  
| TCNM        | 0.0   | 0.0  | 0.2   | 0.0  | 0.028  

1: One-tailed P-value of t-test. P<0.05 indicates that there is significant statistical difference between two extracted EPS
Table 5.5: t-test results of DBP yield (C-DBP and N-DBP) between polysaccharide based EPS and protein based EPS without bromide.

<table>
<thead>
<tr>
<th>Extracted EPS</th>
<th>P. aeruginosa EPS</th>
<th>P. putida EPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave.</td>
<td>SD</td>
</tr>
<tr>
<td>DBP yield (μg mgC⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-DBP</td>
<td>99.6</td>
<td>8.1</td>
</tr>
<tr>
<td>N-DBP</td>
<td>2.6</td>
<td>0.2</td>
</tr>
</tbody>
</table>

¹: One-tailed P-value of t-test. P<0.05 indicates that there is significant statistical difference between two extracted EPS

Table 5.6: t-test results of DBP yield (C-DBP and N-DBP) between polysaccharide based EPS and protein based EPS with bromide.

<table>
<thead>
<tr>
<th>Extracted EPS</th>
<th>P. aeruginosa EPS</th>
<th>P. putida EPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave.</td>
<td>SD</td>
</tr>
<tr>
<td>DBP yield (μg mgC⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-DBP</td>
<td>133.7</td>
<td>7.9</td>
</tr>
<tr>
<td>N-DBP</td>
<td>1.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

¹: One-tailed P-value of t-test. P<0.05 indicates that there is significant statistical difference between two extracted EPS
Chapter 6

Relative Contribution of Biomolecules in Bacterial EPS to DBP Formation

This chapter is adapted from: Z. Wang, O. Choi, Y. Seo, Relative Contribution of Biomolecules in Bacterial Extracellular Polymeric Substances to Disinfection By-products Formation, submitted to *Environmental Science & Technology*.

6.1 Abstract

In this study, detailed chemical compositions of the biomolecules in EPS (amino acids, polysaccharide monomers, and fatty acids) from both pure cultures of bacteria and mixed species biofilm isolated from a water utility were analyzed. Then, based on detailed EPS analysis results, DBP yield experiments were conducted with both extracted EPS and surrogate chemicals to indirectly identify the influence of biomolecules and their structures on DBP formation and speciation. DBP yield results of both extracted EPS and EPS surrogates indicated that proteins in EPS have a greater influence on DBP formation, especially nitrogenous DBPs (N-DBPs), where amino acids containing unsaturated organic carbon or conjugated bonds in R-group produced higher amount of DBPs. For regulated DBPs, HAA yields were higher than THM yields, while haloacetonitriles were the dominant DBP species formed among emerging DBPs. However, DBP yields of
polysaccharide monomers were lower than those of tested amino acids groups and the DBP yields of polysaccharide monomers were not significantly influenced by their structures. Considering the results obtained in this study, biofilm needs to be considered an important precursor to DBP formation and biofilm eradication methods for water distribution systems need to be carefully selected to minimize subsequent DBP formation.

6.2 Introduction

Despite significant knowledge of disinfection by-product (DBP) formation in water treatment plants, where water quality parameters and the formation of DBPs can be monitored simultaneously, the formation of DBPs in water distribution systems is not well understood, due largely to the complex interactions occurring in water distribution systems [35, 39, 234].

While there are various factors (e.g. treated water quality, residence time, hydraulics, etc.) known to affect DBP formation in the water distribution system, it is well accepted that materials deposited on distribution pipes, such as corrosion products and biofilm, not only consume a significant amount of disinfectants but also harbor organic DBP precursors. Since water distribution systems are continuously fed with unremoved natural organic matter (NOM) and biofilm formation is ubiquitous in water distribution systems, high concentrations of organic DBP precursors may accumulate on biofilm surfaces as well as in its internal structures [6, 137, 170] and provide a reservoir of organic material for subsequent DBP formation.
A key feature in this process is extracellular polymeric substance (EPS) secreted by biofilm, which comprise more than 80% of hydrated biofilm [33]. Biofilm EPS are composed of a significant concentration of organic carbon and nitrogen rich compounds (e.g. proteins, amino sugars, polysaccharides, and lipids). Analogously, recent studies investigating the DBP yields of algal extracellular organic matter (EOM), which are reported to possess similar chemical composition as biofilm EPS, reported a wide range of DBP yields [48]. Thus, biofilm EPS may possess similar chemical composition and reactivity to DBP precursors and significantly increase disinfectant demand [47, 48, 62, 137, 174]. Considering the persistence of biofilm and current operational responses to biofilm outbreak in water utilities, which include increasing the concentration of residual disinfectants to eradicate biofilm, biofilm and its EPS may be a significant contributor to DBP formation in distribution systems.

However, DBP formation by biofilm is currently not well understood yet [137, 220]. In our previous study, DBP yields from both total bacteria cells with distinct capsular EPS composition and the extracted EPS were investigated upon chlorine disinfection under relevant conditions in the water distribution system. DBP yield results indicated that bacterial EPS facilitated DBP formation upon chlorination [137]. However, due to the complex chemical compositions of EPS, the relative contributions of biomolecules in EPS to DBP formation were not elucidated.

The main purpose of this study is to evaluate the influence of the major biomolecules (proteins, polysaccharides and lipids) in EPS and their chemical compositions on DBP formation and speciation. Extracted EPS from mixed species
biofilm samples isolated from a water utility as well as two bacterial species (*P. aeruginosa* and *P. putida*) with distinct EPS composition were analyzed to identify detailed biomolecular compositions (amino acids, polysaccharide monomers, and fatty acids) of the EPS [33, 41, 42]. To monitor the influence of biomolecules and their structures on DBP formation and speciation, the DBP yield experiments were conducted with both extracted total EPS and surrogate EPS with pure chemicals representing the relative compositions and structural characteristics of the biomolecules present in the EPS.

### 6.3 Materials and Methods

#### 6.3.1 Culture Preparation and EPS Extraction

Single species bacteria strains with distinct EPS compositions [*P. aeruginosa* (polysaccharide-based EPS) and *P. putida* (ATCC# 12633, wild type strain with protein-based EPS)] as well as mixed species biofilm samples obtained from a water utility were used to harvest and analyze the biomolecular composition of their EPS. Both single and mixed species cultures were regrown in 1/10th strength Luria-Bertani (LB) broth and washed with pH=7 phosphate buffer (0.54 g Na$_2$HPO$_4$•H$_2$O and 0.88 g KH$_2$PO$_4$ for 1 L solution) to remove growth media prior to EPS extraction. Detail preparation methods for bacterial cultures can be found in our previous study [135]. In addition, considering the possible changes of EPS composition due to growth and nutrient conditions, direct EPS extraction was also conducted with biofilm samples isolated from the same water utility
for comparison [235]. EPS extraction was performed using the cation exchange resin (CER) method coupled with high speed vortexing and details can be found elsewhere [137, 225].

6.3.2 Determination of the Biomolecular Compositions of EPS

First, to determine the chemical compositions of EPS, both total protein and polysaccharides content (known major components of bacterial EPS) in extracted EPS were measured [33]. The total protein contents were determined by the modified Lowry Protein Assay Kit (Pierce Biotechnology, IL, USA) with the bovine serum albumin as the standard, while the total polysaccharide concentrations were measured by the phenol-sulfuric acid method with glucose as the standard [46].

The total lipids in concentrated bacterial EPS were extracted by a methanol and chloroform mixture (1:2 v/v) followed by 5 minutes of centrifugation. Then, sodium chloride (0.9% w/v) was added into the solvent phase and mixed. After re-centrifugation, the solvent phase was collected and dried in the oven (45 °C) for 15 minutes to obtain lipid extracts [236]. Then, for fatty acid analysis, extracted lipid samples were methylated by 10% BF₃ in methanol (2 mL) at 100°C for 2 minutes. After the methylation step, all samples were cooled down to the room temperature (22 °C) and 2 mL of isoctane and 1 mL of saturated NaCl were added to samples. Then, the upper isoctane layer was collected for GC analysis in triplicate. Detail GC analysis condition can be found elsewhere [237].
Heterotrophic plate counting (HPC) was conducted with R2A agar to determine total cell numbers for the calculation of the total polysaccharide and total protein contents (µg (10^10 cells)^{-1}; Table 6.1). To enumerate bacteria numbers in the biofilm, the isolated biofilm samples were harvested by being homogenized and vortexed before the HPC test.

Prior to detailed EPS composition analysis, all extracted EPS samples were concentrated using a freeze dryer (Labconco, MO, USA). Then the concentrated samples were filtered by 0.2 µm membranes (Millipore, MA, USA). Due to the complexity of proteins and polysaccharides in EPS, it was difficult to identify and harvest each protein and polysaccharide and their relative contribution to DBP formation. Therefore, proteins and polysaccharides were further broken down to “subunits” (amino acids and polysaccharide monomers) and their relative compositions in EPS were quantified.

For amino acid analysis, extracted EPS samples were dried in glass tubes in a vacuum concentrator and subjected to vapor phase hydrolysis by 6 N HCl at 110°C for 24 hours under argon gas. Then, they were reconstituted in borate buffer and analyzed using a Hewlett-Packard AminoQuant II system (Palo Alto, CA, USA) with a high performance liquid chromatography (HPLC, Hewlett Packard, 1100) according to the manufacturers’ specifications at the Laboratory for Protein Chemistry of the Texas A & M University. Derivatization was performed using o-phthalaldehyde (reacts with primary amino acids) and 9-fluoromethyl-chloroformate (secondary amino acids). Then, the derivatized amino acids were separated by a narrow bore Hypersil AA-ODS reverse phase column (2.1 x 200 mm, 5 mm; Agilent, CA, USA) and detected by a diode array detector. For HPLC mobile phase, a gradient started with 100% solvent A (20 mM Na
acetate buffer with 0.018% triethylamine, 0.05 mM Ethylenediaminetetraacetic acid (EDTA), 0.3% tetrahydrofuran at pH=7.2) for 17 minutes at 0.45 mL min$^{-1}$ and then increased to 60% of solvent B (20% 100 mM Na acetate buffer with 40% acetonitrile and 40% methanol). Two internal standards (norvaline and sarcosine) were used to quantify the amino acids. All samples were analyzed in duplicate [118].

For polysaccharide monomer analysis, extracted EPS samples were treated at higher temperature (125 °C) and set in a water bath for heating at 90 °C (7 hours) to partially break down polysaccharide linkage to monomers [238]. Then samples were filtrated by a 0.2 μm membrane to remove impurities. Filtered samples were analyzed by an HPLC (1200, Agilent, CA, USA) with a refractive index detector. During the HPLC analysis, carbohydrates were separated by a Shodex SH1011 (Shodex, NY, USA) column coupled with a guard column. 5 mM sulfuric acid was used as the mobile phase (flow rate at 0.6 mL min$^{-1}$). The temperature of the column oven was kept constant at 65 °C.

Besides protein and polysaccharide analyses, total lipid contents of EPS were also measured, where only trace amount of lipids were detected.

6.3.3 EPS Surrogate Preparation

After quantifying the detail chemical composition of EPS, EPS surrogates with pure chemicals (amino acids and polysaccharide monomers) were prepared in deionized water (18.2 MΩ) for DBP yield experiments. Due to the solubility differences of amino acids in water, EPS protein surrogates with amino acids from equal molar of amino acid standards (1 mM) were diluted from different concentrations of stock solutions. Then,
EPS protein surrogates solutions were prepared by adding different volume of amino acid standard solutions based on amino acid ratios obtained from EPS analysis results (Table 6.2). To further investigate the influence of chemical structures of biomolecules in EPS on both DBP yields and speciation, grouped amino acid solutions were also prepared based on their chemical structure characteristics in R-groups (acetic, basic, aliphatic, aromatic, polar uncharged, and hydrophobic shown in Table 6.3).

Similar to EPS protein surrogate preparations, EPS polysaccharide surrogates were prepared with 1 mM standard solutions of pure polysaccharide monomers from the Sigma Aldrich and solutions were mixed according to their relative composition ratios obtained from the EPS analysis results (Table 6.4). To further explore the influence of chemical structures of polysaccharide monomers on DBP yields, polysaccharide monomers were grouped based their structural characteristics (pentose, hexose, uronic acid, and sugar amine in Table 6.5). For each prepared surrogate solution, total organic carbon (TOC) content was determined using a TOC analyzer (TOC-V_{SCH}, Shimadzu, Japan) to normalize DBP formation.

### 6.3.4 DBP Yields of Bacterial EPS and Surrogates

To investigate DBP formation from biomolecules in EPS, DBP yield experiments were conducted at pH 7 with both extracted EPS and EPS surrogate solutions using 40 mL amber glass vials. Samples were exposed to excessive Cl₂ doses without head space for 7 days at room temperature (22 ± 2 °C) in the dark following the standard method 5710B[226]. Residual chlorine concentrations were measured by the DPD method via a
spectrophotometer to confirm that extracted EPS and surrogate solutions were completely reacted with Cl$_2$ [226].

6.3.5 DBP Analysis

Both C-DBPs (THMs, HAAs, and HKs) and N-DBPs (HANs and TCNM) were analyzed in this study. Analysis of CF, HAA$_3$ (MCAA, DCAA, and TCAA), HAN$_2$ (DCAN and TCAN), HK$_2$ (1,1-DCP and 1,1,1-TCP), and TCNM were carried out using a gas chromatograph (GC-2010 Plus, Shimadzu, Kyoto, Japan) with dual electron-capture detectors. Detailed DBP analysis method can be found in section 5.2.3 [137]. Spike recoveries were determined during the entire study and the minimal detection limits (MDL) of all DBPs were around 0.1 $\mu$g L$^{-1}$.

6.4 Results

6.4.1 Chemical Characterization of EPS

Table 6.1 shows the total protein and polysaccharide content in the extracted EPS. From the table, $P. \ putida$ EPS showed the highest protein content compared to other EPS [137, 190]. The CER extraction method was selected over other chemical extraction methods to minimize chemical interference on DBP yields. Compared to results from our previous study, the EPS extraction efficiency of CER extraction appeared to be lower than that of the EDTA extraction (chemical) method [219, 239]. The isolated biofilm EPS
from a water utility showed the highest amount of polysaccharide, which may be attributed to the biosorption of NOM on biofilm [137].

**Table 6.1**: Total protein and total polysaccharide contents in extracted EPS.

<table>
<thead>
<tr>
<th>Extracted EPS</th>
<th>EPS characteristics</th>
<th>Total protein content [μg (10^{10} cells)^{-1}]</th>
<th>Total polysaccharide content [μg (10^{10} cells)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> EPS</td>
<td>polysaccharide based (cell bounded)</td>
<td>13.0± 1.0</td>
<td>76.3 ± 3.3</td>
</tr>
<tr>
<td><em>P. putida</em> EPS</td>
<td>protein based (cell bounded)</td>
<td>68.4 ± 0.8</td>
<td>30.0 ± 1.7</td>
</tr>
<tr>
<td>Biofilm EPS (regrown)(^1)</td>
<td>unknown (cell bounded)</td>
<td>33.5 ± 4.7</td>
<td>37.6 ± 1.8</td>
</tr>
<tr>
<td>Biofilm EPS (isolated)(^2)</td>
<td>unknown (biofilm)</td>
<td>18.2 ± 1.0</td>
<td>223.1 ± 17.6</td>
</tr>
</tbody>
</table>

1 Biofilm EPS (regrown): EPS was extracted from mixed culture biofilm regrown in 1/10\(^{th}\) LB broth.

2 Biofilm EPS (isolated): EPS was extracted from mixed culture biofilm isolated from a water utility (without modification).

*Total protein and total polysaccharide were measured in triplicate for each EPS.

Table 6.2 summarizes the relative amino acids composition in the four extracted EPS. From the analysis, only 16 amino acids were identified from all extracted EPS. The asparagine (Asn)/aspartic acid (Asp) and the glutamine (Gln)/glutamic acid (Glu) could not be distinguished by the selected amino acid method since during the acid hydrolysis Gln and Asn were deamidated to Glu and to Asp, respectively. Thus, the Asx represents the combination of the Asn/Asp and Glx represents Gln/Glu. In addition, both cysteine (Cys) and tryptophan (Trp) were not detected during the analysis, which may be attributed to acid hydrolysis [240, 241].
Relative amino acids ratio between the regrown biofilm EPS and isolated biofilm EPS were slightly different, as the growth, nutrient, and environmental condition could influence the chemical composition of EPS [235]. Previous studies indicated that alanine (Ala) and glycine (Gly) were dominant amino acids in bacterial EPS. For example, Kachlany et al. [242] found that Ala was the dominant amino acid in EPS of *P. putida* G7, followed by Gly. Westerhoff et al. [243] also reported that Gly was the dominant amino acids in extracted EPS from activated sludge followed by Ala. From Table 6.2, Ala was the most abundant amino acid in all extracted EPS, with the relative composition ratios of 20.37%, 15.39%, 19.3%, and 11.6% for *P. aeruginosa* EPS, *P. putida* EPS, regrown biofilm EPS, and isolated biofilm EPS, respectively. The second abundant amino acid in the extracted EPS was the Gly, which was slightly higher than 10% for all extracted EPS.

In addition to amino acids analysis, polysaccharide monomers were also analyzed by HPLC. Six sugars, two sugar acids, and one amino sugar were detected from EPS (Table 6.4). *P. aeruginosa* EPS contained D-glucosamine, D-glucuronic acid, D-galacturonic acid, D-glucose, D-mannose, D-arabinose, and L-fucose, except the L-rhamnose and D-ribose, where D-glucosamine, D-glucuronic acid, and L-fucose were main components in its EPS. For *P. putida* EPS, similar compositions were observed compared to *P. aeruginosa* EPS, however less amount of polysaccharide monomers were detected. This was expected as protein is known to be the main component in *P. putida* EPS [190]. For both regrown and isolated biofilm EPS, D-glucosamine was found as the main component. The EPS from regrown biofilm had higher content of D-glucuronic acid but lower D-glucose compared to those from isolated biofilm. In addition to D-glucuronic
acid and D-galacturonic acid, small amount of other organic acids (acetic acid and succinic acid) were also detected from *P. aeruginosa* EPS.

Previous studies reported that glucose, rhamnose, galactose, mannose, glucuronic acid, and glucosamine are the main polysaccharides monomers in biofilm EPS [33, 42, 222, 244]. However, as expected, the compositions of polysaccharide monomers in the bacterial EPS were different, since bacteria species and bacterial growth condition may significantly affect chemical composition of EPS [245].

### 6.4.2 DBP Yields of Extracted EPS

Fig. 6-1a shows both C-DBP and N-DBP yields of extracted EPS. *P. putida* EPS had the highest CF and HAA₃ yields compared to *P. aeruginosa* EPS and biofilm EPS (both the regrown and the isolated). In addition, *P. putida* EPS also possessed higher emerging DBPs yields (HAN₂, HK₂, and TCNM) compared to *P. aeruginosa* EPS displaying consistent trends observed in our previous study [137]. However, the highest HAN₂ yield (4.0 μg mgC⁻¹) and HK₂ yield (7.1 μg mgC⁻¹) were observed in the isolated biofilm EPS.

The highest CF yield (62.9 μg mgC⁻¹) and HAA₃ yield (72.5 μg mgC⁻¹) from *P. putida* EPS was mainly attributed to the highest total protein content compared to *P. aeruginosa* EPS and biofilm EPS (both regrown and isolated) [Table 6-1]. Proteins have been widely reported as important precursors for both THM and HAA formations [48, 179]. Relatively high levels of N-DBP yields from *P. putida* were also observed due to higher organic nitrogen (org-N) content present in protein [56]. The biofilm isolated EPS
contained very high total polysaccharide content compared to all other extracted EPS (Table 6.1). Previous studies have reported that polysaccharides were the major components in biofilm EPS matrix [33, 189], and polysaccharides are known to have lower DBP yields upon chlorination [48, 165, 166]. However, isolated biofilm EPS showed extremely high formation of HAN$_2$ and HK$_2$ among all tested EPS. Compared to isolated biofilm EPS, the EPS from regrown biofilm did not show high level of HAN and HK yields. Since the isolated biofilm EPS hold much higher concentration of polysaccharide than EPS from regrown biofilm (6 times higher), the isolated biofilm EPS appears to be associated with various natural organic matters that have been reported to contribute towards high DBP yields [246] such as known N-DBP precursors (humic acid and fulvic acid) [56].

6.4.3 DBP Yields of EPS Protein Surrogates

Fig. 6-1b shows the DBP yields from tested protein surrogates (16 amino acids according to their relative compositions reported at the Table 6.2). Asn and Gln were selected to represent Asx and Glx for DBP yield experiments. The surrogates for *P. aeruginosa* and *P. putida* EPS had nearly equal CF yields while the surrogate for isolated biofilm EPS had the lowest CF yield (11.7 μg mgC$^{-1}$). In contrast to the CF yield, the surrogate for isolated biofilm EPS had the highest HAA yield (86.3 μg mgC$^{-1}$) followed by the *P. putida* EPS surrogates (69.8 μg mgC$^{-1}$), the *P. aeruginosa* EPS surrogate (57.1 μg mgC$^{-1}$), and the regrown biofilm EPS surrogate (47.9 μg mgC$^{-1}$). For emerging DBPs, *P. putida* EPS surrogate showed higher HK, HAN and TCNM yields compared to the other extracted EPS.
Hong et al. reported that 20 selected amino acids produced the low CF yield (<4.19 μg mgC⁻¹) except tryptophan (Trp) (45.8 μg mgC⁻¹) and tyrosine (Tyr) (147 μg mgC⁻¹) [168]. In our amino acid analysis, only Tyr could be detected, as the Trp was destroyed during the acid hydrolysis pretreatment. Isolated biofilm EPS possessed the lowest Tyr composition (1.2%) compared to those of other extracted EPS, which might be a reason for the lowest CF yield of its surrogate (Fig. 6-1b). The *P. aeruginosa* EPS surrogate showed slightly higher CF yield (27.2 μg mgC⁻¹) compared to that of the *P. putida* EPS surrogate (24.0 μg mgC⁻¹). This may be related to higher Tyr content in the *P. aeruginosa* EPS (3.6 %) than that of *P. putida* EPS (2.7 %). However, the CF yield (17.3 μg mgC⁻¹) of the regrown biofilm EPS surrogate was lower than that of the *P. putida* EPS surrogate, even though the Tyr content in the regrown biofilm EPS (2.9%) was slightly higher than the *P. putida* EPS surrogate (2.7%). This might be related to higher Gln content in the regrown biofilm EPS (13.6%) compared to that of *P. putida* EPS (8.7%), which was previously reported not to produce any CF upon chlorination [161, 168].

For HAA formation, previous studies indicated that aromatic or conjugated structures in amino acids, such as Trp, Tyr, His, and Pro, could provide important reaction sites [161, 166, 168, 178]. Besides, Asp and Asn were also reported to form high level of HAAs upon high Cl₂ dose [168]. The highest Asn content in the isolated biofilm EPS (13.0%) may be a cause for highest HAA yield than that of its surrogate. Similarly, regrown biofilm EPS with the lowest Asn (6.0%) and His (0.6%) contents showed the lowest HAA yield from its surrogate.
For N-DBPs, most EPS protein surrogates possessed higher HAN yields when compared to the extracted EPS (Fig. 6-1b). However, in this study, no TCAN formation was observed from both isolated and regrown biofilm EPS surrogates.

Yang et al. investigated the DCAN yield of individual amino acids [169]. Their results indicated that Trp, Tyr, Asn, and Ala possessed higher DCAN yields among the 20 tested amino acids (Asn>Ala>Tyr>Trp), proposing that amino acids may firstly reacted with HOCl and produce organic n-chloramine to form DCAN [178]. However, Bond et al. reported the Trp had the highest DCAN yield followed by the Asp and the Asn, which showed almost equal DCAN yields (~0.05 μM mmol⁻¹ compound) [166]. In addition, Chu et al. reported that minimum amount of HANs were formed during chlorination of Ala [173]. Slightly different results for DCAN yields from previous studies on the amino acids may be caused by somewhat different experimental conditions applied (e.g. reaction time, temperature, and pH) [166, 169, 180]. In our study, higher DCAN yields from *P. putida* EPS protein surrogate may be attributed to relatively higher Asn, Ala, and Tyr content. In addition, DCAN yields can be further influenced by other factors such as hydrolysis and competitive reactions of intermediates. For example, when Asn reacts with Cl₂, an intermediate [dichlorocyanooacetic acid (HOOC-CCl₂-CN)] can be formed, which leads to the formation of either DCAN (via decarboxylation) or DCAA/TCAA (via hydrolysis)[123, 168]. For the isolated biofilm EPS, although significant Asn content was observed in the EPS, the low content of the Tyr in its EPS may cause a relatively lower HAN yield. In addition, it has been reported that DCAN could be further hydrolyzed to haloacetamides (HAcAms), which may also affect final DCAN levels [134, 180, 247].
For TCNM yields, the protein surrogate for the *P. putida* EPS showed similar TCNM yield (1.0 μg mgC\(^{-1}\)) compared to other EPS surrogates (~0.7 μg mgC\(^{-1}\)). Previous studies reported that amino acids, including Ala, showed very close TCNM formation potential (~4 nM) [120] and that disinfection methods (e.g. preozonation) are more critical for TCNM formation than the chemical structure of amino acids [120, 184].
Figure 6-1: C-DBP and N-DBP yields of EPS and protein and polysaccharide surrogates. (a) DBP yields of extracted EPS; (b) DBP yields of protein surrogates (amino acids); (c) DBP yields of polysaccharide monomer surrogates (error bar represents the standard deviation of duplicate samples).
6.4.4 DBP Yields of EPS Polysaccharide Surrogates

Fig. 6-1c shows the DBP yields of the polysaccharide surrogates for all extracted EPS. Compared to the DBP yield results of both extracted EPS and EPS protein surrogates (Fig. 6-1a, b), all EPS polysaccharide surrogates showed relatively lower C-DBP and N-DBP yields. It was also noticed that HAA and emerging DBP yields of EPS polysaccharide surrogates were much lower than EPS protein surrogates. However, similar CF yields were observed from polysaccharide EPS surrogates compared to those of protein EPS surrogates. For N-DBPs, only DCAN and TCNM were monitored upon the chlorination of glucosamine (the only monomer that contains org-N), where yields were lower than 1 μg mgC⁻¹.

The polysaccharide surrogates (monomers) have five or six saturated carbon ring structures (Table 6.5). Since previous studies widely reported that compounds with phenolic structures and unsaturated/conjugated carbon bonds are major DBP precursors [165, 166, 168, 248], it was expected that the chemical structures of polysaccharide surrogates would possess lower DBP yields. Bond et al. tested the DBP yields of D-xylose, arabinose, and D-mannose and reported that those polysaccharide monomers had low DBP yields and chlorine demands [165, 166]. Similar to their studies, the polysaccharide surrogates tested in this study contributed very analogous DBP yields as they contained similar chemical structures.
Table 6.2: Amino acid compositions in extracted EPS.

<table>
<thead>
<tr>
<th></th>
<th>Asx</th>
<th>Glx</th>
<th>Ser</th>
<th>His</th>
<th>Gly</th>
<th>Thr</th>
<th>Ala</th>
<th>Arg</th>
<th>Tyr</th>
<th>Val</th>
<th>Met</th>
<th>Phe</th>
<th>Ile</th>
<th>Leu</th>
<th>Lys</th>
<th>Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight (Da)</td>
<td>115.1/133.1</td>
<td>129.1/147.1</td>
<td>87.1</td>
<td>137.2</td>
<td>57.1</td>
<td>101.1</td>
<td>71.1</td>
<td>156.2</td>
<td>163.2</td>
<td>99.1</td>
<td>131.2</td>
<td>147.2</td>
<td>113.2</td>
<td>113.2</td>
<td>128.2</td>
<td>97.1</td>
</tr>
<tr>
<td>P. aeruginosa EPS</td>
<td>6.4%</td>
<td>6.0%</td>
<td>2.7%</td>
<td>0.8%</td>
<td>11.1%</td>
<td>3.8%</td>
<td>20.4%</td>
<td>3.9%</td>
<td>3.6%</td>
<td>8.0%</td>
<td>3.6%</td>
<td>2.9%</td>
<td>5.2%</td>
<td>9.6%</td>
<td>4.6%</td>
<td>7.1%</td>
</tr>
<tr>
<td>P. putida EPS</td>
<td>8.8%</td>
<td>8.7%</td>
<td>3.9%</td>
<td>1.5%</td>
<td>12.7%</td>
<td>4.6%</td>
<td>15.4%</td>
<td>5.6%</td>
<td>2.7%</td>
<td>7.5%</td>
<td>1.8%</td>
<td>3.2%</td>
<td>4.9%</td>
<td>8.7%</td>
<td>5.8%</td>
<td>4.2%</td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>6.0%</td>
<td>13.6%</td>
<td>3.1%</td>
<td>0.6%</td>
<td>12.1%</td>
<td>4.5%</td>
<td>19.3%</td>
<td>2.6%</td>
<td>2.9%</td>
<td>8.4%</td>
<td>2.8%</td>
<td>2.9%</td>
<td>5.7%</td>
<td>7.7%</td>
<td>2.5%</td>
<td>4.9%</td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>13.0%</td>
<td>12.9%</td>
<td>8.0%</td>
<td>0.7%</td>
<td>14.7%</td>
<td>7.1%</td>
<td>11.6%</td>
<td>1.2%</td>
<td>1.2%</td>
<td>8.1%</td>
<td>1.2%</td>
<td>2.2%</td>
<td>4.9%</td>
<td>6.2%</td>
<td>1.6%</td>
<td>5.5%</td>
</tr>
</tbody>
</table>

Biofilm EPS (regrown) \(^1\): EPS was extracted from mixed species biofilm regrown in 1/10\(^{th}\) LB broth.

Biofilm EPS (isolated) \(^2\): EPS was directly extracted from mixed species biofilm isolated from a water utility.

Abbreviations: Asx- asparagine/aspartic acid; Glx- glutamine/glutamic acid; Ser- serine; His- histidine; Gly- glycine; Thr- threonine; Ala- alanine; Arg- arginine; Tyr- tyrosine; Val- valine; Met- methionine; Phe- phenylalanine; Ile- isoleucine; Leu- leucine; Lys- lysine; Pro- proline.
### Table 6.3: Amino acid groups based on R-group properties

<table>
<thead>
<tr>
<th>R group property</th>
<th>Compound</th>
<th>Abbreviation</th>
<th>Formula</th>
<th>R-group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acidic</strong></td>
<td>Aspartic acid</td>
<td>Asp</td>
<td>C_4H_7NO_4</td>
<td>-CH_2-COOH</td>
</tr>
<tr>
<td></td>
<td>Glutamic acid</td>
<td>Glu</td>
<td>C_5H_9NO_4</td>
<td>-(CH_2)_2-COOH</td>
</tr>
<tr>
<td><strong>Basic</strong></td>
<td>Arginine</td>
<td>Arg</td>
<td>C_6H_14N_4O_2</td>
<td>-(CH_2)_3-NH-C-</td>
</tr>
<tr>
<td></td>
<td>Histidine</td>
<td>His</td>
<td>C_6H_3N_2O_2</td>
<td>-CH</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>Lys</td>
<td>C_6H_14N_2O_2</td>
<td>-(CH_2)_4-NH_2</td>
</tr>
<tr>
<td><strong>Aliphatic</strong></td>
<td>Serine</td>
<td>Ser</td>
<td>C_3H_7NO_3</td>
<td>-CH_2-OH</td>
</tr>
<tr>
<td></td>
<td>Threonine</td>
<td>Thr</td>
<td>C_4H_9NO_3</td>
<td>-CH</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>Ala</td>
<td>C_3H_7NO_2</td>
<td>-CH_3</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>Gly</td>
<td>C_2H_5NO_2</td>
<td>-H</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>Iso</td>
<td>C_6H_11NO_2</td>
<td>-CH</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>Leu</td>
<td>C_6H_13NO_2</td>
<td>-CH_2-CH-(CH_3)_2</td>
</tr>
<tr>
<td></td>
<td>Proline</td>
<td>Pro</td>
<td>C_5H_9NO_2</td>
<td>-(CH_2)_3-NH_2</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>Val</td>
<td>C_5H_11NO_2</td>
<td>-CH-(CH_3)_2</td>
</tr>
<tr>
<td><strong>Aromatic</strong></td>
<td>Phenylalanine</td>
<td>Phe</td>
<td>C_9H_11NO_2</td>
<td>-CH_2-</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>Tyr</td>
<td>C_9H_11NO_3</td>
<td>-CH_2-3</td>
</tr>
<tr>
<td><strong>Polar Uncharged</strong></td>
<td>Serine</td>
<td>Ser</td>
<td>C_3H_7NO_3</td>
<td>-CH_2-OH</td>
</tr>
<tr>
<td></td>
<td>Threonine</td>
<td>Thr</td>
<td>C_4H_9NO_3</td>
<td>-CH</td>
</tr>
<tr>
<td></td>
<td>Asparagine</td>
<td>Asn</td>
<td>C_5H_10N_2O_3</td>
<td>-CH_2-CO-NH_2</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>Gln</td>
<td>C_5H_10N_2O_3</td>
<td>-(CH_2)_2-CO-NH_2</td>
</tr>
<tr>
<td><strong>Hydrophobic</strong></td>
<td>Alanine</td>
<td>Ala</td>
<td>C_3H_11NO_2</td>
<td>-CH-(CH_3)_2</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>Val</td>
<td>C_3H_11NO_2</td>
<td>-CH-(CH_3)_2</td>
</tr>
<tr>
<td></td>
<td>Isoleucine</td>
<td>Iso</td>
<td>C_6H_13NO_2</td>
<td>-CH</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>Leu</td>
<td>C_6H_13NO_2</td>
<td>-CH_2-CH-(CH_3)_2</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>Met</td>
<td>C_5H_11NO_2S</td>
<td>-(CH_2)_2-S-CH_3</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine</td>
<td>Phe</td>
<td>C_9H_11NO_2</td>
<td>-CH_2-</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>Tyr</td>
<td>C_9H_11NO_3</td>
<td>-CH_2-3</td>
</tr>
</tbody>
</table>
Table 6.4: Polysaccharide monomer compositions in extracted EPS.

<table>
<thead>
<tr>
<th></th>
<th>Sugar amine</th>
<th>Uronic acid</th>
<th>Hexose</th>
<th>Pentose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D-glucosamine</td>
<td>D-glucuronic acid</td>
<td>D-galacturonic acid</td>
<td>D-glucose</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>179.2</td>
<td>194.1</td>
<td>180.6</td>
<td>180.2</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> EPS</td>
<td>22.0%</td>
<td>31.0%</td>
<td>0.3%</td>
<td>0.8%</td>
</tr>
<tr>
<td><em>P. putida</em> EPS</td>
<td>27.2%</td>
<td>33.8%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>83.6%</td>
<td>15.1%</td>
<td>0.0%</td>
<td>1.3%</td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>81.1%</td>
<td>5.9%</td>
<td>0.0%</td>
<td>12.3%</td>
</tr>
</tbody>
</table>

Biofilm EPS (regrown): EPS was extracted from mixed species biofilm regrown in 1/10<sup>th</sup> LB broth.

Biofilm EPS (isolated): EPS was directly extracted from mixed species biofilm isolated from a water utility.
Table 6.5: Polysaccharide monomer groups.

<table>
<thead>
<tr>
<th>Property</th>
<th>Compound</th>
<th>Formula</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentose</td>
<td>D-arabinose</td>
<td>C₅H₁₀O₅</td>
<td><img src="image" alt="D-arabinose" /></td>
</tr>
<tr>
<td></td>
<td>D-ribose</td>
<td>C₅H₁₀O₅</td>
<td><img src="image" alt="D-ribose" /></td>
</tr>
<tr>
<td>Hexose</td>
<td>D-glucose</td>
<td>C₆H₁₂O₆</td>
<td><img src="image" alt="D-glucose" /></td>
</tr>
<tr>
<td></td>
<td>D-mannose</td>
<td>C₆H₁₂O₆</td>
<td><img src="image" alt="D-mannose" /></td>
</tr>
<tr>
<td></td>
<td>L-fucose</td>
<td>C₆H₁₂O₅</td>
<td><img src="image" alt="L-fucose" /></td>
</tr>
<tr>
<td></td>
<td>L-rhamnose</td>
<td>C₆H₁₂O₅</td>
<td><img src="image" alt="L-rhamnose" /></td>
</tr>
<tr>
<td>Uronic acid</td>
<td>D-glucuronic acid</td>
<td>C₆H₁₀O₇</td>
<td><img src="image" alt="D-glucuronic acid" /></td>
</tr>
<tr>
<td></td>
<td>D-galacturonic acid</td>
<td>C₆H₁₀O₇</td>
<td><img src="image" alt="D-galacturonic acid" /></td>
</tr>
<tr>
<td>Amino sugar</td>
<td>D-glucosamine</td>
<td>C₆H₁₃NO₅</td>
<td><img src="image" alt="D-glucosamine" /></td>
</tr>
</tbody>
</table>
6.4.5 Influence of Chemical Structures of Biomolecules in EPS on DBP Yields

To further investigate the influence of chemical structures of biomolecules in EPS on both DBP yields and speciation, amino acids and polysaccharide monomers were grouped based on their structural characteristics.

Table 6.6 shows the DBP yield and the speciation results of the grouped amino acids. Overall, the influence of the chemical structures of amino acids in EPS on DBP yields became more apparent compared to the results from EPS surrogate experiments. For the CF yields, the amino acids in the aliphatic group showed the lowest yields (from 4.3 to 11.8 μg mgC⁻¹) because of less reactive sites (saturated hydrocarbon structures) in the R-groups [166, 168]. In contrast, the aromatic group (Phe and Tyr) showed slightly higher CF yields (from 21.5 to 32.1 μg mgC⁻¹) compared to other amino acid groups, which can be attributed to Tyr contribution to CF formation [168]. However, there were no significant differences in CF yields (in the range of 10-20 μg mgC⁻¹) of the tested hydrophobic groups except the hydrophobic group for *P. putida* EPS (42.0 μg mgC⁻¹).

For HAA₃ yields, DCAA was the main species formed from all amino acid groups except the aromatic group, which showed higher TCAA yield. Previous studies also indicated that Tyr in aromatic group could produce higher TCAA than DCAA [165, 168]. In addition, the acidic group was found to have extremely high DCAA yields (500-600 μg mgC⁻¹) among all groups (~4 to 40 times higher than other groups). Asp has been known to be a crucial precursor for DCAA formation [165, 166], which could explain the results observed. Asn (polar uncharged group), Tyr (aromatic group) and His (basic
group) have been reported as important HAA precursors [168]. Our results also indicated that the polar uncharged, aromatic, and basic groups produced relatively high HAA yields, while the aliphatic group showed the lowest HAA yields.

For emerging DBPs, higher HAN yields were observed in acidic and polar groups (~10 μg mgC\(^{-1}\)) than any other groups. DCAN was the dominant HAN species with much higher yields than TCAN (mostly <1 μg mgC\(^{-1}\)). For HK\(_2\) yields, the hydrophobic groups showed higher 1,1,1-TCP yields compared to those of other groups. For 1,1-DCP yields, low yields (<0.3 μg mgC\(^{-1}\)) were observed for all groups. For TCNM, all amino acid groups showed yields ranging from 0.1 to 1.4 μg mgC\(^{-1}\).

Similar to DBP yield results observed from EPS surrogate tests, no significant differences were found in DBP yields among grouped polysaccharide monomers (Table 6.7). Overall, CF yields ranged from 17.7 to 36.0 μg mgC\(^{-1}\), with an average of 25.0 μg mgC\(^{-1}\). HAA\(_3\) yields were higher than those of CF (1.2 to 2.7 times), where DCAA was the dominant species followed by TCAA and MCAA. For N-DBPs, only glucosamine showed low yields of DCAN and TCNM (~0.5 μg mgC\(^{-1}\))
Table 6.6: DBP yields of grouped amino acids for extracted EPS.

<table>
<thead>
<tr>
<th>Amino acid groups</th>
<th>Extracted EPS</th>
<th>THM</th>
<th>MCAA</th>
<th>DCAA</th>
<th>TCAA</th>
<th>DCAN</th>
<th>TCAN</th>
<th>1,1-DCP</th>
<th>1,1,1-TCP</th>
<th>TCNM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acidic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> EPS</td>
<td>21.7 ± 4.0</td>
<td>15.3 ± 4.5</td>
<td>6249 ± 98.8</td>
<td>27.7 ± 7.1</td>
<td>13.0 ± 0.5</td>
<td>0</td>
<td>0.2 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> EPS</td>
<td>11.9 ± 0.5</td>
<td>8.4 ± 5.6</td>
<td>5733 ± 19.5</td>
<td>15.3 ± 2.8</td>
<td>10.1 ± 3.6</td>
<td>0</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>14.1 ± 1.7</td>
<td>8.8 ± 0.9</td>
<td>4928 ± 79.6</td>
<td>9.1 ± 2.6</td>
<td>8.8 ± 1.0</td>
<td>0</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>15.5</td>
<td>8.0 ± 2.1</td>
<td>5344 ± 61.7</td>
<td>13.9 ± 0.5</td>
<td>8.6 ± 2.3</td>
<td>0</td>
<td>0.1 ± 0</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td><strong>Basic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> EPS</td>
<td>11.3 ± 0.2</td>
<td>7.2 ± 0.7</td>
<td>443 ± 3.6</td>
<td>10.4 ± 1.9</td>
<td>2.2 ± 0.4</td>
<td>0</td>
<td>0</td>
<td>0.7 ± 0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> EPS</td>
<td>12.2 ± 1.5</td>
<td>8.1 ± 0.1</td>
<td>539 ± 0.8</td>
<td>13.4 ± 3.5</td>
<td>2.4 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>0.7 ± 0.4</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>13.5 ± 4.2</td>
<td>7.5 ± 1.4</td>
<td>464 ± 3.4</td>
<td>14.1 ± 8.5</td>
<td>2.0 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>17.6 ± 0.6</td>
<td>2.3 ± 0.4</td>
<td>821 ± 0.1</td>
<td>12.7 ± 1.9</td>
<td>1.7 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td><strong>Aliphatic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> EPS</td>
<td>43 ± 0.7</td>
<td>2.6 ± 0.1</td>
<td>8.4 ± 0.3</td>
<td>4.5 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0</td>
<td>0.2</td>
<td>1.0 ± 0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> EPS</td>
<td>69 ± 0.4</td>
<td>3.4 ± 0.8</td>
<td>112 ± 2.3</td>
<td>10.2 ± 5.0</td>
<td>0.8 ± 0.0</td>
<td>0</td>
<td>0</td>
<td>1.1 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>94 ± 3.1</td>
<td>2.7 ± 0.5</td>
<td>9.9 ± 0.4</td>
<td>8.2 ± 2.9</td>
<td>1.0 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>1.3 ± 0.2</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>118 ± 0.3</td>
<td>2.9 ± 0.1</td>
<td>116 ± 1.2</td>
<td>8.5 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0</td>
<td>0</td>
<td>1.6 ± 0.5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td><strong>Aromatic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> EPS</td>
<td>28.4 ± 1.0</td>
<td>9.2 ± 0.7</td>
<td>47.7 ± 10.8</td>
<td>75.2 ± 9.4</td>
<td>2.2 ± 0.6</td>
<td>0</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> EPS</td>
<td>28.4 ± 0.6</td>
<td>10.9 ± 1.1</td>
<td>42.1 ± 0.6</td>
<td>65.8 ± 2.8</td>
<td>1.6 ± 0.1</td>
<td>0</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>32.1 ± 1.6</td>
<td>10.7 ± 3.1</td>
<td>516 ± 4.3</td>
<td>80.2 ± 5.7</td>
<td>2.2 ± 0.8</td>
<td>0.3 ± 0.1</td>
<td>0.2</td>
<td>0.6 ± 0.7</td>
<td>0.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>21.5</td>
<td>3.6 ± 0.8</td>
<td>218 ± 0.4</td>
<td>32.2 ± 1.7</td>
<td>1.0 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td><strong>Polar Uncharged</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> EPS</td>
<td>11.2 ± 2.1</td>
<td>3.9 ± 1.9</td>
<td>651 ± 3.1</td>
<td>12.7 ± 2.4</td>
<td>8.8 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>0.4 ± 0.3</td>
<td>0.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> EPS</td>
<td>13.7 ± 4.2</td>
<td>6.6 ± 0.2</td>
<td>732 ± 3.4</td>
<td>16.5 ± 2.5</td>
<td>10.4 ± 0.5</td>
<td>0.8 ± 0.1</td>
<td>0</td>
<td>0.3 ± 0.1</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>15.8 ± 5.0</td>
<td>6.0 ± 1.0</td>
<td>633 ± 2.5</td>
<td>19.0 ± 7.6</td>
<td>9.1 ± 0.5</td>
<td>0.6 ± 0.1</td>
<td>0</td>
<td>0.5 ± 0.2</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>20.2 ± 1.4</td>
<td>2.8</td>
<td>1143 ± 5.2</td>
<td>50.1 ± 1.7</td>
<td>10.4 ± 1.5</td>
<td>0.2</td>
<td>0</td>
<td>0.4 ± 0.4</td>
<td>1.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td><strong>Hydrophobic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> EPS</td>
<td>18.4 ± 1.9</td>
<td>8.2 ± 0.2</td>
<td>118 ± 3.5</td>
<td>13.9 ± 4.6</td>
<td>1.2 ± 0.3</td>
<td>0</td>
<td>0.3</td>
<td>1.5 ± 0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> EPS</td>
<td>42.0 ± 7.6</td>
<td>9.3 ± 0.3</td>
<td>133 ± 3.9</td>
<td>17.9 ± 5.1</td>
<td>3.0 ± 0.4</td>
<td>0</td>
<td>0.3 ± 0.1</td>
<td>3.3 ± 0.2</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>15.4 ± 1.5</td>
<td>7.9 ± 0.3</td>
<td>150 ± 0.9</td>
<td>20.5 ± 1.0</td>
<td>0.9 ± 0.1</td>
<td>0</td>
<td>0</td>
<td>1.2 ± 0.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>8.0 ± 0.2</td>
<td>3.4 ± 1.1</td>
<td>108 ± 0.2</td>
<td>12.9 ± 1.0</td>
<td>0.1 ± 0</td>
<td>0</td>
<td>0.1</td>
<td>0.3 ± 0</td>
<td>1.0 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>
Table 6.7: DBP yields of polysaccharide monomers of for extracted EPS.

<table>
<thead>
<tr>
<th>Polysaccharide monomer groups</th>
<th>Extracted EPS</th>
<th>THM CF</th>
<th>MCAA</th>
<th>DCAA</th>
<th>TCAA</th>
<th>DCAN</th>
<th>TCAN</th>
<th>1,1-DCP</th>
<th>1,1,1-TCP</th>
<th>TCNM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa EPS</td>
<td>17.7±2.4</td>
<td>10.5±0.3</td>
<td>14.1±0.1</td>
<td>7.9±0.4</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0.3±0.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P. putida EPS</td>
<td>21.0±2.8</td>
<td>9.8±2.2</td>
<td>18.8±1.6</td>
<td>12.2±1.1</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>17.4±2.0</td>
<td>9.4±1.2</td>
<td>16.1±2.6</td>
<td>8.4±1.0</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hexose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa EPS</td>
<td>32.7±0.5</td>
<td>5.2±1.4</td>
<td>23.2±2.6</td>
<td>10.8±2.4</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>0.7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P. putida EPS</td>
<td>36.0±4.0</td>
<td>14.4±1.2</td>
<td>29.4±2.7</td>
<td>20.8±1.2</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>19.0±1.3</td>
<td>2.1</td>
<td>20.9±1.2</td>
<td>8.3±1.9</td>
<td>0</td>
<td>0</td>
<td>0.4±0.1</td>
<td>0.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>21.4±2.1</td>
<td>2.8±1.0</td>
<td>21.9±0.2</td>
<td>8.3±1.1</td>
<td>0</td>
<td>0</td>
<td>0.4±0.1</td>
<td>0.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Uronic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa EPS</td>
<td>20.4±5.3</td>
<td>8.5±2.7</td>
<td>30.0±5.2</td>
<td>16.6±0.2</td>
<td>0</td>
<td>0</td>
<td>0.2±0.1</td>
<td>0.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P. putida EPS</td>
<td>21.0±1.1</td>
<td>5.0±2.3</td>
<td>33.1±0.9</td>
<td>19.3±3.9</td>
<td>0</td>
<td>0</td>
<td>0.3±0.1</td>
<td>0.3±0.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>24.0±5.3</td>
<td>7.7±1.5</td>
<td>29.7±5.7</td>
<td>18.3±2.6</td>
<td>0</td>
<td>0</td>
<td>0.3±0.1</td>
<td>0.4±0.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>24.8±4.2</td>
<td>6.0±3.8</td>
<td>29.1±4.8</td>
<td>21.1±1.4</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>0.4±0.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Amino sugar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa EPS</td>
<td>21.5±4.4</td>
<td>5.4±0.4</td>
<td>23.8±2.9</td>
<td>12.9±3.1</td>
<td>0.4±0.2</td>
<td>0</td>
<td>0.2±0.1</td>
<td>0.5±0.3</td>
<td>0.5±0.1</td>
<td></td>
</tr>
<tr>
<td>P. putida EPS</td>
<td>22.5±3.0</td>
<td>5.8±0.5</td>
<td>27.6±2.5</td>
<td>10.7±6.3</td>
<td>0.5±0.2</td>
<td>0</td>
<td>0.3</td>
<td>0.5±0.2</td>
<td>0.5±0.1</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (regrown)</td>
<td>23.7±4.8</td>
<td>5.1±0.3</td>
<td>27.5±2.6</td>
<td>10.2±5.6</td>
<td>0.4±0.1</td>
<td>0</td>
<td>0.3</td>
<td>0.5±0.2</td>
<td>0.5±0.1</td>
<td></td>
</tr>
<tr>
<td>Biofilm EPS (isolated)</td>
<td>25.9±1.8</td>
<td>5.5±0.9</td>
<td>25.7±0.1</td>
<td>14.6±0.7</td>
<td>0.5±0.1</td>
<td>0</td>
<td>0.3±0.1</td>
<td>0.7±0.1</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

N/A: DBP data is not available as the extracted EPS did not include the polysaccharide monomer.
6.5 Discussion

In this study, the influence of biomolecules in biofilm EPS on DBP formation was explored with extracted EPS and surrogate chemicals. In addition, the relative contributions of specific EPS constituents to DBP yield and speciation were further investigated by monitoring the effects of chemical structures and compositions of biomolecules in EPS (Table 6.3 and 6.5). According to DBP yield experiments with EPS surrogates, proteins were the major EPS components producing higher DBP yields in congruent with previous studies [48, 137]. Based on DBP yield results, biomolecules containing conjugated chemical structures (His) and phenolic structures (Tyr) were correlated to have high DBP yields (Table 6.6). Results also revealed that Asn and Asp could serve as important DBP precursors.

For THM formation, previous studies reported that Tyr has high CF yields [167, 168], which may be one of the main precursors of THM in biofilm EPS. According to Chu et al., the formation mechanism of CF from Tyr includes the production of 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP), and 2,4,6-trichlorophenol (2,4,6-TCP) intermediates by Cl substitution on phenol rings followed by the ring cleavage reaction [167]. This mechanism can also explain the very low CF yield by another aromatic amino acid (Phe) since benzene structure is more stable than phenolic structure. Trp was also reported to generate relatively high levels of THM [168]. Besides amino acids, THM formation from EPS can also be attributed to a few of the lipids containing conjugated carbon bonds. Joll et al. studied the formation of THM from polyfunctional terpenoids (“lipid” like structure) [172]. They found that relative high THM was formed
from methyl ketone groups of terpenoids. However, in our study, only trace amount of lipids were detected, which implies the main THM yields were from EPS protein.

To explain HAA formation from EPS upon chlorination, several formation pathways can be considered. Among them, first, β-dicarbonyl acid compounds in biomolecules were previously reported as important precursors for HAAs, since carbonyl and carboxyl groups may affect electrons distribution on methanediyl (-CH₂-), where protons can be substituted by Cl [168, 248]. Dickenson et al. investigated HAA yields from model aliphatic β-dicarbonyl acid compounds and reported 3-oxopentanedioic acid (HO–CO–CH₂–CO–CH₂–CO–OH) showed the highest DCAA yields (68% at pH=8 and 96% of HAA₃ at pH=5.5) [248]. Carboxyl groups on β-dicarbonyl acid can withdraw electrons from methanediyl (-CH₂-), which may lead to the electrophilic substitution of Cl on -CH₂- to form HAAs (Fig. 6-2a). Likewise, proteins in EPS may have a chemical structure similar to β-dicarbonyl acid structure and form HAAs (Fig. 6-2b). Second, extremely high HAA yields observed from Asp may indicate the importance of Asp (amine group on α-carbon) in EPS on HAA formation. According to previous studies, cyanoacetic acid (HOOC-CH₂-CN) is a critical intermediate formed during the initial reactions of Asp upon chlorination [175, 176]. Subsequently, the hydrogen atoms on -CH₂- are quickly replaced with Cl due to the electron-withdrawing effect, forming dichlorocyanoacetic acid (HOOC-CCl₂-CN). Then, decarboxylation occurred on HOOC-CCl₂-CN to form HCCl₂-CN because the carboxyl group is not stable. Thus, the addition of HOCl to the nitrile group of HCCl₂-CN leads to the formation of N-chloroamides (HCCl₂-CO-NHCl), which can be rapidly hydrolyzed to the primary product DCAA and the secondary product TCAA (Fig. 6-3) [168]. In addition, Reckhow and Singer reported
that HCCl$_2$-CO-R structure could form DCAA and TCAA by Cl substitution followed by hydrolysis reactions [249]. To further examine the importance of the amine group in Asp for HAA formation, HAA yield of succinic acid (HOOC-CH$_2$-CH$_2$-COOH) was determined and compared to that of Asp (HOOC-CH$_2$-CH(NH$_2$)-COOH). Succinic acid, which lacks an amine group on α-carbon compared to the chemical structure of Asp, showed relatively low HAA yield (43.5 μg mgC$^{-1}$). Our results further illustrate the importance of amine group in Asp for high levels of DCAA formation.

![Figure 6-2: A proposed formation mechanism of DCAA and TCAA. (a) Possible formation pathway of DCAA/TCAA from β-dicarbonyl acid structure. (b) Possible formation pathway of DCAA/TCAA from protein in EPS.](image)

For N-DBPs, it appeared that HANs and TCNM formations could be attributed mainly to the proteins in EPS, since biomolecules containing org-N is the precursor for N-DBPs formation. Specifically, the main contributions to HAN formation came from Asp and Asn (Table 6.6) where both acidic and polar uncharged amino acid groups possessed high DCAN yields (~10 μg mgC$^{-1}$). A possible explanation for this is the
formation of dichlorocyanoacetic acid which is a further decarboxylated to form DCAN [123]. Beside Asp and Asn, Tyr and His also contributed to relatively high HAN yields. For Tyr, the intermediate benzyl cyanide from chlorination could be substituted by Cl on the α-carbon to form DCAN or TCAN after dephenylation [167]. For DCAN formation from His, Li et al. proposed a mechanism with several steps: (1) two protons on amine group connected to the α-carbon was substituted by Cl, followed by dechlorination and decarboxylation to form a nitrile; (2) both the nitrile and heterocycle groups (electron-withdrawing groups) would facilitate electrophilic attack on the α-carbon of the formed 2-(1-chloro-1H-imidazol-4-yl)acetonitrile; and (3) one of the hydrogen atom of the α-carbon on the nitrile group is substituted by HOCl (electrophilic attack) generating 2-chloro-2-(1-chloro-1H-imidazol-4-yl)acetonitrile [179]. Then DCAN could be formed due to the cleavage of the bond of α-carbon and heterocycle. For TCNM formation, chemical structure of precursors appeared not to be important as described in previous studies [184, 250].
Previous studies reported that chemical structure of monosaccharides affect stable agglutinating ability of polysaccharides and influence properties of bacterial EPS [251, 252]. However, unlike their influence on the EPS properties, it was also noticed that aliphatic and alcohol groups in polysaccharide have low reactivity toward chlorine in DBP formation [165]. In addition, the DBP yields were much lower for polysaccharide monomers than amino acids since they contain saturated carbon ring structures (Fig. 6-1). Overall, the results of our study indicated that biomolecules containing conjugated and phenolic structures in bacteria EPS are more prevalent DBP precursors than biomolecules with aliphatic structure. Compositions and structures of biomolecules in protein also
affect DBP formation and speciation. Considering the results obtained in this study, biofilm needs to be considered an important precursor to DBP formation and biofilm eradication methods need to be carefully selected to minimize subsequent DBP formation.
Chapter 7

The Role of Biofilm on the Formation and Decay of DBPs in a Simulated Water Distribution System

This chapter is adapted from: Z. Wang, K. Coburn, Y. Seo, The Role of Biofilm on the Formation and Decay of DBPs in a Simulated Water Distribution System, submitted to Environmental Science & Technology.

7.1 Abstract

This objective aimed to investigate the influence of biofilm on disinfection by-products (DBPs) formation and decay in a simulated water distribution system. After establishing the preformed biofilm, two disinfectants (Cl₂ & NH₂Cl) at increased residual concentration (0.5, 2 and 4 mg L⁻¹) were maintained during the entire operation. For Cl₂ disinfection, during the first phase (0.5 mg L⁻¹ Cl₂ residual), no obvious DBP formation was observed, which mainly due to low DBP formation in combination of DBP volatilization and degradation. Both heterotrophic plate counting and biofilm analysis results suggested formed HAAs were partially biodegraded during this phase. However, when Cl₂ residual reached 2 mg L⁻¹, both carbonaceous DBPs (C-DBPs) and nitrogenous DBPs (N-DBPs) increased dramatically and reached a plateau around 15 days. Then DBP formation decreased, with trihalomethanes and haloacetic acids being the most prevalent
species formed. From biofilm analysis results, average biofilm thickness and total biomass decreased dramatically during this phase, which suggests higher Cl₂ residual not only reacted with humic acid (HA) but also reacted with biofilm to produce DBPs, especially the high formation of N-DBPs (haloacetonitriles). When 4 mg L⁻¹ of Cl₂ residual was maintained in reactor (third phase), there was no further increase of DBPs, which was due to biofilm was already eradicated at 2 mg L⁻¹ Cl₂ residual. DBP formation was mainly from chlorination of HA in bulk phase.

For NH₂Cl disinfection, under different NH₂Cl residuals, the DBP levels were much lower compared to Cl₂ disinfection. In addition, minimal differences were observed of DBP formation under different NH₂Cl residuals. Combined results suggested that both biofilm and associated HA would promote DBP formation in simulated drinking water distribution system in the presence of higher Cl₂ residual, playing a significant role in both the formation and fate of DBPs.

7.2 Introduction

In drinking water distribution systems, maintaining biological stability as well as controlling disinfection by-product (DBP) formation have been great challenges to water utilities [36, 106, 234]. To prevent bacterial regrowth and biofilm formation in drinking water distribution system, water utilities in the U.S. try to maintain minimum disinfectant residuals [16]. However, disinfectant residuals may continuously interact with various untreated DBP precursors and produce DBPs in water distribution systems [4, 36].
According to previous studies for DBP formation in water distribution systems, untreated natural organic matter (NOM) and algal organic matter (AOM) were commonly acknowledged as important DBP precursors [5, 253]. However, the characteristics and concentrations of NOM and AOM entering water distribution systems is largely impacted by water treatment processes [22, 30], which mostly result in lower molecular weight hydrophilic assimilable organic carbon (AOC) and biodegradable dissolved organic carbon (BDOC) that cannot be removed completely [43]. The AOC and BDOC entering water distribution systems not only affect the formation of DBPs but also enhance microbial biofilm regrowth in the distribution system [37-39, 106, 140].

Considering the regulatory presence of disinfectant residual in distribution systems, untreated NOM may not be the sole contributor to DBP formation, given the presence of microbial biofilm [254]. Biofilm can provide a dynamic repository for various organic materials present in the bulk fluid and subsequently utilize them as a primary nutrient source [193, 255]. Heterogeneous structure of biofilm was widely reported as containing voids and channels, which may facilitate absorbing available untreated NOM in distribution system [170]. Moreover, biofilm extracellular polymeric substances (EPS) are comprised of a significant concentration of organic carbon and organic nitrogen rich compounds [proteins, amino acids, polysaccharides, lipids, and nucleic acids], which possess similar chemical composition and reactivity to traditional studied DBP precursors. Thus, structural and chemical characteristics of biofilm EPS and their reactivity with disinfectants may greatly contribute to DBP formation. Previous studies also reported that the concentration of DBPs from tap water were higher than concentrations leaving water treatment plants, which was attributed to biofilm or biofilm
associated NOM reacting with disinfectant residuals [36]. Our previous study with selected bacteria strains also indicated both EPS and biomass could produce relatively high level of DBPs where protein based EPS possessed both higher C-DBPs and N-DBPs [254]. Therefore, besides long residence time and untreated organic matter entering into water distribution systems, the biofilm formation may impact not only the biological stability but also the chemical stability (DBP formation) of water in distribution systems as they continuously reacting with disinfectant residuals. However, since DBPs were firstly discovered, role of biofilm on DBP formation in the water distribution system is not well understood yet.

The aim of this objective is to understand the role of biofilm on the DBP formation and decay in a simulated drinking water distribution system. Two model disinfectants (Cl₂ and NH₂Cl) commonly applied in drinking water distribution systems were selected to continuously monitor both C-DBP and N-DBP formation in both bulk water and biofilm [38]. In addition to monitoring DBP formations, changes of biofilm under different disinfectant type and dose were also examined.

7.3 Materials and Methods

7.3.1 Reactors Operation

Fig.7-1 shows the experimental set-up for a simulated water distribution system. Mixed species biofilm was isolated from a water utility and grown in three continuously
feeding annular reactors (CDC reactor, Boltzmann, MT) for six months to form confluent biofilm. Feed solution used for the reactors was prepared with the City of Toledo tap water which was further treated by a granular activated carbon (GAC) filter to remove both chlorine residual and any existing DBPs. After filtration, GAC filtered tap water was mixed with treated humic acid solution by jar tests coupled with ozonation, which mimicked untreated NOM entering the water distribution system after conventional water treatment processes (coagulation, flocculation, sedimentation, and preozonation). Coagulation was conducted with aluminum sulfate \([\text{Al}_2(\text{SO}_4)_3\cdot18\text{H}_2\text{O}]\) and 2 mg L\(^{-1}\) of ozone was applied to supernatant from jar tests for 5 mins using a lab scale ozonator (OZOTECH, CA, USA). Final TOC concentration of feed solution was maintained around 2 mg L\(^{-1}\) which is close to the condition in many water distribution systems (~2 mg L\(^{-1}\)) [36]. Prepared feed solution was delivered to each reactor at the flow rate of 0.5 mL min\(^{-1}\) [140] using peristaltic pumps (Masterflux, Cole-Parmer, IL). Hydraulic retention time in the reactors was 12 h, which was close to the average retention time of water in a water distribution system [140].
Chlorine ($\text{Cl}_2$) and monochloramine ($\text{NH}_2\text{Cl}$) were applied as model disinfectants for disinfection reactors. In addition, a control reactor (without a disinfectant) was also operated to monitor biofilm formation and growth without disinfectants. For chlorine disinfection, secondary $\text{Cl}_2$ stock solution was prepared by adding concentrated $\text{Cl}_2$ solution in deionized (DI) water. For $\text{NH}_2\text{Cl}$ stock solution preparation, equal volumes of 1 M $\text{NH}_4\text{Cl}$ and 6% NaOCl solution were mixed together, and the pH was adjusted to 8.2 with 1 mM NaOH solution. Disinfectant stock solutions were stored in separate syringes and delivered to reactors by a syringe pump (Fisher Scientific, USA). Concentrations of model disinfectants were determined by DPD methods via a spectrophotometer (HACH, USA, DR 2800).
After obtaining confluent biofilm formation for six months, reactors were operated over 100 days under three different disinfectant doses. For the first experimental phase, reactors were operated around 0.5 mg L\(^{-1}\) of residual disinfectants (both Cl\(_2\) and NH\(_2\)Cl) for 50 days. For the second phase, higher disinfectant residuals (2 mg L\(^{-1}\) of Cl\(_2\) and NH\(_2\)Cl) were applied for 25 days to simulate commonly applied disinfectant residual concentration in water distribution systems. During the third phase, considering biofilm eradication practices in water distribution systems, 4 mg L\(^{-1}\) of Cl\(_2\) & NH\(_2\)Cl residuals were maintained for the last 25 days.

7.3.2 Reactor Sampling

To monitor the influence of biofilm formation on DBP formation, influent, effluent and biofilm (from reactor coupons) samples were periodically collected for various analyses during the entire experiments.

Heterotrophic plate counting (HPC), TOC, pH, disinfectant residuals, and bromide levels were continuously measured for reactor effluents. For total organic carbon (TOC) analyses, samples were filtered by 0.45 μm nitrocellulose membranes and analyzed using a TOC analyzer (TOC\(_{VSH}\), Shimadzu Japan). To evaluate the effect of bromide on DBP speciation, bromide levels of reactor influents and effluents were determined by ion chromatography (Dionex, ICS-1000, USA).

7.3.3 Biofilm Analysis

In order to monitor changes of biofilm formation and growth under disinfectants, biofilm samples were collected and analyzed using both HPC method with R2A agar and
a confocal laser scanning microscope (CLSM). Considering biofilm sloughing as well
planktonic cell growth, bacteria cell numbers in effluents were also monitored. For HPC
analysis, bacteria enumeration results were expressed as CFU (colony forming unit) mL⁻¹
for reactor effluents or CFU (cm²)⁻¹ for biofilm, respectively.

CLSM analyses were conducted to understand the influence of disinfectants on
biofilm formation and structure changes. Biofilm samples from reactor coupons were
stained using BacLight LIVE/DEAD staining kit (Molecular Probes Inc., USA) for
bacteria viability and Alexa 633 conjugated concanavalin A (ConA-Alexa 633) for
polysaccharide (D-glucose and D-mannose residues) components in EPS, respectively
[256]. A mixture of stains was prepared by adding SYTO9 (live), propidium iodide (PI,
dead) and, ConA in 1 mL chlorine demand free buffer with a final concentration of 2.5
μM, 2.5 μM, and 200 μg mL⁻¹, respectively. After that, the mixed stain solution (200 μL)
was added to each biofilm sample grown on a coupon and incubated in the dark for 15
min. Then, biofilm samples were visualized by a Leica TCS SP5 CLSM equipped with
either a 63X oil immersed objective or a 20X dry objective. For each biofilm sample, at
least six positions were randomly selected for z-stack image acquisition and analysis. In
this study, biofilm parameters including thickness of biofilm, total biomass, viability ratio
(biomass of live cells divided by biomass of total cells), and roughness coefficient (a
measure of how the thickness of biofilm varies) were quantitatively analyzed using the
COMSTAT program [257].
7.3.4 DBP Formation and DBP Yield Tests

To monitor the DBP formation and speciation in reactors, 40 mL of samples were collected from reactor effluents in every 3-4 days and analyzed for DBP formation. Before DBP analysis, Cl₂ residual in each sample was quenched by adding 40 mg NH₄Cl. Samples were analyzed according to the US EPA methods using a gas chromatograph with dual electron capture detectors [228-230].

To examine DBP formation from biofilm as well as biofilm associated HA, DBP yield (formation potential) tests were also conducted with biofilm samples grown on removable couples of the reactors. In details, biofilm grown coupons were carefully removed from the reactors and soaked in a 50 mL facon tubes containing 25 mL phosphate buffer solution. Biofilm was removed from coupons using sterilized surgical blades. Then, high speed vortex and a mechanical homogenizer were applied to extracted biofilm samples for homogenizations. Then, extracted biofilm solution was transferred and mixed with phosphate buffer solution (pH=7) in 40 mL amber glass vials [54]. Samples were exposed to excessive Cl₂ doses following the standard method 5710B [226] and incubated in the dark at room temperature 22 ± 2°C for 7 days. After that, free chlorine concentrations were examined and then residuals were quenched by NH₄Cl before the samples were ready for DBP analysis.

To quantify DBP formation from biofilm, DBP formation tests with feed solution were also conducted using different residual disinfectant concentrations (0.5, 2, and 4 mg L⁻¹) for each experimental phase and results were compared to DBP formations from bulk solutions.
7.3.5 DBP Analysis Methods

THM₄ (CF, BDCM, DBCM, and BF), HAA₉ (MCAA, MBAA, DCAA, BCAA, TCAA, BDCAA, DBAA, CDBAA, and TBAA), HAN₄ (TCAN, DCAN, BCAN, and DBAN), HK₂ (1,1-DCP and 1,1,1-TCP), TCNM and chloral hydrate (CH) were analyzed in this study. Detailed analysis methods can be found elsewhere [254]. For quality assurance and quality control, spike recovery was calculated to verify the validity of entire analysis in this study. Both average spike recoveries and method detection limits (MDL) for all tested DBPs were determined for quality assurance and quality control.

7.3.6 Fluorescent Excitation-Emission Matrix (EEM) Analysis

Fluorescent EEM spectra were measured using a Spectra Max M5 microplate reader (Molecular Devices, USA) with a xenon lamp as the excitation source. To minimize the effect of fluorescence quenching, which may result from relatively high concentration of DBP precursors, TOC levels of analyzed samples were diluted to be around 10 mg L⁻¹ by 0.01 M KCl solution before the fluorescence measurements. EEM spectra were generated by scanning each sample on excitation wavelength from 250 to 450 nm at intervals of 10 nm and emission wavelengths between 290 and 500 nm at 10 nm steps.

According to previous studies, EEM peaks (flourphores) can be correlated to the presence of humic-like, tyrosine like, tryptophan-like, or phenol-like organic compounds [258, 259]. Peaks at 250 < λ < 280 nm excitation wavelengths and λ < 380 nm emission wavelengths are related to soluble microbial byproduct-like material (such as amino
acids) (region I). Peaks at longer excitation wavelengths ($\lambda > 280$ nm) and longer emission wavelengths ($\lambda > 380$ nm) are related to humic acid-like organics (region II). The peaks at shorter excitation wavelengths ($250 < \lambda < 280$ nm) and longer emission wavelengths ($\lambda > 350$ nm) are related to fulvic acid-like materials (region III) [62, 260].

7.4 Results and Discussion

7.4.1 Biofilm Analysis Results

Figs 7-2, 7-3, and 7-4 show the change stacked CLSM images during the entire operation of control reactor (R1), Cl$_2$ applied reactor (R2), and NH$_2$Cl applied reactor (R3), respectively. Besides, biofilm analysis results are shown in Table 7.1. For the R1, both total biomass and average biofilm thickness increased slightly during 100 day operation (after initial biofilm incubation for 6 month) suggesting further biofilm growth. Without a disinfectant, the cell viability ratios were around 80% during the entire operation. For disinfectant applied reactors (R2 & R3), total biomass and average biofilm thickness decreased continuously during the entire experiments, especially under the presence of higher disinfectant residuals (2 & 4 mg L$^{-1}$). For the chlorine disinfected R2, the total biomass and average biofilm thickness decreased significantly, which suggested that biofilm was eradicated under higher Cl$_2$ residual. Especially, rapid decrease of total biomass [from 4.05 to 0.24 $\mu$m$^3$ ($\mu$m$^2$)$^{-1}$] and average biofilm thickness (from 8.75 to 0.59 $\mu$m) were observed from the beginning to the end of the second phase.
Table 7.1: Biofilm analysis results from three reactors \( ^a \).

<table>
<thead>
<tr>
<th></th>
<th>Sampling Date</th>
<th>Total Biomass ( [\mu m^3 (\mu m^2)^{-1}] )</th>
<th>Average Thickness (( \mu m ))</th>
<th>Roughness Coefficient</th>
<th>Viability Ratio ( ^b ) (% )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R1 (control)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First phase</td>
<td>25th day</td>
<td>8.5 ± 1.2</td>
<td>34.5 ± 2.8</td>
<td>1.0 ± 0.1</td>
<td>79.7 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>50th day</td>
<td>9.4 ± 2.9</td>
<td>41.6 ± 12.2</td>
<td>0.9 ± 0.4</td>
<td>72.5 ± 13.7</td>
</tr>
<tr>
<td>Second phase</td>
<td>75th day</td>
<td>12.6 ± 2.2</td>
<td>45.0 ± 4.4</td>
<td>0.7 ± 0.1</td>
<td>75.1 ± 14.6</td>
</tr>
<tr>
<td>Third phase</td>
<td>100th day</td>
<td>14.9 ± 3.4</td>
<td>53.6 ± 6.6</td>
<td>0.6 ± 0.1</td>
<td>81.0 ± 13.4</td>
</tr>
<tr>
<td><strong>R2 (Cl(_2))</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First phase (0.5 mg L(^{-1}))</td>
<td>25th day</td>
<td>7.4 ± 3.3</td>
<td>16.1 ± 4.9</td>
<td>1.2 ± 0.2</td>
<td>14.2 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>50th day</td>
<td>4.1 ± 1.1</td>
<td>7.9 ± 1.0</td>
<td>1.8 ± 0.2</td>
<td>10.0 ± 5.6</td>
</tr>
<tr>
<td>Second phase (2.0 mg L(^{-1}))</td>
<td>75th day</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.4</td>
<td>2.0</td>
<td>2.3 ± 4.3</td>
</tr>
<tr>
<td>Third phase (4.0 mg L(^{-1}))</td>
<td>100th day</td>
<td>0</td>
<td>0</td>
<td>2.0</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td><strong>R3 (NH(_2)Cl)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First phase (0.5 mg L(^{-1}))</td>
<td>25th day</td>
<td>7.7 ± 2.8</td>
<td>19.6 ± 9.1</td>
<td>1.2 ± 0.3</td>
<td>45.5 ± 13.4</td>
</tr>
<tr>
<td></td>
<td>50th day</td>
<td>5.0 ± 2.3</td>
<td>12.6 ± 5.8</td>
<td>1.4 ± 0.3</td>
<td>28.4 ± 14.4</td>
</tr>
<tr>
<td>Second phase (2.0 mg L(^{-1}))</td>
<td>75th day</td>
<td>5.0 ± 2.2</td>
<td>11.0 ± 4.4</td>
<td>1.4 ± 0.3</td>
<td>11.4 ± 5.8</td>
</tr>
<tr>
<td>Third phase (4.0 mg L(^{-1}))</td>
<td>100th day</td>
<td>2.6 ± 0.8</td>
<td>6.7 ± 1.7</td>
<td>1.6 ± 0.1</td>
<td>3.6 ± 4.3</td>
</tr>
</tbody>
</table>

\( ^a \): Values represent average ± standard error (\( N=6 \)). \( ^b \): Cell viability was expressed as the biomass of live cells (stained with SYTO 9) divided by the biomass of total cells (SYTO 9 stained live cells and PI stained dead cells) \( \times 100\% \).
**Figure 7-2**: CLSM images of biofilm in R1 (control reactor). (a) CLSM image at 25\textsuperscript{th} day; (b) CLSM image at 50\textsuperscript{th} day; (c) CLSM image at 75\textsuperscript{th} day; (d) CLSM image at 100\textsuperscript{th} day (green indicates live cells; red indicates dead cells, blue indicates EPS).
Figure 7-3: CLSM images of biofilm in R2 (Cl$_2$ applied reactor). (a) CLSM image at 25$^{th}$ day; (b) CLSM image at 50$^{th}$ day; (c) CLSM image at 75$^{th}$ day; (d) CLSM image at 100$^{th}$ day (green indicates live cells; red indicates dead cells, blue indicates EPS).
Figure 7-4: CLSM images of biofilm in R3 (NH$_2$Cl applied reactor). (a) CLSM image at 25$^{th}$ day; (b) CLSM image at 50$^{th}$ day; (c) CLSM image at 75$^{th}$ day; (d) CLSM image at 100$^{th}$ day (green indicates live cells; read indicates dead cells, blue indicates EPS).
For the R3, compared to the Cl2 disinfected R2, the average thicknesses of biofilm decreased slowly under NH2Cl disinfection. During the entire third phase, where highest NH2Cl residual was maintained, the total biomass and average biofilm thickness changed from 4.97 to 2.56 µm³ (µm²⁻¹) and 10.98 to 6.65 µm, respectively. Although the total biomass and average biofilm thickness were also found decreased, at the end of operation, 2.56 µm³ (µm²⁻¹) of total biomass and 6.65 µm of average biofilm thickness were still observed. For viability ratio changes in disinfected reactors, under the presence of Cl2, 14.48% and 0.31% of viability ratios were observed from the end of the first phase and the end of entire operation, respectively. Overall, viability ratios of R3 were higher than those in R2, since Cl2 disinfection is more efficient compared to NH2Cl disinfection. The changes of the biofilm structural parameters and viability ratios in R2 and R3 suggested that chlorine was more effective for biofilm control.

7.4.2 DBP Formation Results from Reactor Effluents

7.4.2.1 DBP Formation upon Chlorination

Fig. 7-5 (a) shows the formation of bulk phase DBPs during Cl2 disinfection. During the first phase (0.5 mg L⁻¹ of free Cl2 residual), minimal THMs (~1-2 µg L⁻¹) and HAAs (~5 µg L⁻¹) formations were observed. Besides the regulated DBPs, the formations of emerging DBPs were very low. The low DBP formation during the first phase may be attributed to (1) low free Cl2 residual for DBP formation, (2) DBP volatilization during reactor operation, and (3) biodegradation of HAAs by biofilm. To confirm potential DBP biodegradation and volatilization, we conducted DBP formation batch experiments with treated HA at the initial Cl2 dose (0.5 mg L⁻¹) for 12 h (hydraulic retention time in this
study). The batch experiment results revealed that DBP levels of reactor effluent were much lower than those of DBP batch experiments (Fig 7-6 a). During the operation, the reactor was not gas tight and with free head space. In addition, a magnetic stir bar was continuously stirring to simulate mixing in the distribution system. Thus, the volatilization of DBPs became inevitable. Previous studies reported the volatilization of THM, HAN, HK, CH and TCNM [261, 262], which may explain the low DBP formation during the first phase.
Three concentrations of disinfectant residual were maintained throughout the entire operation: (1) first phase, 0.5 mg L\(^{-1}\) of Cl\(_2\) or NH\(_2\)Cl residual; (2) second phase, 2.0 mg L\(^{-1}\) of Cl\(_2\) or NH\(_2\)Cl residual; (3) 4.0 mg L\(^{-1}\) of Cl\(_2\) or NH\(_2\)Cl residual. Error bar represents duplicate sample analysis.
For HAAs, the reason for low formation may be related to biodegradation, which was reported in previous studies [12, 53]. From batch experiment DBP formation results (Fig. 7-6 a), around 12 μg L\(^{-1}\) of HAA was observed, which was 58% higher than those in the bulk phase of the R2 (~5 μg L\(^{-1}\)). During the first phase, where residual Cl\(_2\) concentration was low, HPC results for biofilm between control and Cl\(_2\) disinfected reactors showed that significant amount of biofilm was still present [\(~10^5\) (R1) and \(~10^3\)-\(10^4\) CFU (cm\(^2\))^\(-1\) (R2)]. Furthermore, the biofilm analysis results with CLSM also confirmed that the presence of live biofilm (14.18%) could partially degrade HAAs. Considering obtained results and non-volatile nature of HAAs, the relatively HAA formation in the reactor can be attributed to the biodegradation [53].

DBP levels dramatically increased at the beginning of the second phase, when 2 mg L\(^{-1}\) Cl\(_2\) residual was applied. The levels of regulated DBPs significantly increased and reached a plateau (observed the highest concentration of 63 μg L\(^{-1}\) for THMs and 80 μg L\(^{-1}\) for HAAs). In addition, the levels of HANs and CH exhibited comparatively similar trends to those of the THMs and HAAs, with the highest concentrations observed around 20 μg L\(^{-1}\). Formations of HKs and TCNM also increased slightly. After reaching the plateau, all DBP concentrations were decreased until the end of the second phase. To evaluate biofilm contribution to DBP formation in the presence of 2 mg L\(^{-1}\) Cl\(_2\) residual, batch DBP formation experiments with feed solution were also conducted. Compared to results from batch DBP formation experiments with treated HA under 2 mg L\(^{-1}\) Cl\(_2\) dose (Fig. 7-6 b), higher DBP formation was found in the reactor.
For the second phase, initial boost of DBP levels beyond the formation potential of HA (Fig. 7-6 c and d) suggested the presence of biofilm facilitated DBP formation under higher free Cl₂ residual. According to previous studies, biofilm and EPS could harbor available AOC or BDOC (low molecular weight) and subsequently utilize them for their growth [31, 135, 193, 197]. Compared to reactor influent, TOC levels of
effluents were observed to slightly decrease during experiments (data not shown), which may suggest biosorption and degradation of HA by biofilm. Thus, higher Cl$_2$ residual could react with biofilm associated HA as well as HA in the bulk phase simultaneously, which would explain increasing DBP formation reach the plateau. After that, the decrease of DBP levels suggests biofilm was continuously eradicated by high Cl$_2$ residual [263] and lost its ability to further harbor HA. Therefore, at the end of second phase, DBP formation was mainly contributed by the DBP formation of HA in bulk solution. Constant DBP formation during the third phase (4 mg L$^{-1}$ Cl$_2$ residual) further confirmed treated HA in bulk water was the only precursor. Results of batch DBP yield test suggested only treated HA in bulk phase reacted with high Cl$_2$ residual to produce DBPs after biofilm eradication during the third phase.

Higher levels of HAN formation were also observed during the second phase, which also suggest that biofilm involve in reaction and produce N-DBPs. Oliver reported that nitrogenous precursors from algae or its effluent organic matter have been related to N-DBP formation, especially HANs [115]. Since organic nitrogen content is rich in biofilm and EPS than that of treated HA [33, 115, 190, 254], the increase of N-DBPs further indicated that biofilm could enhance DBP levels in the water distribution system.

During the third phase, constant DBP formation was observed in the presence of 4 mg L$^{-1}$ Cl$_2$ residual. For regulated DBPs, the levels of HAAs (~45 μg L$^{-1}$) were found higher than THMs (~20 μg L$^{-1}$). For emerging DBPs, the levels of HANs and CH formation remained low in the third phase (~4 μg L$^{-1}$). For HKs and TCNM, low formation (~1.0 μg L$^{-1}$) was also found during this phase. From the beginning to the end
of third phase, total biomass and average biofilm thickness decreased from 0.24 to 0.03 \( \mu \text{m}^3 \text{ (}\mu \text{m}^2\text{)}^{-1} \) and 0.59 to 0.04 \( \mu \text{m} \), respectively (Table 7.1). Biofilm analysis results for the third phase indicated that biofilm got mostly removed losing reaction sites for DBP formation. DBP formation results from bulk solution, which is similar to batch experiment results, also suggest only HA contributed the DBP formation in the presence of 4 mg L\(^{-1}\) Cl\(_2\) residual.

### 7.4.2.2 DBP Formation upon Chloramination

Fig. 7-5 (b) shows the DBP formation under different NH\(_2\)Cl residuals (0.5, 2.0, and 4.0 mg L\(^{-1}\)). In comparison to Cl\(_2\) disinfection, DBP formation upon chloramination was much lower as expected [8, 20, 139]. For regulated DBPs, overall, the levels of HAAs were higher than THMs. In addition, the formation of emerging DBPs was almost not observed except for 1,1-DCP.

HAA formation was slightly higher than THM levels in the presence of 0.5 mg L\(^{-1}\) NH\(_2\)Cl residual. Compared to Cl\(_2\) disinfection, increase in THMs and HAAs levels was not observed at 2.0 mg L\(^{-1}\) NH\(_2\)Cl residual, which may be attributed to the low reactivity of NH\(_2\)Cl to DBP precursors compared to that of Cl\(_2\) [38, 142]. However, regulated DBP levels were increased (~2 \( \mu \text{g} \text{ L}^{-1} \) for THMs and ~5 \( \mu \text{g} \text{ L}^{-1} \) for HAAs) under the presence of 4.0 mg L\(^{-1}\) NH\(_2\)Cl residual (the third phase). Unlike Cl\(_2\), which could significantly eradicate biofilm over 2.0 mg L\(^{-1}\) residual concentrations, NH\(_2\)Cl residual could not remove biofilm completely (Table 7.1) even under 4.0 mg L\(^{-1}\) residual concentration. This remaining biofilm could facilitate DBP formation reacting with increased NH\(_2\)Cl residual.
Many water utilities recently have adapted NH$_2$Cl over Cl$_2$ to meet the stringent DBP regulations [264]. Previous studies reported that NH$_2$Cl produces less amount of DBPs [142, 265]. For example, Bougeard et al. [142] investigated the DBP level of 11 water treatment utilities in UK. They reported the level of both THM$_4$ and HAA$_9$ were less than 10 μg L$^{-1}$. Hua and Reckhow [141] also reported that using NH$_2$Cl as disinfectant produces much less DBPs compared to Cl$_2$ disinfection. For emerging DBP formation, except 1,1-DCP, HAN and TCNM formations by NH$_2$Cl were lower than that of Cl$_2$, which is consistent with results from previous studies [13, 62, 141, 142].

7.4.2.3 Statistically Analysis of DBPs from Reactor Effluents

Fig. 7-7 shows the occurrence (box-and-whisker plots) of the analyzed DBPs from reactor effluents. In Fig. 7-7 a, during the entire operation, the median levels of THM$_4$ and HAA$_5$ were around 10 μg L$^{-1}$ upon chlorination. Low median levels of regulated DBPs were mainly related to the minimal formation, volatilization, and biodegradation of DBP during the first phase. From the 10$^{th}$ to 90$^{th}$ percentile, the THM$_4$ and HAA$_5$ levels were in the range of 1.3 to 61.5 μg L$^{-1}$ and 2.2 to 70.7 μg L$^{-1}$, respectively for the entire experiment. In addition, high levels of DBPs (95$^{th}$ percentiles) was mainly from the second phase when biofilm participated in reaction producing extra DBPs. In this study, highest THM$_4$ and HAA$_5$ concentrations (outliers) can be found as 73 and 105 μg L$^{-1}$, respectively. From the information provided by the American Water Works Association (AWWA) Water Industry Database, which is based on 815 American water utilities serving a total of 141 million people, THM$_4$ levels in the studied water distribution systems ranged from 1.5 to 71 μg L$^{-1}$, with a median value 34 μg L$^{-1}$ [266].
For HAA₅ formation, in another survey conducted by the AWWA with 35 U.S. water utilities, the mean and median HAA₅ levels were 17.3 and 12.8 μg L⁻¹, respectively [36]. In addition, case studies also indicated that for the 25th and 75th percentile of HAA₅, 7.1 and 22.6 μg L⁻¹ were monitored [267]. It was also reported that the 90th percentile of HAA₅ was below 50 μg L⁻¹, but the range of concentrations can be as high as 280 μg L⁻¹ [268].

For emerging DBPs, the formation of HAN₄ and CH was higher than HK₂ and TCNM. The median of HAN₄, CH, HK₂ and TCNM were 0.8, 1, 0.5, and 0.3 μg L⁻¹, respectively. For HAN₄ and CH, from 25th to 75th percentile, the formation was observed as 0.3 to 3.7 μg L⁻¹ and 0.3 to 5.2 μg L⁻¹, respectively. For emerging DBP outliers, HAN₄, CH, levels were around 20 μg L⁻¹, which was mainly attributed to Cl₂ interaction with biofilm. The HK₂ and TCNM formation were observed in small level ranges under different chlorine residual. However, less information was available from actual drinking water utilities for emerging DBPs.
Figure 7-7: The formation of THM$_4$, HAA$_5$, HAN$_4$, HK$_2$, CH, and TCNM during operation of annular reactors (100 days). (a) Cl$_2$ as disinfectant, (b) NH$_2$Cl as disinfectant. Median (solid line), 10th, 25th, 75th and 90th percentiles as vertical boxes with error bars, and 5$^{th}$/95$^{th}$ percentiles (●)

In Fig. 7-7 b, compared to DBP formation upon chlorination, only low levels of THM$_4$, HAA$_5$ and HK$_2$ were observed under NH$_2$Cl. For THM$_4$, from 10$^{th}$ percentile to 90$^{th}$ percentile formation was ranged between 0 and 1.7 μg L$^{-1}$, with the median at 0.4 μg
L⁻¹. For HAA₅, from 10th percentile to 90th percentile, the range of levels was between 1.5 and 6.1 μg L⁻¹, with a median at 2.8 μg L⁻¹. For HK₂, the 10th percentile to 90th percentile was from 0 to 0.3 μg L⁻¹.

7.4.2.4 DBP Speciation

In addition to chlorinated DBPs, brominated DBPs were also observed in this study, since trace amount of bromide (~45 μg L⁻¹) was detected in the feed solution. The pH changes have been known to affect the DBP speciation [36], however, in this experiment, pH levels were steady during the entire study (~7.1). All detailed DBP speciation results were summarized in the (Fig. 7-8 through 7-11). In Fig. 7-8 a, for THMs speciation, CF had the highest formation during the entire operation compared to other THMs. In Fig. 7-9 a, for HAAs speciation, DCAA was the dominant species formed, which was around two times higher than TCAA during the entire study. The lower concentrations of MCAA formed may be partly attributed to biodegradation during the first phase, since one halogen HAA species have been reported to be easily utilized by bacteria [12, 53]. Previous studies reported that, the formation and speciation of brominated THMs and HAAs were determined by bromide concentration in water [129, 269]. During this study, since low bromide concentration was observed, low formation of brominated DBPs was expected. For emerging DBPs (Fig.7-10 a), DCAN was the dominate species of HANs in the presence of higher chlorine residual (>2mg L⁻¹). For brominated HAN species, the levels of BCAN were slightly higher than DBAN. In addition, TCAN showed the lowest formation during the entire study. For HKs, 1,1,1-TCP formation was always higher than 1,1-DCP (Fig. 7-11 a).
For NH₂Cl disinfection, DBP speciation (Fig 7-8 b, 7-9 b, and 7-11 b) was similar to that of Cl₂ disinfection except for HK₂. 1,1-DCP possessed higher formation compared to 1,1,1-TCP, which was consistent with previous studies [13, 62].

**Figure 7-8:** Detailed THM₄ speciation from reactor effluents (a) Cl₂ as disinfectant; (b) NH₂Cl as disinfectant. [Three different concentrations of disinfectant residual were maintained throughout the entire operation: (1) first phase, 0.5 mg L⁻¹ of Cl₂ or NH₂Cl; (2) second phase, 2.0 mg L⁻¹ of Cl₂ or NH₂Cl; (3) 4.0 mg L⁻¹ of Cl₂ or NH₂Cl.] Error bar represents duplicate sample analysis.
Figure 7-9: Detailed HAA₉ speciation from reactor effluents (a) Cl₂ as disinfectant; (b) NH₂Cl as disinfectant. [Three different concentrations of disinfectant residual were maintained throughout the entire operation: (1) first phase, 0.5 mg L⁻¹ of Cl₂ or NH₂Cl; (2) second phase, 2.0 mg L⁻¹ of Cl₂ or NH₂Cl; (3) 4.0 mg L⁻¹ of Cl₂ or NH₂Cl.] Error bar represents duplicate sample analysis.
Figure 7-10: Detailed HAN₄ and TCNM speciation from reactor effluents during chlorination. [Three different concentrations of disinfectant residual were maintained throughout the entire operation: (1) first phase, 0.5 mg L⁻¹ of Cl₂; (2) second phase, 2.0 mg L⁻¹ of Cl₂; (3) 4.0 mg L⁻¹ of Cl₂.] Error bar represents duplicate sample analysis.
Figure 7-11: Detailed HK$_2$ and CH speciation from reactor effluents (a) Cl$_2$ as disinfectant; (b) NH$_2$Cl as disinfectant. [Three different concentrations of disinfectant residual were maintained throughout the entire operation: (1) first phase, 0.5 mg L$^{-1}$ of Cl$_2$ or NH$_2$Cl; (2) second phase, 2.0 mg L$^{-1}$ of Cl$_2$ or NH$_2$Cl; (3) 4.0 mg L$^{-1}$ of Cl$_2$ or NH$_2$Cl.] Error bar represents duplicate sample analysis.
7.4.3 DBP Yields of Biofilm and Biofilm Associated HA

DBP yield results of biofilm and biofilm associated HA from all reactors upon chlorination and chloramination are shown in Figs. 7-12 through 7-14. Overall, biofilm and biofilm associated HA from R1 (control reactor) upon chlorination showed the highest DBP yields compared to biofilm samples from other reactors. In contrast to the original method to express DBP yields as $\mu$g mgC$^{-1}$, in this experiment, the DBP yields were expressed as $\mu$g (dm$^2$)$^{-1}$, which can highlight the contribution of DBPs from both biofilm and associated HA on each coupon.

Fig. 7-12a shows both CF and HAA$_3$ yields of biofilm samples from the control reactor (R1) upon chlorination. Overall, the DBP yields were continuously increased during the operation. Before the start of experiment, all reactors were operated for six months to obtained confluent biofilm. However, during this study, total biomass increased (~40%) in R1 according to biofilm analysis data (Table 7.1). Simultaneously, the new developed biofilm can provide the adsorption sites for HA in bulk water. Since DBP precursors (biofilm and associated HA) were increased on coupons, which would explain the continuously rise of DBP yields. For emerging DBP yields (Fig. 7-12c), upon chlorination, CH yields was observed greatly increased from 2$^{nd}$ to 14$^{th}$ week, which may suggest more HA was adsorbed on biofilm. Since previous studies reported that NOM produce higher CH than biomolecules such as algae [47, 48]. The significant increase of CH yield may further confirm biofilm consistently adsorbed HA from bulk phase. For other emerging DBP, the increase was much less significant than those of CH.
DBP yield results of biofilm and associated HA in R2 (Cl\textsubscript{2} as disinfectant) upon chlorination are represented in Fig. 7-13a and c. On the contrary to R1, both DBP yields decreased during the operation, which may be attributed to detachment of biofilm as well as to pre-occurred interactions between Cl\textsubscript{2} residuals and biofilm associated HA in the reactor. From 2\textsuperscript{nd} to 6\textsuperscript{th} week, the yields of CF and HAA\textsubscript{3} were around 30 and 40 μg (dm\textsuperscript{2})\textsuperscript{-1}, respectively. However, both CF and HAA\textsubscript{3} yields of biofilm and biofilm associated HA decreased to around 10 μg (dm\textsuperscript{2})\textsuperscript{-1} during the second phase, which may suggest that more DBP precursors (biofilm and biofilm associated HA) on coupons were already depleted under increased Cl\textsubscript{2} residual (2 mg L\textsuperscript{-1}). In addition, based on the biofilm analysis data, increased Cl\textsubscript{2} residual significantly affected biofilm structure and began to eradicate preformed biofilm under applied shear stresses in the reactor. Even lower CF and HAA\textsubscript{3} yields [~5 μg (dm\textsuperscript{2})\textsuperscript{-1}] in third phase further confirmed our hypothesis that decreased DBP formation was correlated to biofilm detachment (Table 7.1). For emerging DBPs, the highest yields were observed at the 2\textsuperscript{nd} week, which may be related to the abundance of biofilm on reactor coupon during the early phase of disinfection. From the second to third phase, very minimal of DBPs yields were observed with the increased Cl\textsubscript{2} residuals.

Fig. 7-14a and c shows the DBP yields of biofilm and biofilm associated HA in R3 (NH\textsubscript{2}Cl as disinfectant) coupons upon chlorination. Similar trends for both regulated and emerging DBPs were observed compared to R2, but slightly higher CF and HAA\textsubscript{3} yields were found [around 10 μg (dm\textsuperscript{2})\textsuperscript{-1} higher]. The obtained results suggested under NH\textsubscript{2}Cl disinfection, less biofilm and biofilm associated HA were depleted in R3, which was also confirmed by biofilm analysis data.
For DBP yield results of coupons upon chloramination [Fig 7-12 (b and d), Fig 7-13 (b and d), and Fig. 7-14 (b and d)], precursors from R1 showed higher DBP yields compared to the other two reactors (R2 & R3), due to higher amount of DBP precursors on reactor coupons. In Fig. 7-12b and d, overall, regulated and emerging DBP yields were lower than 10 and 2.5 μg (dm$^2$)$^{-1}$, respectively. During the entire study, relative low regulated DBP yields were for all tested samples, the differences of emerging DBP yields among collected samples were not significant under chloramination (Fig. 7-12 d, 7-13 d, 7-14 d). For the emerging DBP speciation, HKs were shown to exhibit slightly higher yields compared to those of DCAN, 1,1,1-TCP, and TCNM, since 1,1-DCP is the main DBP produced upon chloramination.
**Figure 7-12**: DBP yields of biofilm and biofilm associated HA in R1 (control); (a) regulated DBP yields upon chlorination (Cl₂); (b) regulated DBP yields upon chloramination (NH₂Cl); (c) emerging DBP yields upon chlorination (Cl₂); (d) emerging DBP yields upon chloramination (NH₂Cl). Error bar represents standard deviation of duplicated samples.
Figure 7-13: DBP yields of biofilm and biofilm associated HA in R2 (Cl₂ disinfected); (a) regulated DBP yields upon chlorination (Cl₂); (b) regulated DBP yields upon chloramination (NH₂Cl); (c) emerging DBP yields upon chlorination (Cl₂); (d) emerging DBP yields upon chloramination (NH₂Cl). Error bar represents standard deviation of duplicated samples.
Figure 7-14: DBP yields of biofilm and biofilm associated HA in R3 (NH$_2$Cl disinfected); (a) regulated DBP yields upon chlorination (Cl$_2$); (b) regulated DBP yields upon chloramination (NH$_2$Cl); (c) emerging DBP yields upon chlorination (Cl$_2$); (d) emerging DBP yields upon chloramination (NH$_2$Cl). Error bar represents standard deviation of duplicated samples.
7.4.4 Fluorescence EEM Spectra Analysis Results

Fig. 7-15a shows the fluorescent EEM spectra of treated HA (feed solution). Most peaks were mainly located in region (em > 380 nm, 250 nm < ex < 380 nm) for humic and fulvic like substances (region II and III). For the fluorescent EEM spectra of both isolated and regrown EPS (Fig. 7-15b and 7-15c), the main difference is Fig. 7-15b (isolated EPS spectra) possess more peaks in region II and III. The isolated EPS sample has more peaks in the region for humic and fulvic like substances, which may suggest isolated EPS are associated with unremoved natural organic matter. In addition, the regrown EPS sample possessed more peaks in the regions of soluble microbial byproduct-like material (amino acids, region I) than treat HS samples.
Figure 7-15: The fluorescent EEM spectra of treated HA, isolated EPS, and regrown EPS.
Fig. 7-16 (a-g) shows EEM spectra composition changes for biofilm samples (at the sampling interval of every two weeks) from the R1 (control) during the entire operation. Overall, it seems that during the entire operation, the peak numbers and intensity in region II and III were increased, which may suggest that HA was continuously adsorbed on biofilm from bulk water. At the second week (Fig 7-16a), there were fewer peaks in region II and III compared to those of other weeks, which indicated that less humic and fulvic like material (HA) were adsorbed on reactor coupon and soluble microbial byproduct-like material (biofilm EPS) might be the dominant substances. Thus, less regulated DBP yields but relative higher N-DBP yields at the 2\textsuperscript{nd} week (Fig. 7-12c) may be attributed to higher content of microbial byproduct-like substances.

After the second week, more peaks were found in region II and III, simultaneously and higher THM, HAA and CH yields were also observed. Biofilm analysis results (Table 7.1) suggested that the increase of total biomass can provide more adsorption sites for HA in bulk solution. Besides the biofilm analysis data, fluorescent EEM spectra results also suggested that more HA was associated by biofilm, which contributed to the increase of DBP yields.
Figure 7-16: The fluorescent EEM spectra of R1 (control) during the entire operation. (a) 2nd week; (b) 4th week; (c) 6th week; (d) 8th week; (e) 10th week; (f) 12th week; (g) 14th week.
Fig. 7-17 provides the fluorescent EEM spectra change of DBP precursors in R2 during the entire operation. Compared to Fig. 7-16, the number of peaks and intensities in Fig. 7-17 were lower than those in Fig. 7-16, especially at the regions II and III. Since Cl₂ residual was maintained in this reactor, some biofilm and associated HA may be depleted during the disinfection processes. Under lower disinfectant residual, in the humic-like and humic like region, the presence of peaks suggested the adsorbed HA was not completely reacted with low Cl₂ residual(Fig 7-17a,b). However, in the second and third phase [Fig. 7-17 (d-h)], fluorescent peaks were mainly located in region I, which confirmed that associated HA was consumed by higher Cl₂ residual, simultaneously, the DBP yields from biofilm and biofilm associated HA in R2 became much lower compared to those in the first phase (Fig 7-13). At the end of operation (Fig 7-17h), less peaks were observed, which indicated that most of biofilm was eradicated.
Figure 7-17: The fluorescent EEM spectra of R2 (Cl₂ as disinfectant) during the entire operation. (a) 2\textsuperscript{nd} week; (b) 4\textsuperscript{th} week; (c) 6\textsuperscript{th} week; (d) 8\textsuperscript{th} week; (e) 10\textsuperscript{th} week; (f) 12\textsuperscript{th} week; (g) 14\textsuperscript{th} week.
Fig. 7-18 shows the fluorescent EEM spectra of DBP precursors change in R3 (NH$_2$Cl as disinfectant). Similar trend was observed compared to those in Fig. 7-17, which suggested that under high NH$_2$Cl dose, part of biofilm and associated HA had been reacted. However, under NH$_2$Cl disinfection, more peaks and higher intensities were observed in Fig. 7-18, which was attributed to the lower reactivity of NH$_2$Cl compared to Cl$_2$. Since more biofilm were available in R3 compared to R2, the DBP yields from the biofilm associated HA in R3 (Fig. 7-13) were higher than those in R2 (Fig. 7-14).
Figure 7-18: The fluorescent EEM spectra of R3 (NH₂Cl as disinfectant) during the entire operation. (a) 2^{nd} week; (b) 4^{th} week; (c) 6^{th} week; (d) 8^{th} week; (e) 10^{th} week; (f) 12^{th} week; (g) 14^{th} week.
7.5 Conclusion

The results from this study revealed that biofilm could facilitate DBP formation in drinking water distribution systems upon chlorination. Specific findings are as follow: (1) DBP formation was dramatically increase in the presence of 2 mg L$^{-1}$, especially THMs, HAAs, HANs, and CH. Not only the HA in bulk solution, but also biofilm and biofilm associated HA can react with higher Cl$_2$ residual and produce extra DBPs; (2) Higher organic nitrogen content in biofilm/EPS contributed to higher formation of toxic N-DBPs (HANs and TCNM), which increases public health risk; (3) In contrast to Cl$_2$ disinfection, higher NH$_2$Cl residual did not increase significant DBP; (4) Hydraulic flushing coupled with high disinfectant does appears to be a solution to minimize biofilm associated DBP formation.
Chapter 8

Conclusion & Future Work Recommendation

8.1 Conclusions

The four objectives of this study indicated that the presence of biofilm in drinking water distribution could play an important role on DBP formation and decay, which can be attributed to the following reasons:

- Biofilm EPS was shown to have the biosorption capacity to untreated NOM in drinking water distribution system. Those associated NOM could not only serve as nutrient sources for biofilm, but could also act as DBP precursors to enhance DBP levels.

- Biofilm and EPS, which are mainly comprised by biomolecules (polysaccharides, proteins, lipids, and nucleic acids), could also be considered as DBP precursors. Proteins in EPS have been shown to produce higher level of DBPs, especially N-DBPs, upon chlorination.

- Biofilm in drinking water distribution systems can degrade HAAs in the presence of low Cl₂ residual.
In details, the first objective of this study demonstrated that bacterial EPS could serve as important biosorption sites for untreated NOM in water distribution systems. The effect of divalent ions (Ca$^{2+}$ and Mg$^{2+}$) was highlighted to significantly enhance the biosorption capacity compared to other influencing factors of adsorbents and adsorbate (NOM hydrophobicity and EPS properties). Two mechanisms could be used to explain this phenomena: (1) the presence of divalent ions could suppress the electric double layer then decrease the potential energy between NOM and biofilm EPS; (2) The presence of divalent ions could also bind the functional groups (such as the carboxyl group) in both EPS and NOM (“bridge effect”). In addition to the effect of divalent ions, hydrophobic interactions between hydrophobic NOM and EPS were found to enhance the biosorption. However, the impact of hydrophobic interaction is not as significant as the divalent ion bridging effect.

The results of the second objective reflected the effect of bacterial EPS on the formation of C-DBPs and N-DBPs. At a chlorine limited condition (biomass in excess), both C-DBPs and N-DBPs were formed without preference for speciation. In contrast to the impact of EPS quantity and characteristics, DBP formation was determined by other influencing factors, such as pH, chlorine dose, and contact time. Moreover, time based experiments revealed that DBP formation from biomass occurs rapidly, reaching a plateau in less than four hours. However, under an excess of chlorine compared to biomass (DBP yield experiments), DBP formation was proportional to EPS content and mainly determined by EPS chemical characteristics. Protein based EPS showed higher DBP yields compared to polysaccharide based EPS. The N-DBP yield from *P. putida* EPS was two times higher than that of *P. aeruginosa* EPS, which suggested that higher
org-N content in protein based EPS contributed to higher N-DBP yield. HAA levels were higher than those of THM where DCAA was dominant in HAA species.

Based on the conclusion obtained from the second objective, bacterial EPS could contribute considerable formation of both C-DBPs and N-DBPs in water distribution systems upon disinfection. However, the relative contributions of biomolecules in EPS to DBP formation were not well understood due to the complex chemical composition of EPS. In this objective, the DBP formation results of "subunits" (amino acids and polysaccharide monomers) of major chemical components (polysaccharide and protein) in EPS indicated the protein is the major DBP precursor in biofilm EPS. Results also indicated that protein EPS surrogates possessed similar regulated DBP and slightly higher N-DBP yields compared to those of extracted EPS. In addition, group study of amino acids and polysaccharide monomers suggested conjugated and phenolic structure in amino acids are critical for higher DBP formation compared to the aliphatic structure in polysaccharide.

To investigate the influence of both NOM and biofilm on the fate of DBPs in a simulated drinking water distribution system (the fourth objective), DBP formation and decay were measured in lab scaled annular reactors upon both Cl₂ and NH₂Cl disinfection. In the presence of low Cl₂ residual, minimal DBP formation was observed, which may be mainly attributed to DBP volatilization and HAA biodegradation by biofilm during operation. At higher Cl₂ residual, biofilm was found to enhance the DBP formation. Both regulated (THMs and HAAs) and emerging DBPs (HANs and CH) were increased significantly at the beginning. The decrease of the average thickness of biofilm
and total biomass, and corresponding changes of DBP formation (especially HANs) suggest that biofilm was significantly involved in the reaction and formation of DBPs. However, biofilm could be eradicated by higher chlorine residual and DBP formation was only contributed by HA in bulk solution. On the other hand, higher NH₂Cl residuals did not contribute to higher DBP levels.

8.2 Implication of This Research and Future Work Recommendation

8.2.1 Implication of This Research

In drinking water distribution systems, it is very difficult to control biological activities and DBP levels simultaneously. In other words, maintaining the biological stability of drinking water could also bring the unwanted DBPs. DBP levels may be further increased due to the inevitable interaction between biofilm and disinfectants in water utilities. Although some advanced disinfection techniques (such as O₃, H₂O₂, peracetic acid) may reduce halogenated DBP levels, more organic and inorganic DBPs (aldehyde, bromate, and chlorate) will be produced after the switching of disinfection methods.

Based on the obtained results from this study, two suggestions may be applied to reduce unwanted DBPs in drinking water distribution systems. First, physical disinfection methods (such as UV disinfection) may need to be considered at the treatment plant to minimize chlorine does, since no DBPs are produced. Second, for both biofilm and DBP
formation control, hydraulic flushing may be used periodically to minimize biofilm formation and DBP precursor accumulations in drinking water distribution systems.

8.2.2 Future Work Recommendations

Future work recommendations of this study could be summarized in the following aspects:

- Further evaluate the biosorption using other isotherm models besides Freundlich and Langmuir isotherms. Other isotherm models such as BET, modified Langmuir-Freundlich isotherms can be applied to assess NOM biosorption on bacteria cultures.

- Further determine the role of biofilm and EPS on DBP formation in water distribution system. For this study, biofilm and associated NOM have shown to have a significant impact on DBP formation, especially N-DBPs. However, due to the complexity in drinking water distribution systems, it is very difficult to elucidate the DBP contribution from biofilm and EPS exclusively. Other factors which were discussed in the literature review section could also influence DBP formation. To further elucidate the role of biofilm and EPS, fluorescent EEM spectra and PARAFAC modeling technique based on Matlab could be used to differentiate amino acids (biofilm or EPS) and humic-like substances (NOM) [260, 270]. By analyzing fluorescent EEM spectra of biofilm associated NOM sample, it may be feasible to track the DBP contribution of some important precursors from biofilm and EPS.
• Although this research provides some insight for HAA biodegradation, only HPC and CLSM analysis results may not be enough to provide solid evidence of HAA biodegradation. Investigate the biodegradation capacity of biomass could be determined by enzyme (dehalogenase) activity evaluation techniques. Bench-scale studies may need to be conducted in future to estimate biodegradation activity and to isolate the enzymes that are responsible for this activity.

• The impact of corrosive pipe materials on DBP formation and decay in simulated drinking water distribution systems need to be investigated. The corrosion of pipe material was widely reported in North America, because of the age of the water distribution pipelines [271, 272]. In addition, corrosion may increase the disinfectant doses, further inhibit biofilm disinfection, and provide the sites to harbor available NOM [6, 273]. Therefore, it is necessary to investigate the impact of corrosive pipe materials on DBP formation and decay in simulated drinking water distribution system.
References


Appendix

Appendix A provides the detailed DBP analysis methods, calibration curves and method detection limits of all tested DBPs.

Appendix B shows detailed EPS extraction (EDTA and CER) methods (B.1) and procedure of total protein and polysaccharide measurement (B.2).

Appendix C outlines the detailed NOM (SRHA, SRNOM, and SRFA) composition in Chapter 4 (C.1) and detailed humic acid feed solution preparation in Chapter 7 (C.2).
Appendix A

A.1 THM/HAN/HK/TCNM/CH Analysis Methods

A.1.1 Stock Solution Preparation

- Prepare a clean volumetric flask (10 mL), then filled with 7 mL pure MTBE to volumetric flask.
- For adding DBP standard (purchased at Sigma Aldrich), typically, 100 µL of standard EPA 551A (THM; 2000 µg mL⁻¹), 20 µL of EPA 551B (HAN, HK, and TCNM; 2000 µg mL⁻¹), and 40 µL of chloral hydrate standard (1000 µg mL⁻¹) were added in this volumetric flask.
- Then fill the rest of the flask with pure MTBE.

A.1.2 Standard and Calibration Curve Preparation

- Prepare seven 40 mL vials, and then fill 20 mL of DI water to each of them.
- Transfer different volume (usually 0, 10, 25, 50, 75, 100, 150, and 200 µL) of prepared stock solution into the individual vials to obtain the 0, 10, 25, 50, 75, 100, 150, and 200 µg L⁻¹ standard solution.
- Calibration curves preparation can be followed by the steps in A.1.3.
**A.1.3 DBP analysis**

- Add 40 mg of ammonium chloride (NH$_4$Cl) to quench any existing Cl$_2$ residual.
- Add approximately 1 g buffer salt (Na$_2$HPO$_4$ and KH$_2$PO$_4$) to maintain the pH in the range of 4.5-5.5.
- Add 4 mL MTBE with internal standard (IS) in each vial. Detailed MTBE with IS solution preparation can be found in U Mass SOP.
- Add approximately 10 g of anhydrous sodium sulfate (Na$_2$SO$_4$) to each vial to increase the extraction efficiency.
- Shake vigorously (2 mins) by hand.
- Freeze to the samples around 1-2 hours until the organic and water layer completely separated.
- Transfer 700 µL of organic layer into 1.5 mL GC autosampler vials (avoid transferring water).
- Analysis by GC-ECD as soon as possible.

**A.1.4 Method Detection Limit (MDL) determination**

- Please follow the U mass SOP for more details. The MDLs should be around 0.1 µg L$^{-1}$. 
A.1.5 Spike Recovery Determination

- Select 10% of the samples then add 20 µL of the prepared standard stock solution in it (spiked sample).
- After obtaining the DBP concentration in spiked sample, spike recovery can be calculated based on U Mass SOP or EPA standard methods. Spike recovery should be in the range of 80-120%.

A.2 HAA Analysis Methods

A.2.1 Surrogate Stock Solution Preparation

- Prepare a clean volumetric flask (10 mL), then filled with 7 mL pure MTBE to volumetric flask.
- Add 100 µL of 2-bromobutyric acid (1000 µg mL⁻¹) into the 10 mL volumetric flask.
- Then fill the rest of the flask with pure MTBE.

A.2.2 HAA Stock Solution Preparation

- Prepare a clean volumetric flask (10 mL), then filled with 7 mL pure MTBE to volumetric flask.
- Typically, 100 µL of standard EPA 552.2 (2000 µg mL⁻¹) were added in this volumetric flask.
- Then fill the rest of the flask with pure MTBE.
A.2.3 Standard and Calibration Curve Preparation

- Prepare seven 60 mL vials, and then fill 20 mL of DI water to each of them.
- Transfer different volume (usually 0, 10, 25, 50, 75, 100, 150, and 200 µL) of prepared stock solution into the individual vials to obtain the 0, 10, 25, 50, 75, 100, 150, and 200 µg L\(^{-1}\) standard solution.
- Calibration curve preparation can be followed by the steps in A.2.4.

A.2.4 DBP Analysis

- Add 40 mg of ammonium chloride (NH\(_4\)Cl) to quench any existing Cl\(_2\) residual.
- Add 20 µL of surrogate stock solution to each vial.
- Add 1.0 mL concentrated H\(_2\)SO\(_4\) to each vial.
- Add 3 mL MTBE with internal standard (IS) in each vial.
- Add approximately 10 g of anhydrous sodium sulfate (Na\(_2\)SO\(_4\)) to each vial to increase the extraction efficiency.
- Shake vigorously (2 mins) by hand.
- Freeze to the samples around 1-2 hours until the organic and water layer completely separated. Prepare the acidic methanol (5% H\(_2\)SO\(_4\)) solution. Slowly add H\(_2\)SO\(_4\) into methanol with a glass rod. Place 2 mL of this solution into 40mL vials.
- Transfer 1 mL from first extract then placed into the vials contain 2 mL acidic methanol.
• Place the vials in 50 °C water bath for 2 hrs, temperature and reaction time has to be carefully controlled.
• After methylation step, prepare the saturated NaHCO₃ solution; add 5 mL of this solution into each vial.
• Shake the vials then release the produced CO₂, then add 1 mL pure MTBE to each vial.
• Shake the vials around 30 seconds.
• Freeze to the samples around 1-2 hours until the organic and water layer completely separated.
• Transfer 600 µL of organic layer into 1.5 mL GC autosampler vials (avoid transferring water).
• Analysis by GC-ECD as soon as possible.

**A.2.5 MDL Determination**

• Please follow the U mass SOP for more details.

**A.2.6 Spike Recovery Determination**

• Select 10% of the samples then add 20 µL of the prepared standard stock solution in it (spiked sample).
• After obtaining the DBP concentration in spiked sample, spike recovery can be calculated based on U Mass SOP or EPA standard methods. Spike recovery should be in the range of 80-120%.
A.3 Calibration Curves of Measured DBPs

Figure A-1: Calibration curve of chloroform (CF).

Figure A-2: Calibration curve of dichlorobromomethane (DCBM).
Figure A-3: Calibration curve of dibromochloromethane (DBCM).

Figure A-4: Calibration curve of bromoform.
Figure A-5: Calibration curve of dichloroacetonitrile (DCAN).

Figure A-6: Calibration curve of trichloroactetonitrile (TCAN).
Figure A-7: Calibration curve of bromochloroacetonitrile (BCAN).

Figure A-8: Calibration curve of dibromoacetonitrile (DBAN).
Figure A-9: Calibration curve of 1,1-dichloropropane (1,1-DCP).

Figure A-10: Calibration curve of 1,1,1-trichloropropane (1,1,1-TCP).
Figure A-11: Calibration curve of trichloronitromethane (TCNM).

Figure A-12: Calibration curve of chloral hydrate (CH).
Figure A-13: Calibration curve of monochloroacetic acid (MCAA).

Figure A-14: Calibration curve of monobromoacetic acid (MBAA).
Figure A-15: Calibration curve of dichloroacetic acid (DCAA).

Figure A-16: Calibration curve of trichloroacetic acid (TCAA).
Figure A-17: Calibration curve of bromochloroacetic acid (BCAA).

Figure A-18: Calibration curve of bromodichloroacetic acid (BDCAA).
Figure A-19: Calibration curve of dibromoacetic acid (DBAA).

Figure A-20: Calibration curve of chlorodibromoacetic acid (CDBAA).
Figure A-21: Calibration curve of tribromoacetic acid (TBAA).
### A.4 MDL Results

#### Table A.1: MDL of THM and HAN.

<table>
<thead>
<tr>
<th>DBPs (μg L⁻¹)</th>
<th>CHCl₃</th>
<th>CHCl₂Br</th>
<th>CHClBr₂</th>
<th>CHBr₃</th>
<th>TCAN</th>
<th>DCAN</th>
<th>DBAN</th>
<th>BCAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>2.2</td>
<td>0.4</td>
<td>0.9</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>2.2</td>
<td>0.5</td>
<td>0.9</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>2.2</td>
<td>0.4</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>2.2</td>
<td>0.4</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>2.2</td>
<td>0.4</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>2.2</td>
<td>0.4</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

s₇ = standard deviation of 7 replicate analyses where the mean is no more than 10 times the MDL.

\[ t(n-1,1-\alpha = 0.99) = 2.998 \]

<table>
<thead>
<tr>
<th>DBPs (μg L⁻¹)</th>
<th>CHCl₃</th>
<th>CHCl₂Br</th>
<th>CHClBr₂</th>
<th>CHBr₃</th>
<th>TCAN</th>
<th>DCAN</th>
<th>DBAN</th>
<th>BCAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>0.5</td>
<td>0.9</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.9</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Table A.2: MDL of HK, TCNM, and CH.

<table>
<thead>
<tr>
<th>DBPs (μg L⁻¹)</th>
<th>1,1-DCP</th>
<th>TCNM</th>
<th>1,1,1-DCP</th>
<th>CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>0.5</td>
<td>0.9</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.9</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

s₇ = standard deviation of 7 replicate analyses where the mean is no more than 10 times the MDL.

\[ t(n-1,1-\alpha = 0.99) = 2.998 \]

<table>
<thead>
<tr>
<th>DBPs (μg L⁻¹)</th>
<th>1,1-DCP</th>
<th>TCNM</th>
<th>1,1,1-DCP</th>
<th>CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>0.5</td>
<td>0.9</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.9</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.4</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

s₇ = standard deviation of 7 replicate analyses where the mean is no more than 10 times the MDL.

\[ t(n-1,1-\alpha = 0.99) = 2.998 \] = Students’ t-value for a one-sided 99% confidence level and a standard deviation estimate with n-1 degrees of freedom.
Table A.3: MDL of HAA.

<table>
<thead>
<tr>
<th>DBPs (μg L⁻¹)</th>
<th>MCAA</th>
<th>MBAA</th>
<th>DCAA</th>
<th>BCAA</th>
<th>TCAA</th>
<th>BDCAA</th>
<th>DBAA</th>
<th>CDBAA</th>
<th>TBAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>1.3</td>
<td>2.7</td>
<td>8.8</td>
<td>2.6</td>
<td>3.3</td>
<td>5.8</td>
<td>4.8</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>1.1</td>
<td>2.6</td>
<td>8.5</td>
<td>2.5</td>
<td>3.2</td>
<td>5.7</td>
<td>4.8</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>1.1</td>
<td>2.9</td>
<td>8.5</td>
<td>2.6</td>
<td>3.2</td>
<td>5.7</td>
<td>4.7</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>1.1</td>
<td>2.9</td>
<td>8.4</td>
<td>2.5</td>
<td>3.2</td>
<td>5.7</td>
<td>4.7</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>1.0</td>
<td>2.5</td>
<td>8.7</td>
<td>2.5</td>
<td>3.2</td>
<td>5.7</td>
<td>4.7</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>1.0</td>
<td>2.5</td>
<td>8.5</td>
<td>2.9</td>
<td>3.2</td>
<td>5.6</td>
<td>4.7</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>1.2</td>
<td>2.7</td>
<td>8.5</td>
<td>2.8</td>
<td>3.6</td>
<td>5.8</td>
<td>5.0</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>s⁷</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>t(n-1,1-α=0.99)</td>
<td>2.998</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDL</td>
<td>0.4</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

s⁷ = standard deviation of 7 replicate analyses where the mean is no more than 10 times the MDL

\( t(n-1,1-\alpha=0.99) \) = Students’ t-value for a one-sided 99% confidence level and a standard deviation estimate with n-1 degrees of freedom.
Appendix B

B.1 EDTA Extraction Method

- Prepare as much as bacteria (9 flasks) to ensure to obtain enough EPS.
- Harvest the bacteria by centrifugation (2, 500 rpm, 15 mins) then washed twice with DFB buffer.
- EDTA was added to each culture suspension in 1:1 ratio, and then shaken for 3 hrs at 4 °C.
- Then the solutions were vortexed, then centrifuged at 14, 000×g for 20 min at 4 °C.
- The supernatant was collected as extracted EPS.

B.2 CER Extraction Method

- Prepare as much as bacteria (9 flasks) to ensure to obtain enough EPS
- Harvest the bacteria by centrifugation (2, 500 rpm, 15 mins) then washed twice with DFB buffer.
• CER was purchased from Sigma Aldrich. Resins were carefully washed by methanol or ethanol two times to remove the organic impurities, and then washed by DI water three times to remove the inorganic impurities.

• Transfer the bacteria culture to four 50 mL falcon tubes, then added approximately 10 g of CER and 10 mL DI water to each falcon tubes.

• Apply the high vortex then centrifuged at 5,000 rpm for 30 min at 4 °C.

• The supernatant was collected as extracted EPS.

B.3 Total Protein and Polysaccharide Measurement

B.3.1 Total Protein Analysis

• BSA standards were purchased from Thermo Scientific.

• Prepare the standards from 0-1000 µg mL⁻¹ according to the procedure within the BSA standard package.

• Add the coomassie (Bradford) protein Assay Kit to each vial and react for 15 mins.

• Measure the standards by using Hach spectrophotometer in our lab (595 nm) and record the absorbance.
Figure B-1: The calibration curve of total protein measurement.

B.3.2 Total Polysaccharide Analysis

- Weigh 0.1 g of glucose and then dissolved in 100 mL volumetric flask by DI water (stock solution).
- Prepare the standards from 0-300 µg mL\(^{-1}\) by diluting the stock solution.
- Add 1 mL of 5% phenol solution to each vial.
- Add 5 mL concentrated H\(_2\)SO\(_4\) to each vial.
- Incubate for 15 mins.
- Measure the standards by using Hach spectrophotometer in our lab (490 nm) and record the absorbance.
Figure B-2: The calibration curve of total polysaccharide measurement.
Appendix C

C.1 Chemical Composition of Tested NOM from Suwannee River

Table C.1 shows the detailed chemical composition of SRFA, SRHA, and SRNOM.

Table C.1: chemical composition of SRFA, SRHA, and SRNOM (adapted from International Humic Substances Society’s website)

<table>
<thead>
<tr>
<th>Sample</th>
<th>H$_2$O</th>
<th>Ash</th>
<th>C</th>
<th>H</th>
<th>O</th>
<th>N</th>
<th>S</th>
<th>P</th>
<th>$\delta^{13}$C</th>
<th>$\delta^{15}$N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suwannee River I</td>
<td>10</td>
<td>3.1</td>
<td>52.55</td>
<td>4.4</td>
<td>42.53</td>
<td>1.19</td>
<td>0.58</td>
<td>&lt;0.01</td>
<td>-27.7</td>
<td>-1.41</td>
</tr>
<tr>
<td>Standard FA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suwannee River I</td>
<td>8.8</td>
<td>0.46</td>
<td>52.44</td>
<td>4.31</td>
<td>42.2</td>
<td>0.72</td>
<td>0.44</td>
<td>&lt;0.01</td>
<td>-27.6</td>
<td>-1.85</td>
</tr>
<tr>
<td>Aquatic NOM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suwannee River</td>
<td>8.15</td>
<td>7</td>
<td>52.47</td>
<td>4.19</td>
<td>42.69</td>
<td>1.1</td>
<td>0.65</td>
<td>0.02</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

Source: Elemental analyses by Huffman Laboratories, Wheat Ridge, CO, USA; Isotopic analyses by Soil Biochemistry Laboratory, Dept. of Soil, Water, and Climate, University of Minnesota, St. Paul, MN, USA

H$_2$O content is the % (w/w) of H$_2$O in the air-equilibrated sample (a function of relative humidity).

Ash is the % (w/w) of inorganic residue in a dry sample.

C, H, O, N, S, and P are the elemental composition in % (w/w) of a dry, ash-free sample.

$\delta^{13}$C and $\delta^{15}$N are the abundances of the respective stable isotopes in units of per mil, or o/oo.

The data for bulk source materials are reported on an as-stored basis, except that %H and %O are corrected for water content.

nd means that item was not detected.
C.2 Steps of Humic Acid Feed Solution Preparation

- Humic acid (HA) was purchased from Sigma Aldrich (Catalog #: 53680-50G).
- Dissolve 20 g of humic acid in 1000 mL DI water, use a stir bar to ensure good mixing.
- Wash the granular activated carbon (GAC) three times to remove the impurities, and then bake the GAC in oven at 90°C for three hours.
- Pack the GAC in column reactor, regenerate every six months.
- Tap water from City of Toledo (see table C.2 for detailed information) was treated by GAC column to remove any existing DBP and disinfectant residuals.
- Added 50 mL prepared HA solution into 12 L treated tap water.
- Prepare six beakers (for jar test) and divide the 12 L water in each beaker (2 L)
- Prepare 10000 mg L⁻¹ aluminum sulfate [Al₂(SO₄)₃] as coagulant, add 8 mL of coagulant to each beaker.
- Start the jar tester at 150 rpm in 2 mins, then decrease to 30 rpm and keep 30 mins.
- Turn off the jar tester and let the aggregated HA to precipitate.
- Transfer the supernatant to a clean beaker; turn on the air for ozonator, ozonated the sample for 5 mins (a stir bar should be put to ensure good mixing of O₃).
Table C.2: Contaminants in City of Toledo’s tap water (adapted from drinking water quality report from the City of Toledo in 2012).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Level</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorite</td>
<td>ppm</td>
<td>0.37</td>
<td>0.04-0.37</td>
</tr>
<tr>
<td>Fluoride</td>
<td>ppm</td>
<td>1.13</td>
<td>0.85-1.13</td>
</tr>
<tr>
<td>Nitrate</td>
<td>ppm</td>
<td>2.45</td>
<td>0.2-2.45</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Synthetic Organic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine</td>
</tr>
<tr>
<td>Simazine</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DBPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>THM₄</td>
</tr>
<tr>
<td>HAA₅</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microbiological Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbidity</td>
</tr>
<tr>
<td>TOC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Residual Disinfectants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Chlorine</td>
</tr>
<tr>
<td>Alkalinity*</td>
</tr>
<tr>
<td>Hardness*</td>
</tr>
</tbody>
</table>

**nd:** item was not detected.

*: data obtained from A Case Study-The City of Toledo 20 year master plan and needs assessment