

**Structural Characterization of Trichloroacetic Acid
Precursors Originating from Freshwater Raphidophytes
and Their Treatability in Drinking Water Treatment**

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List of Abbreviations

1D NMR	One-dimensional nuclear magnetic resonance
2D NMR	Two-dimensional nuclear magnetic resonance
AC	Activated carbon
AOM	Algal organic matter
APCI	Atmospheric pressure chemical ionization
bEOM	Bound extracellular organic matter
BSA	Bovine serum albumin
C-DBPs	Carbonaceous disinfection by-products
CCL	Contaminant Candidate List
COSY	Correlation spectroscopy
DBPFP	Disinfection by-product formation potential
DBPs	Disinfection by-products
DCAA	Dichloroacetic acid
DCAAFP	Dichloroacetic acid formation potential
DCAN	Dichloroacetonitrile
DCANFP	Dichloroacetonitrile formation potential
dEOM	Dissolved extracellular organic matter
DEPT	Distortionless enhancement by polarization transfer
DOBr	Dissolved organic bromine
DOC	Dissolved organic carbon
DOCl	Dissolved organic chlorine
DOM	Dissolved organic matter
DON	Dissolved organic nitrogen
DPD	<i>N, N</i> -Diethyl- <i>p</i> -phenylenediamine

EDTA	Ethylenediamine- <i>N, N, N', N'</i> -tetraacetic acid
EEM	Excitation-emission matrix
EOM	Extracellular organic matter
ESI	Electrospray ionization
FT-IR	Fourier transform infrared spectroscopy
HAA5	Sum of five haloacetic acids (MCAA, DCAA, TCAA, monobromoacetic acid, and dibromoacetic acid)
GAC	Granular activated carbon
HAAFP	Haloacetic acid formation potential
HAAs	Haloacetic acids
HILIC	Hydrophilic interaction liquid chromatography
HMQC	Heteronuclear multiple quantum correlation
HPLC	High-performance liquid chromatography
HPTFE	Hydrophilic polytetrafluoroethylene
HRMS	High-resolution mass spectrometry
IOM	Intracellular organic matter
IR	Infrared
JWWA	Japan Water Works Association
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LC-Orbitrap/MS	Liquid chromatography-Orbitrap mass spectrometer
MAX	Mixed-mode anion exchange
MCAA	Monochloroacetic acid
MCL	Maximum contaminant level
MCX	Mixed-mode cation exchange
MES	2-Morpholinoethanesulfonic acid
N-DBPs	Nitrogenous disinfection by-products
NDMA	<i>N</i> -Nitrosodimethylamine
NMR	Nuclear magnetic resonance
NOM	Natural organic matter
NTU	Nephelometric turbidity unit
PAC	Powdered activated carbon

PACl	Polymeric aluminum chloride
PBS	Phosphate buffer solution
RP	Reversed phase
SEC	Size-exclusion chromatography
SPE	Solid-phase extraction
TCAA	Trichloroacetic acid
TCAAFP	Trichloroacetic acid formation potential
THMs	Trihalomethanes
TOC	Total organic carbon
TON	Total organic nitrogen
TOSCY	Total correlation spectroscopy
TTHMs	Total trihalomethanes
TU	Turbidity unit
UF	Ultrafiltration
USEPA	U.S. Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization
<i>A. flos-aquae</i>	<i>Anabaena flos-aquae</i>
<i>C. muelleri</i>	<i>Chaetoceros muelleri</i>
<i>G. semen</i>	<i>Gonyostomum semen</i>
<i>M. aeruginosa</i>	<i>Microcystis aeruginosa</i>
<i>S. quadricauda</i>	<i>Scenedesmus quadricauda</i>

Chapter 1

Introduction

1.1 General background

1.1.1 Disinfection by-products (DBPs) in drinking water

In drinking water treatment, disinfection is an essential process to ensure the microbiological safety of finished water. Among various disinfection processes (e.g., chlorination, chloramination, ozonation, disinfection with chlorine dioxide, and ultraviolet irradiation), chlorination is one of the most widely used owing to its high effectiveness and stability to provide a residual effect that inhibits microbial regrowth in the distribution systems.¹ In Japan, as in many other countries, chlorine is routinely applied during the treatment process to inactivate pathogens, and its concentration is maintained throughout the distribution system to prevent microbial contamination. According to the *Ordinance Enforcement of the Water Supply Act*, a minimum free chlorine concentration of 0.1 mg/L must be maintained in the distribution system to suppress pathogen growth and recontamination.²

Although chlorine disinfection has been widely employed to control pathogenic microorganisms and significantly reduce the risk of acute waterborne diseases, it also entails undesirable side effects. Chlorine reacts with naturally occurring and anthropogenic constituents in water, leading to the formation of unintended chemical compounds referred to as disinfection by-products (DBPs), some of which are of health concern. Epidemiological investigations have indicated that chronic exposure to chlorinated drinking water containing DBPs may increase the risk of cancer, whereas *in vitro* bioassays have

demonstrated mutagenic and genotoxic effects for several DBPs.³ Since the first discovery of trichloromethane in chlorinated water in 1972,⁴ more than 1,000 DBPs have been reported,⁵ and extensive research has been conducted on their occurrence, formation mechanisms, and toxicological properties.⁶ Consequently, guideline values for frequently detected DBPs, such as trihalomethanes (THMs) and haloacetic acids (HAAs), have been established by the World Health Organization (WHO), and regulatory limits or target concentrations have been established in many countries.¹ In Japan, 11 DBP species are regulated,² and to comply with these regulations, water utilities have implemented multiple strategies, including source water management, optimization of treatment processes, and, in some cases, the introduction of advanced treatment or the substitution of drinking water sources.

Once formed, DBPs are difficult to remove because chlorine is typically added in the final stages of the treatment process, and a residual chlorine concentration is maintained in distribution systems, allowing DBP formation to continue. This indicates that, to reduce DBP formation, the decomposition or removal of DBP precursors is essential. Dissolved organic matter (DOM) in source waters is one of the main precursors of DBPs. The physicochemical complexity of DOM poses challenges to understanding the formation mechanisms of DBPs and treatability of DBP precursors. To develop effective mitigation strategies for DBP precursors in drinking water systems, it is crucial to expand scientific knowledge on the relationships between DBP formation and its precursors, specifically, which compounds in source waters act as DBP precursors, under what disinfection conditions they form DBPs, and how these precursors can be removed or transformed into less reactive forms.

1.1.2 Algae as DBP precursors

Algae, particularly microalgae that float freely in drinking water sources, are key contributors to source water quality and DBP formation. Algal blooms can develop rapidly over short periods and substantially alter both the composition and concentration of DOM in source water, imposing significant operational challenges on drinking water treatment facilities. As detailed below, numerous studies have demonstrated that algae play a major role in producing DBP precursors in surface waters and subsequently in forming DBPs

during drinking water treatment. Field observations have frequently associated algal proliferation with elevated DBP formation in both natural and drinking water sources.

For example, Jack et al. reported a positive correlation between chlorophyll-a concentrations and THM formation potential in Ohio river waters, suggesting that organic matter derived from algae can serve as an important source of DBP precursors.⁷ Similarly, Pan et al. observed that halobenzoquinone concentrations, a class of DBPs, increased during algal bloom periods.⁸ Aziz et al. also found that chlorination of lake water dominated by *Microseira* and *Phormidium* generated approximately twice as much DBPs as under normal conditions, accompanied by an increase in cytotoxicity.⁹ Likewise, Lee et al. reported that the concentrations of microcystins and total THMs were significantly higher during *Microcystis aeruginosa* (*M. aeruginosa*) blooms.¹⁰ Moreover, Khan et al. demonstrated that reservoirs with higher total organic carbon and chlorophyll-a concentrations, where cyanobacteria were dominant, exhibited greater formation potentials for THMs and HAAs.¹¹ Collectively, these findings demonstrate that algal blooms markedly increase the concentrations of DBP precursors in source waters, ultimately leading to greater DBP formation in finished drinking water.

Recently, a specific group of microalgae known as raphidophytes has attracted increasing attention in Japan.¹²⁻¹⁴ They have been associated with elevated levels of trichloroacetic acid (TCAA), one of the regulated HAAs, in drinking water.¹²⁻¹⁴ In several cases, raphidophyte bloom have raised concerns over potential exceedances of the regulatory limit for TCAA.^{12,13} Despite this, limited information is available regarding the chemical nature and treatability of TCAA precursors originating from raphidophytes. Consequently, practical countermeasures at water treatment plants have so far been limited to simple operational adjustments, such as temporarily suspending pre-chlorination. A better understanding of the chemical characteristics and treatment behavior of TCAA precursors derived from raphidophytes is therefore essential for developing effective and sustainable control strategies.

1.2 Objectives

The overall objective of this study is to propose strategies for reducing TCAA formation associated with raphidophytes, based on an understanding of the chemical structures and treatability of their TCAA precursors. The specific objectives are as follows:

- To develop a method for isolating TCAA precursors originating from raphidophytes in large-volume raw water samples or algal cultures for subsequent analyses.
- To elucidate the chemical structures and functional groups of TCAA precursors originating from raphidophyte.
- To evaluate the treatability of these precursors and identify effective control methods and operational conditions.

1.3 Structure of this thesis

This thesis consists of six chapters. Chapter 1 provides the general background, states the research objectives, and presents an overview of the thesis. Chapter 2 reviews current knowledge on algae and algal-derived organic matter, with a focus on DBP formation, the characteristics of DBP precursors, and their treatability in water treatment processes. Furthermore, as a representative example of issues related to algae and DBPs, it introduces the problem of raphidophytes and TCAA, which is also the focus of this study, and highlights the existing research gaps. Chapter 3 describes the characterization of TCAA precursors from raphidophytes by fractionation based on their hydrophobicity, molecular size, and functional group properties, and presents the development of a large-scale isolation method derived from these findings. Chapter 4 details the analysis of water quality parameters and the isolated TCAA precursors using nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared spectroscopy (FT-IR), and high-resolution mass spectrometry (HRMS), and discusses the structural features responsible for TCAA formation. Chapter 5 evaluates the treatability of TCAA precursors through individual and integrated treatment processes to identify effective control strategies. Finally, Chapter 6 summarizes the main findings, discusses the limitations of the study, and outlines directions for future research.

Chapter 2

Literature Review

2.1 Introduction

This chapter reviews the current understanding of the relationships among algae, algal organic matter (AOM), and DBP formation. The DBPs formed from algal sources and the factors influencing AOM characteristics and DBP formation, including algal taxonomy, growth stage, and metabolic pathways, are summarized. Furthermore, analytical approaches used to characterize AOM contributing to DBP formation and examples of their applications are introduced, along with the treatability of algal cells and AOM during water treatment processes. Based on these findings, the current challenges in controlling AOM as DBP precursors are discussed. Finally, this chapter focuses on the formation of TCAA, one of the regulated DBPs, from raphidophytes, highlighting the ecological features of raphidophytes and identifying the research gaps that this study aims to address.

2.2 Algae and algal organic matter (AOM)

Algae frequently proliferate in reservoirs and rivers used as drinking water sources. The “*Aquatic Organisms in Water Supply Systems*” published by the Japan Water Works Association (JWWA) lists approximately ten algal groups, including cyanobacteria (Cyanophyceae), green algae (Chlorophyceae), diatoms (Bacillariophyceae), chrysophytes (Chrysophyceae), and raphidophytes (Raphidophyceae), encompassing around 100 genera and several hundred species that may occur in drinking water sources.¹⁵ Rapid algal growth

can quickly degrade source water quality, causing problems such as musty odors,^{16,17} and the production of toxins, including cyanotoxins.¹⁸ Although AOM itself may not be directly harmful to humans, it can still pose concerns for drinking water safety by reducing treatment efficiency (e.g., interference with coagulation and membrane fouling).^{19,20} Moreover, one of the most critical issues associated with AOM is its role as a precursor of DBPs.²¹ Additionally, evaluating variations in the quantity and quality of AOM and its transformations during drinking water treatment is becoming increasingly important, as global environmental issues, particularly climate change, are shifting species distributions and increasing the frequency of algal blooms in water sources.²²

AOM is a complex mixture of extracellular and intracellular materials derived from algal metabolism. Analyzing AOM as a whole is impractical due to its diverse and complex components, so it is typically fractionated by metabolic pathways in advance. It can be broadly classified into three fractions: extracellular organic matter (EOM), which is actively released into the surrounding water; intracellular organic matter (IOM), which remains within cells and is released upon cell lysis; and cell debris, consisting of residual cell structures such as cell membranes and cell walls.²³ Past studies often did not separately extract IOM, and analyses were limited to EOM and the remaining cell fraction, which implicitly included IOM (often simply referred to as algal cells).²⁴ More recently, the EOM fraction itself has been further subdivided into bound EOM (bEOM) and dissolved EOM (dEOM), depending on whether it is loosely attached to the cell surface or freely dissolved in water.^{25,26}

Moreover, the composition of AOM varies depending on the algal growth phases.²⁷ Algal growth generally proceeds through the lag (adaptation to a new environment), log/exponential (rapid increase in cell population), stationary (balance between growth and death), and death/decline (cell decay) phases, corresponding to different physiological states. During the log/exponential phase, algae actively excrete low molecular weight metabolites, whereas in the stationary or decline phases, the breakdown of cell walls releases higher molecular weight intracellular materials, such as chlorophylls and proteins.^{27,28}

The characteristics of AOM also differ markedly among algal taxa. Each algal group exhibits unique growth strategies and biochemical compositions,²⁹ leading to distinct

AOM profiles and DBP formation potentials (DBPFPs). In the following section, the formation of DBPs from AOM is discussed in relation to algal taxonomy, metabolic pathways, and growth phases, providing a comprehensive understanding of the occurrence and behavior of AOM-derived DBPs in water treatment.

2.3 DBP formation from AOM

2.3.1 Carbonaceous and nitrogenous DBPs produced from AOM

The formation of both carbonaceous DBPs (C-DBPs) and nitrogenous DBPs (N-DBPs) has been reported from AOM during chlorination. The C-DBP and N-DBP species frequently detected are summarized in Figure 2.1. Among various DBPs, the formation of THMs and HAAs, particularly chloroform, dichloroacetic acid (DCAA), and TCAA, has been the most extensively studied, as detailed in the next subsection (see Subsection 2.3.2 and Table 2.1). These C-DBPs are regulated in many countries worldwide because of their frequent detection at relatively high concentrations.¹ For instance, the U.S. Environmental Protection Agency (USEPA) has set the maximum contaminant levels (MCLs) for total THMs (TTHMs, the sum of chloroform (CHCl_3), bromodichloromethane (CHBrCl_2), dibromochloromethane (CHBr_2Cl), and bromoform (CHBr_3)) and HAA5 (the sum of chloroacetic acid (referred to as monochloroacetic acid (MCAA) in this study), DCAA, TCAA, bromoacetic acid, and dibromoacetic acid) at 0.08 and 0.06 mg/L, respectively.³⁰ In Japan, the standard values for chlorinated HAAs were revised in 2015 to more stringent levels (i.e., MCAA, 0.02 mg/L; DCAA, 0.03 mg/L; TCAA, 0.03 mg/L).² Consequently, TCAA has become the species of greatest concern regarding potential exceedance of these regulatory limits.

In addition, various N-DBPs such as haloacetonitriles, trichloronitromethane, haloacetamides, *N*-nitrosamines, and haloacetaldehydes have been reported to form during chlorination of AOM (see Subsection 2.3.2 and Table 2.1). Since algae contain a high proportion of nitrogenous compounds such as proteins and chlorophylls, N-DBPs are generally produced in greater amounts than those formed from natural organic matter (NOM).²⁴ Although N-DBPs are known to be more toxic than C-DBPs,³¹ no clear regulatory limits

have been established to date. In the United States, several N-DBPs are listed only in the Contaminant Candidate List (CCL),³² while in Japan, a few compounds have been included as items under review or target items for water quality management, which are non-enforceable monitoring categories.²

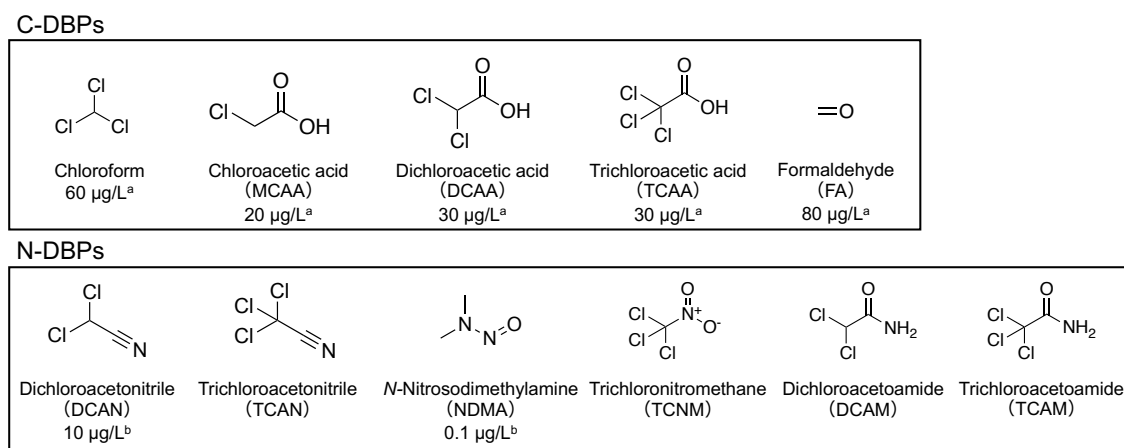


Figure 2.1. Examples of C-DBPs and N-DBPs.

^a represents the regulatory values defined under the drinking water quality standards in Japan, while ^b represents the target values set for water quality management objectives in Japan.

2.3.2 DBP yields from AOM

The available data on DBP formation from the chlorination of algal samples are summarized in Table 2.1, organized by algal species. The characteristics of AOM, such as growth phase, chlorination conditions, and fractions (EOM, IOM, or algal cells), are also included as factors influencing DBP formation. For C-DBPs, THMs were reported in all studies, ranging from 0 to 177 µg/mg C, while HAAs were the second most reported group, with DCAA ranging from 0.8 to 79 µg/mg C and TCAA from 6.3 to 93 µg/mg C. Considering current drinking water quality standards, if algae proliferate in source water, an increase of a few mg C/L in dissolved organic carbon (DOC) concentration may raise concerns about the potential exceedance of regulatory limits for DBPs. This scenario is quite realistic, as such DOC increases can readily occur during algal blooms. In Japan, for instance, the drinking water standards for individual HAAs such as DCAA and TCAA are 30 µg/L (see Figure 2.1). Given that DOC levels can reach several mg C/L during algal blooms, there is a concern that the concentrations of these HAAs in treated water could

approach or exceed the regulatory thresholds, underscoring the urgent need to develop appropriate treatment strategies, preventive measures, and predictive approaches to mitigate algal impacts on DBP formation.

For N-DBPs, the formation of dichloroacetonitrile (DCAN) has been frequently observed. High yields were often detected for green algae, with the IOM fraction of *Chlorella sorokiniana* producing a maximum of 16.7 $\mu\text{g}/\text{mg C}$.³³ Although direct quantitative data were not reported in some studies, figures indicated that DCAN and TCAN could be formed at approximately 30 $\mu\text{g}/\text{mg C}$ after 24 h of reaction.³⁴ In other studies, the formation of TCAN was often negligible, likely due to its lower stability compared with DCAN.³⁵ In addition, TCNM has also been detected, although its formation was not as pronounced as that of DCAN. The formation of *N*-nitrosodimethylamine (NDMA) has been also confirmed from *M. aeruginosa* in chlorination.³⁶

2.3.3 Influence of algal properties on DBP formation

Significant differences in DBP formation were observed among algal species, AOM fractions, chlorination conditions, and growth phases (see Table 2.1). Regarding algal species, distinct variations in DBP yields were reported even under similar experimental conditions. For instance, *Oscillatoria prolifera*, *Chaetoceros muelleri* (*C. muelleri*), and *Scenedesmus quadricauda* (*S. quadricauda*) produced 30, 30, and 48 $\mu\text{g}/\text{mg C}$ of THMs, respectively, with *S. quadricauda* showing the highest formation potential.³⁷ Similarly, *Nitzschia* sp. and *Chlamydomonas* sp. yielded 48 and 34 $\mu\text{g}/\text{mg C}$ of THMs, respectively, indicating inter-group differences in DBP formation.³⁸ Even within the same algal group, the DBP yields varied considerably. For example, *M. aeruginosa* produced higher amounts of both THMs and HAAs than *Anabaena flos-aquae* (*A. flos-aquae*) under identical chlorination conditions,³⁹ and among diatoms, *C. muelleri* showed nearly twice the THM formation of *Melosira* sp.^{37,40}

Differences were also evident among AOM fractions. For *M. aeruginosa* and *A. flos-aquae*, algal cells generated higher amounts of THMs and TCAA than EOM,³⁹ whereas in *Chlorella* sp., the values were nearly comparable between these fractions.⁴¹

Chlorination conditions also affected DBP formation. In one study, THM yields from *M. aeruginosa* did not change substantially with reaction time,³⁴ whereas in *Chlamy-*

domonas sp., both THM and HAA concentrations increased markedly as the chlorination period was extended.^{38,42} Even among different studies, the reported THM yields from *M. aeruginosa* ranged widely from 4.4 to 64 $\mu\text{g}/\text{mg C}$,^{34,39} likely due to differences in reaction time (1 vs. 3 days) as well as other factors such as algal growth conditions, nutrient availability, or experimental setup (e.g., cculture volume, vessel type and shape, or mixing conditions).

Growth phase also played an important role. *M. aeruginosa* exhibited the highest THM yield per unit carbon during the lag phase, which decreased and then increased again toward the death phase, suggesting compositional changes in AOM during growth.⁴³ Consistent trends were observed in other studies, where the highest THM formation was reported when cultures were grown until the death phase,^{41,44} likely due to the release of IOM following cell lysis.

Finally, even slight differences in culture conditions can lead to variability in DBP formation. For example, *S. quadricauda* showed approximately 20% variation in THM yield between experiments conducted under nearly identical conditions, except for the culture volume.³⁷ These findings indicate that the DBPFP can vary depending on subtle differences in growth and experimental conditions. This has also been demonstrated in *M. aeruginosa*, where variations in growth conditions significantly influenced the THM formation potential, highlighting the importance of considering environmental and experimental factors when assessing DBP formation.⁴⁵

2.3.4 Environmental and non-algal factors influencing DBP formation

The types of DBPs formed from AOM can be influenced by environmental factors such as the presence of bromide (Br^-).⁴⁶ Under conditions where Br^- is present, brominated DBPs are preferentially produced over chlorinated species.⁴⁶ Although their concentrations are generally lower than those of chlorinated DBPs, their toxicities are often reported to be higher, warranting careful attention.⁴⁷ Iodinated DBPs are often formed during chloramination in the presence of iodide ions (I^-), whereas their formation is generally limited under conventional chlorination.⁴⁸ Furthermore, the incorporation of bromine into AOM

has been demonstrated.⁴⁹ Hua et al. showed that algae can absorb halogens and form organically bound halogens, suggesting that in environments where Br^- is present, not only should the influence of bromide on chlorination reactions be considered, but AOM containing bromine may also serve as a precursor of brominated DBPs.

In addition, metal ions and oxides can affect DBP formation. Ge et al. found that iron and manganese ions decreased DCAA formation potentials (DCAAFPs) and TCAA formation potentials (TCAAFPs) from *Chlorella vulgaris* cells, likely because their oxides coated the algal cells during chlorination.⁵⁰ Similarly, in pipelines using lead dioxide (PbO_2), the presence of iodide can promote the formation of iodinated DBPs even under normal chlorination conditions.⁵¹ Moreover, xenobiotics, chemicals that are foreign to living organisms, can also influence DBP formation. For instance, the presence of nitrobenzene, a typical xenobiotic compound, was reported to enhance the formation potential of THM precursors derived from *M. aeruginosa*.⁵² This enhancement might be attributed to the increased protein production and the biodegradation of nitrobenzene by *M. aeruginosa*, which consequently altered the composition of AOM and its reactivity toward chlorine. These observations indicate that non-algal-derived environmental factors are also important determinants of DBP formation and should be considered when predicting DBP yields from AOM.

Table 2.1. Summary of published DBP data from AOM in chlorination.

Algal species	Group	Growth phase	Chlorination condition							IOM/EOM /Algal cells	C-DBPs (μg/mg C)			N-DBPs (μg/mg C)				Ref.
			pH	Time (h)	Temp. (°C)	Cl ₂ dose (mg Cl ₂ /L)	DOC (mg C/L)	Cl ₂ /DOC	THM		DCAA	TCAA	DCAN	TCAN	TCNM	NDMA		
<i>Anabaena flos-aquae</i>	cyanobacteria	Stationary	7	168	21	>0.5 ^a	—	—	Algal cells	50	29	49	—	—	—	—	[39]	
<i>Anabaena flos-aquae</i>	cyanobacteria	Stationary	7	168	21	>0.5 ^a	—	—	EOM	26	26	22	—	—	—	—	[39]	
<i>Anabaena flos-aquae</i>	cyanobacteria	Onset of stationary	7	168	20	—	—	5	EOM	26.6	18.7 ^b		0.39	N.D. ^c	0.16	—	[40]	
<i>Aphanizomenon flos-aquae</i>	cyanobacteria	Onset of stationary	7	168	20	—	—	5	EOM	56.6	24 ^b		0.12	N.D. ^c	0.11	—	[40]	
<i>Microcystis aeruginosa</i>	cyanobacteria	Stationary	7	168	21	>0.5 ^a	—	—	Algal cells	61	71	93	—	—	—	—	[39]	
<i>Microcystis aeruginosa</i>	cyanobacteria	Stationary	7	168	21	>0.5 ^a	—	—	EOM	28	42	24	—	—	—	—	[39]	
<i>Microcystis aeruginosa</i>	cyanobacteria	Late exponential	7.5	72	22–24	3–5 ^a	—	—	IOM	64	60	51	1.2	—	—	—	[53]	
<i>Microcystis aeruginosa</i>	cyanobacteria	10 days cultivation	7	24, 72	—	—	2.0	—	EOM	156, 177	—	—	—	—	—	—	[34]	
<i>Microcystis aeruginosa</i>	cyanobacteria	Onset of stationary	7	168	20	—	—	5	EOM	42.6	28.7 ^b		1.32	N.D. ^c	0.13	—	[40]	
<i>Microcystis aeruginosa</i>	cyanobacteria	Late exponential	7.5	24	21	40	2.0	20	IOM	4.4	—	—	—	—	—	—	[54]	
<i>Microcystis aeruginosa</i>	cyanobacteria	Late exponential	7	72	25	—	—	5	EOM	—	—	—	—	—	—	3.70	[36]	
<i>Microcystis aeruginosa</i>	cyanobacteria	Late exponential	7	72	25	—	—	5	IOM	—	—	—	—	—	—	2.81	[36]	
<i>Microseira wollei</i>	cyanobacteria	Natural water	7	24	25	1–2 ^a	—	—	Algal cells	0	3.84 ^b		0.1	—	—	—	[9]	
<i>Microseira wollei</i>	cyanobacteria	Natural water	7	24	25	1–2 ^a	—	—	Algal cells	0	3.92 ^b		1.13	—	—	—	[9]	
<i>Microseira wollei</i>	cyanobacteria	Natural water	7	24	25	1–2 ^a	—	—	Algal cells	0	3.23 ^b		0.68	—	—	—	[9]	
<i>Lyngbya</i> sp.	cyanobacteria	Late exponential	7.5	72	22–24	3–5 ^a	—	—	IOM	38	63	32	1	—	—	—	[53]	
<i>Oscillatoria prolifera</i>	cyanobacteria	Exponential	7	168	20	—	—	5	EOM	30	—	—	—	—	—	—	[37]	
<i>Oscillatoria</i> sp.	cyanobacteria	Exponential	7	96	20	—	—	5	Algal cells	26	34	39	—	—	—	—	[38]	
<i>Oscillatoria</i> sp.	cyanobacteria	Late exponential	7.5	72	22–24	3–5 ^a	—	—	IOM	47	79	35	0.7	—	—	—	[53]	
<i>Synechicoccus</i> sp.	cyanobacteria	Late exponential	7.5	24	21	40	2.0	20	IOM	6.7	—	—	—	—	—	—	[54]	
<i>Phormidium</i> spp.	cyanobacteria	Natural water	7	24	25	1–2 ^a	—	—	Algal cells	3.4	9.43 ^b		1.42	—	—	—	[9]	
Natural water in Taihu Lake ^d	cyanobacteria	Natural water	7	168	22	—	—	5	AOM	16	31 ^b		—	—	—	—	[55]	
<i>Alexandrium tamarense</i>	dinoflagellate	Late exponential	7.5	24	21	40	2.0	20	IOM	6.7	—	—	—	—	—	—	[54]	
<i>Asterionella formosa</i>	diatom	Onset of stationary	7	168	20	—	—	5	EOM	18.7	25 ^b		0.53	N.D. ^c	0.24	—	[40]	
<i>Chaetoceros muelleri</i>	diatom	Onset of stationary	7	168	20	—	—	5	EOM	30	—	—	—	—	—	—	[37]	
<i>Melosira</i> sp.	diatom	Onset of stationary	7	168	20	—	—	5	EOM	19	13.2 ^b		0.87	N.D. ^c	0.36	—	[40]	
<i>Nitzschia</i> sp.	diatom	Exponential	7	96	20	—	—	5	Algal cells	48	25	19	—	—	—	—	[38]	
<i>Chlamydomonas</i> sp.	green algae	Exponential	7	96	20	—	—	5	Algal cells	34	29	33	—	—	—	—	[38]	
<i>Chlamydomonas</i> sp.	green algae	Exponential	7	0–2	20	1000	50	20	Algal cells	3.0–22	0.89–10.6	3.7–8.2	0.043–8.38	N.D. [≤] 1.82		—	[42]	
<i>Chlorella sorokiniana</i>	green algae	Stationary	8	168	20	—	—	5	IOM	57.9	—	—	16.7	—	—	—	[33]	
<i>Chlorella</i> sp.	green algae	Late exponential	7	168	20	—	—	5	EOM	8.3	11	8.2	—	—	—	—	[41]	
<i>Chlorella</i> sp.	green algae	Late exponential	7	168	20	—	—	5	IOM	9.7	18	6.3	—	—	—	—	[41]	
<i>Chlorella vulgaris</i>	green algae	Death	7	168	20	—	—	10	EOM	23.9	13.4	14.2	13.9	—	9.3	—	[44]	
<i>Scenedesmus quadricauda</i>	green algae	Onset of stationary	7	168	20	—	—	5	EOM	48	—	—	—	—	—	—	[37]	
<i>Scenedesmus quadricauda</i> ^e	green algae	Onset of stationary	7	168	20	—	—	5	EOM	64	35	23	—	—	—	—	[37]	
<i>Scenedesmus subspicatus</i>	green algae	Onset of stationary	7	168	20	—	—	5	EOM	19*	35.8		1.1	N.D. ^c	—	—	[40]	

^a Cl_2 dose was adjusted based on residual chlorine.^b The value was the sum of the formation of HAAs.^c N.D. means not detected.^d The priority species were *Oscillatoria* sp., *Anabaena* sp., *Aphanizomenon* sp., and *Microcystis* sp.^e Large scale culture at 20L.

2.4 Characterization of AOM as DBP precursors

As discussed above, AOM has been shown to be a major precursor of DBPs. In this section, the characteristics of AOM as a DBP precursor are summarized. Detailed information on the physicochemical properties and chemical structures of DBP precursors within the AOM pool is essential for optimizing treatment conditions and identifying more effective strategies to reduce DBP formation.

Several studies have highlighted the high biodegradability of algal-derived DOC. For example, Nguyen et al. reported that more than 60% of algal DOC was decomposed in five days.³⁷ Similarly, the ratio of biodegradable DOC to total DOC (BDOC/DOC) after five days ranged from 52.5% to 67.4%, indicating a high biodegradability of algal DOC.⁵⁶ The biodegradation of AOM likely cause temporal variations in DBP formation, depending on microbial activity and degradation stages. This suggests that during algal growth, AOM itself should be considered, whereas for evaluating DBP formation after growth, it is necessary to take into account the AOM that has been altered through biodegradation.

The relationship between AOM characteristics and DBP formation has been widely investigated using bulk parameters such as water quality indicators (e.g., total organic carbon (TOC) or DOC, total or dissolved organic nitrogen (TON/DON), and their ratios) and biochemical composition (e.g., lipids, proteins, carbohydrates, and humic substances).^{57,58} Among these, proteins, lipids, and humic substances have been identified as reactive DBP precursors, whereas glycans, which exhibit low reactivity toward chlorine, are typically not considered important precursors.^{57,58} AOM is a component of NOM, but its composition differs from that of bulk NOM. Compared with bulk NOM, AOM generally contains higher proportions of reactive proteins and peptides and has elevated DON concentrations.⁵⁵ Consequently, chlorination of AOM tends to produce larger amounts of N-DBPs and C-DBPs than that of NOM.⁵⁵

Additionally, simple optical analyses such as ultraviolet (UV) absorbance and excitation-emission matrix (EEM) spectroscopy have frequently been used to detect specific compounds (e.g., proteins and humic substances) and key structural features (e.g., unsaturated carbon bonds and aromaticity).^{59–61} These studies consistently concluded that proteinaceous compounds containing reactive amino acids such as tyrosine and tryptophan, as

well as unsaturated carbon structures, are major contributors to DBP formation.

Fractionation studies based on molecular weight have shown that both low- and high-molecular-weight fractions can act as DBP precursors, depending on the algal species and origins (i.e., EOM and IOM).^{61–63} In addition, from the fractionation based on the differences of hydrophilicity of AOM, hydrophilic AOM often contribute more to DBP formation than hydrophobic fractions.^{40,56}

Beyond bulk characterization, recent studies have increasingly applied molecular-level techniques for AOM analysis. Advanced analytical methods such as infrared (IR) spectroscopy, X-ray photoelectron spectroscopy (XPS), HRMS, and NMR spectroscopy have been employed to elucidate the molecular structures of AOM.^{41,64–67} These studies have identified specific molecular features as key determinants of DBP precursor reactivity. For example, van Krevelen diagrams generated from HRMS spectra of *M. aeruginosa* suggested that peptide-like compounds and organic chloramines act as important precursors of DBPs.⁶⁴ Similarly, van Krevelen analyses of *M. aeruginosa*, *Dolichospermum circinale*, and *Cylindrospermopsis raciborskii* revealed that compounds with relatively high O/C (0.5–0.8) and low H/C (0.9–1.8) ratios serve as key precursors of DCAN.⁶⁶ Solid-state ¹⁵N NMR analysis of extracts from *M. aeruginosa* further detected abundant aliphatic and heterocyclic N-containing groups, supporting the role of proteinaceous materials in N-DBP formation.⁶⁵ ¹³C NMR and X-ray Photoelectron Spectrometer analyses of *Chlorella* sp. showed that its IOM contained a complex mixture of aliphatic, amino acid, carbohydrate, aromatic, and carbonyl moieties, whereas EOM fractions are dominated by carboxylic groups,⁴¹ leading to the different DBP formation profiles. In natural waters, HRMS and NMR profiling demonstrated that major shifts in DOM composition were attributable to algal proliferation, highlighting the ecological relevance of these molecular signatures.⁶⁷

Despite the development of various AOM characterization and fractionation methods, these approaches are typically designed based on the separation techniques themselves rather than the specific behaviors of DBP precursors. As a result, the obtained fractions often contain substances unrelated to DBP formation, making it difficult to extract information specific to DBP-forming components. This limitation could be overcome by preparing solutions containing highly purified DBP precursors at high concentrations;

however, to our knowledge, no study has yet succeeded in doing so.

2.5 Treatability of AOM and algal cells

One effective strategy to control algae-derived DBPs is to minimize the concentration of AOM prior to disinfection. AOM removal methods can be broadly classified into two major approaches: (1) the removal of algal cells and (2) the removal of AOM including EOM and IOM released by cell lysis.

The former approach focuses on IOM by removing cells without rupture, using coagulation, flocculation, filtration, or membrane filtration. Several studies have explored treatment methods for the removal of algal cells and their interactions with flocculants and membranes.^{68,69} The efficiency of coagulation and flocculation is influenced by algal morphology and extracellular polymer content.⁶⁸ For instance, filamentous species tend to form larger and more stable flocs compared with unicellular species, facilitating removal by sedimentation or filtration.⁶⁸ In addition, AOM biopolymer components such as proteins and polysaccharides can be partially removed along with algal cells during coagulation, contributing to a reduction in DBP precursors.⁶⁸ However, when the concentration of AOM is high, floc formation may be inhibited, leading to lower overall removal efficiency.⁶⁸

Pre-oxidation with chlorine, ozone, or permanganate is commonly employed to enhance the efficiency of these processes by altering the chemical properties and zeta potential of algal cell surfaces. However, excessive oxidant doses can increase DBP formation due to cell lysis and the release of IOM.^{70,71} Although ozonation was found to be more effective than pre-chlorination in reducing DBP formation, excessive ozone doses led to higher DBP yields.⁷⁰ Another study also reported that ozonation could enhance DBP formation, while permanganate was more effective for DBP control.⁷¹ Pre-oxidation with permanganate can improve subsequent coagulation and sedimentation when the dosage is properly controlled,⁷² although elevated Mn concentrations may pose additional concerns. Maintaining cell integrity during pre-oxidation is crucial, and determining the optimal oxidant dose remains a key challenge. Recently, machine learning techniques have been applied to optimize pre-chlorination conditions.⁷³

In addition to conventional approaches, various alternative methods have been investigated. Electrochemical methods have also been proposed. For example, an electrochemical system using graphite felt and a Pt electrode successfully removed more than >90% of algal cells.⁷⁴ However, the cost of such systems may limit their practical application. Flotation techniques, which generate bubbles by adding surfactants or injecting compressed air, have also shown promising results. The addition of coagulants facilitates floc formation, achieving removal efficiencies exceeding 90%.⁷⁵ These methods could be feasible for application in water sources. Ultrasound treatment has been reported to inactivate algal cells and partially disrupt their antioxidant systems. The coagulation performance of algal cells improved substantially after ultrasonication,⁷⁶ indicating its potential as a pretreatment method. Although these techniques are highly effective, their implementation generally involves higher costs compared with conventional pre-oxidation processes.

The second approach targets the removal of EOM and IOM released by ruptured cells. The effectiveness of various treatment methods, including coagulation, sedimentation, ozonation, UV irradiation, activated carbon (AC) adsorption (both powdered and granular), and membrane filtration, has been extensively evaluated.^{57,77–80} Granular activated carbon (GAC) has been reported to remove multiple types of algal EOM with moderate efficiency (23–58%), which is limited by the highly hydrophilic nature of AOM.⁸¹ Adsorption can be enhanced under slightly acidic conditions (pH 5) and at elevated temperatures (20–40°C).⁸¹ Aluminum-based coagulants also remove AOM, but their capacity for EOM/IOM removal is limited. Optimal coagulant dosages are required, as both under- and over-dosing reduce treatment efficiency.⁷⁹ Polymeric aluminum chloride (PACl) has been reported to achieve better coagulation performance than the others.⁸² UV irradiation preferentially decomposes protein-like and humic-like substances, leading to decreased HAA formation after chlorination.⁷⁷ Although ozonation alone may increase DBP formation, subsequent AC treatment effectively mitigates this issue.⁷⁸ Additionally, aluminum-based coagulants significantly remove protein-like compounds, chlorophyll, and phycocyanin in AOM, although their capacity to precipitate AOM is limited.⁷⁹

The treatability of AOM is generally evaluated by measuring DBP formation before and after treatment, in combination with AOM characterization to elucidate removal

mechanisms. If the structures and properties of DBP precursors were clearly identified, the interpretation and predictive accuracy of such analyses would be greatly improved. As discussed in the previous chapter, the ability to isolate highly purified DBP precursors at elevated concentrations would also facilitate a more mechanistic evaluation of treatability.

2.6 Raphidophytes as TCAA precursors

Raphidophytes are single-celled, motile, and phototactic algae characterized by two flagella and are known to adversely affect human health and microbial ecosystems.^{83,84} They inhabit both freshwater and marine environments. Marine raphidophytes (e.g., *Chattonella* and *Heterosigma*) contain chloroplast pigments that give them a reddish color and can cause red tides during bloom events worldwide, often resulting in massive fish kills.⁸⁴ Freshwater raphidophytes, typically green in color (e.g., *Gonyostomum* and *Merotrichia*), are also known to negatively impact human health and aquatic ecosystems.⁸³ Among them, *Gonyostomum semen* (*G. semen*) has been the most extensively studied (Figure 2.2). Because metabolites released from *G. semen* can cause skin inflammation upon contact, ecological investigations have been conducted since the 1980s.⁸³ *G. semen* cells are approximately 45–75 μm long, 20–30 μm wide, and 10–20 μm thick, lacking a cell wall and exhibiting a soft, leaf-like morphology.^{15,85} Unlike many other phytoplankton, *G. semen* is relatively large and thrives in cooler waters. Thus, when it becomes dominant, it can constitute a substantial proportion of the phytoplankton community, partly due to the absence of natural predators.⁸⁶

Blooms of *G. semen* have been primarily reported worldwide in northern Europe and North America, with frequent occurrences in Scandinavia,^{87,88} the United States,⁸⁹ South America,⁹⁰ and Japan.^{91,92} Over the past decade, raphidophytes have been increasingly detected in water sources in the Kansai region of Japan, including Lake Biwa (Shiga Prefecture),¹² Muro Dam (Nara Prefecture),¹³ and the Sengari Reservoir (Hyogo Prefecture),¹⁴ where their presence had not been previously reported. Previous studies demonstrated that raphidophyte occurrence in source water markedly increased TCAA concentrations in drinking water, approaching the Japanese regulatory limit of 0.03 mg/L.^{12,13} Furthermore, strong correlations ($R > 0.9$, $p < 0.05$) were observed between the cell pop-

ulation of *G. semen* and the formation potentials of chloroform, DCAA, and TCAA in chlorination, with the TCAAFP of isolated cells being approximately three times higher than that of chloroform or DCAA.¹² Notably, recent comprehensive DBP formation profiling using various algal cultures has demonstrated that *G. semen* possesses an high TCAAFP, not only exceeding its own other DBPFPs but also significantly surpassing the DBPFPs of other common algae.⁹³ These findings indicate that raphidophytes produce AOM that serves as a highly efficient precursor for TCAA formation. However, the specific algal metabolites contributing to TCAA formation and the most effective treatment strategies have not been fully investigated. Therefore, establishing effective methods for removing TCAA precursors derived from raphidophytes is essential.

Raphidophyte cells are fragile and easily ruptured because they lack a cell wall. As discussed in the section on treatability, it is extremely challenging to remove algal cells without cell disruption. Even a small amount of chlorine can cause cell lysis,¹² suggesting that the removal of AOM, rather than intact cells, is a more practical approach for controlling DBP formation. This study focused on the relationship between raphidophytes and TCAA precursors as part of a broader investigation into AOM and algal contributions to DBP formation. Unlike previous studies, TCAA precursors were extracted at high concentrations and purities, their structural characteristics were elucidated, and their treatability was evaluated to propose practically feasible and optimized treatment strategies. A logical and effective approach was adopted to achieve these objectives.

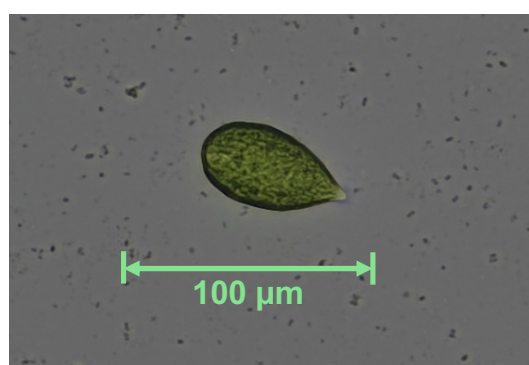


Figure 2.2. Vegetative cells of *G. semen*.

2.7 Summary

This chapter reviewed the occurrence, characteristics, and treatment of AOM with respect to its role as a precursor of DBPs. AOM differs from NOM in terms of composition, hydrophilicity, and molecular weight distribution, resulting in distinct DBP formation behaviors during chlorination. Both C-DBPs and N-DBPs are formed from AOM, and their yields vary substantially depending on algal species, growth phase, and the proportions of EOM and IOM.

Various analytical and fractionation studies have demonstrated that proteins and aromatic compounds in AOM act as key DBP precursors, whereas carbohydrates tend to be less reactive. Although molecular-level analyses have increasingly elucidated individual precursor compounds, challenges remain in distinguishing true DBP precursors from coexisting nonreactive substances.

In terms of treatability, AOM removal can be categorized into two major approaches: the removal of algal cells and the removal of dissolved AOM. The treatability of AOM and algal cells varies depending on the treatment method, with coagulation and sedimentation, ozonation, and AC adsorption being the most widely studied. However, the efficiency of these processes can differ according to algal taxa and their metabolic products, and in some cases, inappropriate pre-oxidation conditions can even enhance DBP formation. These findings highlight the need to develop optimized treatment strategies tailored to the characteristics of each algal group.

Finally, among the various algal taxa, attention has been directed toward raphidophytes such as *G. semen*, which have recently been detected in source waters in the Kansai region of Japan. Their occurrence has been associated with elevated TCAA levels in drinking water, underscoring the necessity of establishing effective removal methods for TCAA precursors derived from raphidophytes. Addressing this issue forms the primary focus of the present study.

Chapter 3

Characterization and Isolation of Trichloroacetic Acid Precursors Originating from Freshwater Raphidophytes upon Chlorination

3.1 Introduction

In recent years, raphidophytes have been detected in drinking water sources in the Kansai region of Japan, including Lake Biwa (Shiga Prefecture), Muro Dam (Nara Prefecture), and the Sengari Reservoir (Hyogo Prefecture), where they had previously been unreported.^{12–14} This leads to increase in TCAA concentrations in finished water.^{12,13}

As mentioned in Chapter 2, minimizing DBP formation from algae typically requires the removal of algal cells without decomposition to prevent the release of IOM. However,

The results of the present chapter are based on the following peer-reviewed publications:

(1) Tada, Y., Kosaka, K., Echigo, S., and Itoh, S. (2023). High formation of trichloroacetic acid from high-molecular-weight and ultra-hydrophilic components in freshwater raphidophytes upon chlorination. *Science of the Total Environment*, 879, 163000. <https://doi.org/10.1016/j.scitotenv.2023.163000>

(2) Tada, Y., Hori, K., Kosaka, K., Echigo, S., and Itoh, S. (2025). Structural estimation of high-molecular-weight and ultra-hydrophilic trichloroacetic acid precursors originated from raphidophytes. *Environmental Science & Technology*, 59(42). <https://doi.org/10.1021/acs.est.5c05108>

because raphidophyte cells lack cell walls,⁸⁵ they are fragile and easily disrupted by slight physical shocks or environmental fluctuations in salinity. Consequently, intact removal is difficult, and direct treatment of dissolved TCAA precursors is necessary. The properties of these precursors, however, have not been fully investigated, and such information is critical for developing effective treatment strategies.

The objective of this chapter is to investigate the physicochemical properties of the unique TCAA precursors from raphidophytes and to establish effective isolation methods for further studies, including precursor identification (Chapter 4) and treatability experiments (Chapter 5). First, the TCAA precursors in natural water (collected from Muro Dam) containing raphidophytes, as well as from *G. semen* cultures, were characterized in terms of their spectral and retention properties using several high-performance liquid chromatography (HPLC) columns, solid-phase extraction (SPE) cartridges, ultrafiltration (UF) membranes, and HPLC fractionation with a size-exclusion chromatography (SEC) column. Based on this information, an isolation method for the TCAA precursors was developed.

3.2 Materials and methods

3.2.1 Reagents and solutions

Ethylenediamine-*N*, *N*, *N'*, *N'*-tetraacetic acid (EDTA) disodium salt dihydrate and 2-morpholinoethanesulfonic acid (MES) monohydrate were purchased from Dojindo Laboratory (Kumamoto, Japan). Standard stock solutions of HAAs (a mixture of nine brominated or chlorinated acetic acids) and anion mixed standard solutions (a mixture of seven anions) were obtained from Kanto Chemical Corporation (Tokyo, Japan). Bovine serum albumin (BSA) and myoglobin from equine skeletal muscle were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Sigma-Aldrich (St. Louis, USA), respectively. The other reagents and standard solutions were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Their chemical grades and purities are summarized in Table 3.1. All aqueous solutions were prepared with ultrapure water purified using a Milli-Q-Plus system (Merck, Darmstadt, Germany) and stored at 4°C.

Table 3.1. Characteristics of all reagents.

Compounds	Purity (%)	Grade
Sodium nitrate	>99	Guaranteed reagent
Ammonium nitrate	>99	Guaranteed reagent
Magnesium sulfate heptahydrate	>99.5	Guaranteed reagent
Potassium dihydrogen phosphate	>99.5	Guaranteed reagent
Dipotassium hydrogen phosphate	>99	Guaranteed reagent
Calcium chloride dihydrate	99–103	Guaranteed reagent
Iron(III) citrate <i>n</i> -hydrate	16–20 (as Fe)	Wako 1st grade
Citric acid	>98	Wako special grade
Biotin (vitamin H)	>97	Wako 1st grade
Thiamine (vitamin B1) hydrochloride	>98	Wako special grade
Pyridoxine hydrochloride (vitamin B6)	>98	Wako special grade
Cyanocobalamine (vitamin B12)		Wako special grade
Ethylenediamine- <i>N, N, N', N'</i> -tetraacetic acid disodium salt dihydrate	>99.5	
Iron(III) chloride hexahydrate	>99	Guaranteed reagent
Manganese(II) chloride tetrahydrate	>99	Guaranteed reagent
Zinc sulfate heptahydrate	>99.5	Guaranteed reagent
Cobalt(II) chloride hexahydrate	99–102	Guaranteed reagent
Disodium molybdate(VI) dihydrate	>99	Guaranteed reagent
2-Morpholinoethanesulfonic acid monohydrate	>99	
Potassium dihydrogen phosphate	>99.5	Guaranteed reagent
Disodium hydrogenphosphate	>99	Guaranteed reagent
Ammonium chloride	>99.5	Guaranteed reagent
Sodium hypochlorite	>5 (available chlorine)	Practical grade
<i>N, N</i> -Diethyl- <i>p</i> -phenylenediamine sulfate	>98	for residual chlorine analysis
Sulfuric acid	>95	Guaranteed reagent
Potassium permanganate solution (0.005 mol/L)		for volumetric analysis
Hydrochloric acid	35.0–37.0	Super special grade
Potassium hydrogen phthalate standard solution	0.97–1.03 mg C/mL	for water analysis
Nitrogen standard solution	99.0–100.9 mg N/L	for photometric analysis
Formic acid	>99.5	for LC/MS
Methanol	>99.7	for LC/MS
Acetic acid	>99.5	for LC/MS

3.2.2 Algal materials

Both laboratory-cultured and wild raphidophyte samples were used. The cultured raphidophytes were grown under optimized laboratory conditions, whereas the growth of wild raphidophytes may have been influenced by environmental stressors and nutrient limitations. Including both types of samples allowed us to assess whether findings obtained under controlled conditions are applicable to natural environments. It should be noted that both samples were characterized using a mixture of EOM and IOM because it was challenging to completely separate them as they are highly fragility owing to the lack of rigid cell walls.⁸⁵

3.2.2.1 Environmental waters containing raphidophytes

The first sample was reservoir water collected under Shimoto bridge in Muro Dam (34.56 °N, 136.01 °E) during a raphidophyte bloom in October 2017 and October 2023. The reservoir serves as a source water for a nearby drinking water treatment plant, with the intake located slightly downstream of the sampling site. The species of raphidophytes in the 2017 sample was unidentified, whereas the 2023 sample was identified as *Gonyostomum* spp based on DNA sequencing.⁹⁴ After collection, the samples were immediately filtered using glass fiber filter papers (0.6 µm, GA-55, Advantec, Tokyo, Japan; 0.7 µm, GF/F, Cytiva Whatman, Marlborough, USA) and stored at 4 °C until use.

3.2.2.2 Laboratory-cultured raphidophytes

The second type of sample was the *G. semen* (a species of raphidophytes) cultured in the laboratory. The *G. semen* strain (NIES-1380) was purchased from the National Institute for Environmental Studies, Japan. All glassware and plasticware used for the cultivation were sterilized in advance by dry heat at 250 °C for 2 h or autoclave at 121 °C for 20 min, respectively. Cultivation was conducted in 15 mL of AF-6 medium (NIES collection Microbial Culture Collection⁹⁵) in a 25 mL flask with a vent cap (Falcon® Cell Culture Flasks, Corning, NY, USA) or in 200 mL of Lake Biwa water supplemented with the same nutrients as the AF-6 medium in a 300 mL conical flask capped with a culture stopper (BioSilico, As One, Osaka, Japan), to enhance the growth of raphidophytes and their

production of TCAA precursors. These media were sterilized in advance by filtration with 0.2 μm sterilized filter (Advantec) or Stericup filtration unit (Merck, Darmstadt, Germany). The pH of the media was adjusted to 6.6 by the addition of 400 mg/L of MES monohydrate and sodium hydroxide. The constituents of the AF-6 medium are listed in Table 3.2. Initial cell number were set at 50 cells/mL. The culture flasks were maintained at 20 °C in an incubator at 2000 lux under daylight-type fluorescent lighting on a 14:10 h light:dark photo-cycle for approximately 3–5 weeks (exponential phase to beginning of stationary phase).

After the growth of *G. semen*, the cell population was counted three times using an inverted microscope (TS100-F, Nikon, Tokyo, Japan) with a plankton counter (MPC-200, Matsunami, Osaka, Japan). Then, the culture was ultra-sonicated for 15 min at 42 kHz with a sonicator (Branson 2510 or M2800-J, Emerson, MO, USA) to disrupt the cells and filtered with 0.2 μm hydrophilic polytetrafluoroethylene (HPTFE) membrane filter (Advantec) to remove the cell debris. Although Yokoi et al. used a centrifuge (300 rpm, 10 min) for the separation,⁹⁶ this process did not work for the *G. semen* cultures. The cell population decreased by approximately 10% and the swimmability of *G. semen* was lost after the centrifugation, indicating that the condition of raphidophytes deteriorated after the centrifugal separation.

3.2.3 DBPFPs in chlorination

DBPFP represents the maximum amount of DBPs that can be produced under chlorination conditions, after allowing sufficient reaction time while maintaining an adequate residual free chlorine level. In this study, DBPFP was used to evaluate DBP formation. The DBPs targeted for measurement in this study included TCAA, as well as MCAA and DCAA, and N-DBPs (DCAN and NDMA), which are commonly detected from algal samples.

Sodium hypochlorite solution was used as the free chlorine solution. It was standardized using the *N, N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method.⁹⁷ The conditions of chlorination were as follows: sample volume, 4–10 mL (in amber vial); reaction time, 24 h; pH 7 (5 mM phosphate buffer); temperature, 20 °C in the dark. Sample concentration, dilution factor, and chlorine dose were determined to ensure a sufficient residual free chlorine level ($> 0.1 \text{ mg Cl}_2/\text{L}$) after the reaction. For the *G. semen* cul-

Table 3.2. Components of AF-6 medium.

Compounds	Molecular weight	Concentration
NaNO ₃	84.995	140 mg/L
NH ₄ NO ₃	80.043	22 mg/L
MgSO ₄ ·7 H ₂ O	246.47	30 mg/L
KH ₂ PO ₄	136.09	10 mg/L
K ₂ HPO ₄	174.18	5 mg/L
CaCl ₂ ·2 H ₂ O	147.01	10 mg/L
Citric acid (C ₆ H ₈ O ₇)	192.12	2 mg/L
Iron (III) citrate n-hydrate (FeC ₆ H ₅ O ₇ ·nH ₂ O)	244.94+	2 mg/L
EDTANa ₂ (C ₁₀ H ₁₄ N ₂ Na ₂ O ₈ ·2 H ₂ O)	372.24	5 mg/L
Vitamin		
Biotin (C ₁₀ H ₁₆ N ₂ O ₃ S)	244.31	2 µg/L
Thiamine Hydrochloride (C ₁₂ H ₁₇ ClN ₄ OS·HCl)	337.27	10 µg/L
Pyridoxine hydrochloride (C ₈ H ₁₁ NO ₃ ·HCl)	205.64	1 µg/L
Cyanocobalamine (C ₆₃ H ₈₈ CoN ₁₄ O ₁₄ P)	1355.37	1 µg/L
Metal		
FeCl ₃ ·6 H ₂ O	270.30	0.98 mg/L
MnCl ₂ ·4 H ₂ O	197.91	0.18 mg/L
ZnSO ₄ ·7 H ₂ O	287.55	0.11 mg/L
CoCl ₂ ·6 H ₂ O	237.93	20 µg/L
Na ₂ MoO ₄ ·2 H ₂ O	241.97	12.5 µg/L
Organic buffer		
MES·H ₂ O (C ₆ H ₁₃ NO ₄ S)·H ₂ O	213.25	400 mg/L

ture, the original samples, fractions, and samples after each pretreatment process were uniformly diluted 10 times (concentrated samples were further diluted to the extent that they were concentrated), and the chlorine dose was 100 mg Cl₂/L or 30 mg Cl₂/L (after the desalination by UF) for ensuring enough residual chlorine. High initial chlorine doses were employed because of high background free chlorine consumption (e.g., the chlorine consumption of an AF-6 medium: 6.2×10^2 mg Cl₂/L). The range of residual free chlorine was 1–40 mg Cl₂/L. For reservoir water, the dilution factor was identical to that of the *G. semen* culture, and the chlorine dose was 30 mg Cl₂/L. The range of residual free chlorine was 1–30 mg Cl₂/L. For the chlorination conditions for DCAN formation potential (DCANFP), the chlorine dose was separately adjusted so that the residual free chlorine concentration after 24 h was maintained at 1–2 mg Cl₂/L, because DCAN is susceptible to decomposition under high residual chlorine conditions.

After the reaction, residual free chlorine was measured using the DPD colorimetric method to confirm that there was sufficient residual chlorine, and quenched with 50 mg N/L of ammonium chloride solution for HAAs, and ≥ 100 mg/L sodium ascorbate for N-DBPs.^{98,99} The samples were stored in a dark refrigerator at 4 °C and analyzed within one week using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (4000QTRAP, Sciex, MA, USA). DBPFPs were calculated by subtracting the DBP concentrations in the control sample. The control samples were obtained by the chlorination of culture media without *G. semen* or ultrapure water during each measurement, with sample pretreatment performed as necessary.

3.2.4 Characterization of TCAA precursors originating from raphidophytes by fractionation techniques

Although various DBPs were targeted in this study, N-DBPs and MCAA were almost undetectable, while TCAA was the predominant species formed. Therefore, TCAA was selected as the primary target compound for detailed analysis (see Results and Discussion 3.3.1). In addition, since DCAA can be simultaneously quantified during the measurement of TCAA, it was also analyzed. Accordingly, the following characterization focused on the formation of TCAA and DCAA and the properties of their precursors.

3.2.4.1 HPLC fractionation

The hydrophobic-hydrophilic properties of the TCAA precursors from raphidophytes were evaluated using HPLC fractionation with two different LC columns. First, the samples were concentrated 10 times under a gentle stream of nitrogen gas at 40 °C, followed by HPLC fractionation with an alkyl reversed phase (RP) column (ZORBAX SB-Aq, 150 × 4.6 mm, 5 μm, Agilent Technologies, CA, USA), which retains some hydrophilic compounds at a high percentage of an aqueous mobile phase. The analytical conditions are listed in Table 3.3. Fractions were collected every minute from 1 to 10 min because the recovery rates of DCAA formation potential (DCAAFP) and TCAAFP were sufficiently high (103–110% and 82–101%, respectively). The recovery rates were defined as the ratios of HAA formation potentials (HAAFPs) of the injected samples to the sum of the HAAFPs of their fractions. Each fraction was free of organic solvents, requiring no additional pretreatment. The fractions were then diluted with ultrapure water to the original sample concentration and subjected to chlorination.

Owing to the non-retention of the HAA precursors on the RP column (see Section 3.3.2.1), the fractions collected at 1–2 min were further fractionated using a hydrophilic interaction liquid chromatography (HILIC) column (InfinityLab Poroshell 120 HILIC-OH5, 150 × 4.6 mm, 2.7 μm, Agilent Technologies) after 40 times concentration by the nitrogen gas spraying at 40 °C. The UV absorbance at 254 nm measurements were also performed. The analytical conditions are listed in Table 3.4. Fractions were collected every minute from 17 to 30 min. The recovery rates of DCAAFP and TCAAFP were 30–80% and 81–133%, respectively. They were then concentrated 20 times under a gentle stream of nitrogen gas at 40 °C, diluted to the initial volume to replace acetonitrile with ultrapure water, and the HAAFPs were finally measured.

3.2.4.2 SPE fractionation

The HAAFPs of the reservoir water and *G. semen* culture before and after passing through the three SPE cartridges were compared to investigate the acid-base properties of the HAA precursors (Figure 3.1). Oasis mixed-mode cation exchange (MCX) and mixed-mode anion exchange (MAX) cartridges (sorbent weight: 500 mg, particle size: 60 μm, barrel

Table 3.3. HPLC conditions for the RP fractionation.

Category	Conditions
LC	Prominence 20A (Shimadzu)
Fraction collector	FRC-10A (Shimadzu)
Column	ZORBAX SB-Aq, 150 × 4.6 mm, 5 μm (Agilent Technologies)
Column temperature	25 °C
Injection volume	100 μL
Mobile phase	A: ultrapure water, B: methanol
Flow rate	1.0 mL/min
Gradient condition	B: 0% (0–10 min) → 30% (15 min) → 80% (18–30 min) → 0% (35–45 min)
Number of fractions	40 (0.33–1 min, every 1 min fractions to 40 min)

Table 3.4. HPLC conditions for the HILIC fractionation.

Category	Conditions
LC	Prominence 20A (Shimadzu)
Fraction collector	FRC-10A (Shimadzu)
UV detector	SPD-M20A (Shimadzu)
Column	Poroshell120 HILIC-OH5, 150 × 4.6 mm, 2.7 μm (Agilent Technologies)
Column temperature	25 °C
Injection volume	50 μL
Mobile phase	A: 1.5 mM phosphoric acid, B: methanol
Flow rate	0.5 mL/min
Gradient condition	B: 95% (0–15 min) → 5% (25–42 min) → 95% (45–60 min)
Number of fractions	14 (every 1 min fractions from 17 to 41 min)

size: 6 cc, Waters, MA, USA) were used. Although the two cartridges can capture both polar ionic compounds and hydrophobic compounds,¹⁰⁰ hydrophobic adsorption was not considered because the HAA precursors from raphidophytes are hydrophilic as observed from the HPLC fractionations (see Section 3.3.2.1). The MCX cartridge was preconditioned by sequentially passing 20 mL of methanol, 20 mL of 1% formic acid solution, and 20 mL of ultrapure water. Then, 10 mL of the samples were passed through the cartridges at a flow rate of 1 mL/min. The MAX cartridge was preconditioned by sequentially passing 20 mL of methanol, 20 mL of 10% ammonium solution, and 20 mL of ultrapure water, followed by loading 10 mL of the sample at 1 mL/min. Sample pH was not adjusted to avoid possible degradation of the HAA precursors. The effluents were collected and diluted 10 times with ultrapure water for HAAFP testing.

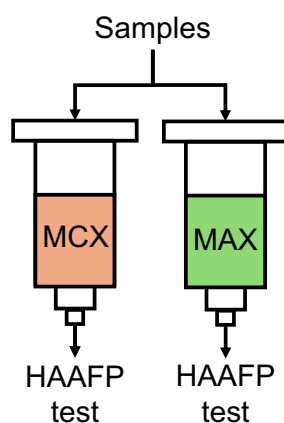


Figure 3.1. An overview of the fractionation by SPE cartridges.

3.2.4.3 Fractionation by centrifugal UF devices

Commercially available centrifugal UF device, Amicon Ultra–4 mL (Merck), with molecular weight cutoffs of 3, 10, and 30 kDa, was used to evaluate the molecular weights of the TCAA precursors. The experiment was performed according to the manufacturer’s instructions. The overview is shown in Figure 3.2. First, 4 mL of the samples was placed in the devices pre-rinsed with ultrapure water to remove trace amounts of glycerin in the UF membranes that may interfere with the following experiments. They were then centrifuged at $7,500 \times g$ at 20 °C for 40 (3 kDa), 15 (10 kDa), and 10 (30 kDa) min using

a high-speed refrigerated microcentrifuge (MX-305, TOMY, Tokyo, Japan). The filtrates were collected and diluted 10 times with ultrapure water, followed by HAAFP testing. HAAFPs of the four molecular weight fractions (<3, 3–10, 10–30, and >30 kDa) were calculated based on the HAAFP of each filtrate.

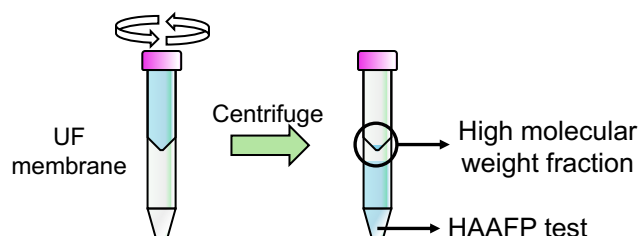


Figure 3.2. An overview of the fractionation by UF membranes.

3.2.4.4 HPLC fractionation with SEC

To determine molecular weights of TCAA precursors, the retentate on 3 kDa centrifugal UF device (Amicon Ultra-4 mL, Merck) was further fractionated by HPLC with an SEC column (Ultrasphere 250, 300×7.8 mm, 6 μ m, Waters) in parallel with UV absorbance at 254 nm and fluorescence measurements at 280/330 and 350/450 nm (excitation/emission wave length). The analytical conditions are listed in Table 3.5. Fractions were collected every minute between 8 and 17 min, because the holdup time of this HPLC system was 8 min, and sufficient recovery was obtained from the fractions till 17 min. Each fraction was desalted using a centrifugal UF devices (Amicon Ultra-4 mL) with a molecular weight cutoff of 3 kDa before the HAAFP test.

The retentate was prepared as follows. 4 mL of the *G. semen* culture was placed in the centrifugal UF devices (Amicon Ultra-4 mL) with a molecular weight cutoff of 3 kDa. The samples were then centrifuged at 7500 $\times$ g at 20 $^{\circ}$ C for 40 min using a high-speed refrigerated microcentrifuge. After centrifugation, the filtrate was discarded and ultrapure water was added to the concentrate to make the final volume of 4 mL. This process was repeated three times. The final volume of the concentrate was adjusted to 200 μ L (i.e., the concentrated ratio was 20) by adding ultrapure water.

Retention times of the standards (BSA (66.4 kDa), myoglobin (17 kDa), aprotinin (6512 Da), and cyanocobalamin (1355 Da)) were measured as references. They were

determined by fluorescence at 275/310 nm for BSA, myoglobin, and aprotinin, and UV absorbance at 360 nm for cyanocobalamin.

Table 3.5. HPLC conditions for the SEC fractionation.

Category	Conditions
LC	Prominence 20A (Shimadzu)
Fraction collector	FRC-10A (Shimadzu)
UV detector	SPD-M20A (Shimadzu)
Fluorescence detector	RF-10AXL (Shimadzu)
Column	Ultrahydrogel 250, 7.8 × 300 mm, 6 μm (Waters)
Column temperature	30 °C
Injection volume	60 μL
Mobile phase	A: 1 M NaCl+250 mM phosphoric acid (pH 7), B: methanol
Flow rate	0.6 mL/min
Gradient condition	B: 0% (0–30 min) → 50% (32.5–42.5 min) → 0% (43–95 min)
Number of fractions	9 (every 1 min fractions from 8 to 17 min)

3.2.5 Isolation method of TCAA precursors from raphidophytes

Based on the properties of TCAA precursors elucidated by characterization experiments, large scale isolation method was developed using a combination of UF and MCX SPE. Initially, raphidophyte cells were disrupted via sonication (M2800-J, Emerson) for 15 min, followed by cell debris removal through successive filtrations using glass fiber filter paper (GF/F, 0.7 μm, Cytiva Whatman) and two HPTFE membrane filters (0.2 μm and 0.1 μm, Advantec). Next, high-molecular-weight fractions were subsequently isolated using an UF membrane with a nominal molecular weight limit of 3 kDa (Ultracel regenerated cellulose, 90 mm or 150 mm diameter, Merck). An overview of the UF system is shown in Figure 3.3. A 3 kDa ultrafiltration membrane filter was installed in a filtration unit (UHP-90K or UHP-150K, Advantec) connected to a water reservoir tank (RP-2 or DV-10, Advantec). 0.6 or 2 liters of the samples and 2 or 10 liters of ultrapure water were added to the filtration unit and reservoir tank, respectively. UF was initiated under high pressure (0.3–0.4 MPa) using nitrogen gas. Ultrapure water from the reservoir tank flowed into the filtration unit, where the high-molecular-weight fraction was retained by the membrane, whereas low-molecular-weight salts and other substances permeated through and were

expelled from the filtration unit. When the residual liquid volume in the unit decreased to approximately 100 mL (for UHP-90K) or 500 mL (for UHP-150K), the unit and tank were refilled with ultrapure water, and filtration was restarted. This cycle was repeated until the residual volume decreased to approximately 30 mL (for UHP-90K) or 100 mL (for UHP-150K). This procedure was performed thrice, resulting in the collection of purified and concentrated residues (20-fold concentration). The fractions were effectively desalted (Table 3.6) and concentrated 20-fold, with their pH values approximately 3.

The high-molecular-weight fractions without pH adjustment (approximately 3) were loaded through a mixed-mode MCX cartridge at a flow rate of 1 mL/min to remove basic and hydrophobic impurities. Low pH facilitated the retention of weakly acidic impurities through hydrophobic interactions with the lipophilic moieties of the stationary phase. MCX cartridges were preconditioned in advance by sequentially passing 20 mL of methanol and 20 mL of 1% formic acid solution through the cartridges at a flow rate of 5 mL/min. To prevent the breakthrough of the adsorbates, the sample passage was limited to 100 mL per cartridge. The load effluents were used for the following analyses or further pretreatment. For each pretreatment method, a blank test was performed in ultrapure water or a medium without raphidophytes, and no significant increases in DOC or HAAFP were observed. The HAAFPs of each fraction were measured after each processing step to evaluate the isolation efficiency of the TCAA precursors from the samples.

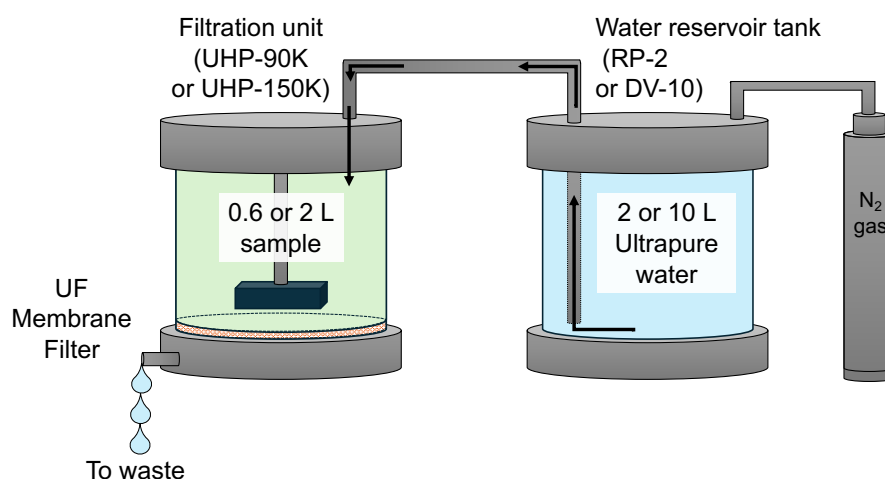


Figure 3.3. An overview of the ultrafiltration system.

Table 3.6. Concentrations of metal (Fe, Mn, Ca, and Mg) and inorganic ions (Cl^- , NO_3^- , PO_4^{3-} , SO_4^{2-}) before and after the ultrafiltration.

The concentrations before filtration were calculated based on the nutrient concentration added to the AF-6 medium (the concentration originally contained in Lake Biwa water was not taken into account.). Concentrations after ultrafiltration were expressed as average $\pm$ maximum and minimum values from duplicates. N.D. means not detected.

	Before UF	After UF	Removal rate (%)
Cl^- ($\mu\text{g/L}$)	5.28×10^3	10 ± 0.2	99.81
NO_3^- ($\mu\text{g/L}$)	1.19×10^5	15 ± 0.0	99.99
PO_4^{3-} ($\mu\text{g/L}$)	9.70×10^3	43 ± 0.1	99.56
SO_4^{2-} ($\mu\text{g/L}$)	1.17×10^4	610 ± 1.0	94.79
Fe ($\mu\text{g/L}$)	6.59×10^2	0.75 ± 0.25	99.89
Mn ($\mu\text{g/L}$)	50.0	N.D.	100
Ca ($\mu\text{g/L}$)	2.73×10^3	2.8 ± 0.4	99.90
Mg ($\mu\text{g/L}$)	2.96×10^3	0.40 ± 0.0	99.99

Lyophilization was essential to obtain samples for molecular-level structural analyses in the following chapters; however, the lyophilized sample obtained from *G. semen* cultures could not be redissolved owing to impurities, despite the hydrophilic nature of TCAA precursors. To address this issue, the load effluent from the SPE cartridge was further purified by preparative HPLC fractionation (Shimadzu, Kyoto, Japan) using a large-size HILIC column (Inertsil WP300 Diol, GL Science, Tokyo, Japan). The analytical conditions are listed in Table 3.7. The injected sample was prepared using a 20-fold concentration of the load effluent from the SPE cartridges. Fractions were collected every 2 or 3 min over a retention time range of 0.33 to 25 min. After the organic solvent (i.e., acetonitrile) was evaporated under a gentle stream of nitrogen gas at 40 °C (MG-2000, EYELA, Tokyo, Japan) and the remaining solution was diluted, HAAFPs were measured to identify the retention time of the TCAA precursors. The fraction with the highest TCAAFP was collected and freeze-dried using a lyophilizer (FDU-540, EYELA, Tokyo, Japan). For the reservoir water samples, the load effluent from the SPE cartridge was directly lyophilized for subsequent NMR and FT-IR analyses.

Table 3.7. Preparative HPLC fractionation conditions with an HILIC column.

Category	Conditions
LC	Prominence 20AR (Shimadzu)
Fraction collector	FRC-10A (Shimadzu)
UV detector	SPD-20A (Shimadzu)
Column	Inertsil WP300 Diol, 14 × 150 mm, 5 μm (GL Science)
Guard Column	Inertsil WP300 Diol, 14 × 50 mm, 5 μm (GL Science)
Column temperature	Room temperature (approximately 25 °C)
Injection volume	100 μL
Mobile phase	A: 1.5 mM phosphoric acid (pH 3.5), B: methanol
Flow rate	10 mL/min
Gradient condition	B: 99% (0–6 min) → 75% (8–14 min) → 5% (16–26 min) → 99% (28–38 min)
Number of fractions	12 (0.33–2.5, 2.5–5, 5–7, 7–9, 9–11, 11–13, 13–15, 15–17, 17–19, 19–21, 21–23, 23–25 min)

3.2.6 Analytical methods

3.2.6.1 HAA

The HAA concentrations were determined by LC-MS/MS (4000 QTARP, Sciex) using three different columns; thus, three methods were used for technical reasons. Two of these methods had been previously developed.^{101,102} The other was developed based on the previous method.¹⁰¹ The operating conditions of LC-MS/MS and multiple reaction monitoring (MRM) conditions for all methods are listed in Table 3.8. The calibration curves were prepared from five levels of working standard solutions containing MCAA, DCAA, and TCAA at the same concentration (3, 10, 30, 100, and 300 μg/L). The limits of quantification (LOQs) of MCAA, DCAA, and TCAA were 10, 3, and 3 μg/L, respectively.

Table 3.8. LC-MS/MS condition for HAAs.

LC System		Prominence 20A (Shimadzu)		
		VP-ODS	Acclaim Trinity P1	ZORBAX SB-Aq
Column		5 μ m, 150 $\times$ 4.6 mm (Shimadzu)	3 μ m, 2.1 $\times$ 100 mm (Thermo Fisher Scientific, MT, USA)	80Å, 150 $\times$ 4.6 mm (Agilent Technologies)
Column temperature (°C)		50	35	50
Sample cool temperature (°C)		4	4	4
Inject volume (μ L)		10	10	40
Mobile phase A		0.3% formic acid	200 mM ammonium acetate + 0.5% acetate	0.3% formic acid
Mobile phase B		methanol	acetonitrile	methanol
Flow rate (mL/min)		0.3	0.4	0.4
Measurement time (min)		38	18	35
Gradient condition of organic solvent (B%)		1 (0–0.5 min)→50 (5 min) →99 (25–35 min)→1 (36 min)	95 (0–2 min)→80 (9.5 min) →5 (10–16 min)→95 (17 min)	1 (0–0.5 min)→50 (20 min) →99 (21–31 min)→1 (32 min)
MS/MS System		4000 Q TRAP (Sciex)		
Ionization mode		ESI negative		
Curtain gas (psi)		5	30	50
Ionspray voltage (V)		-5500	-4500	-3000
Turbo ionspray temp. (°C)		300	310	300
Nebulizer gas pressure (psi)		80	40	80
Turbo gas pressure (psi)		50	50	50
Collision gas (psi)		6	6	6
		92.889/35.0	93.0/35.0	92.889/35.0
MRM condition		Q1/Q3	Q1/Q3	Q1/Q3
		126.779/82.8	127.0/83.0	126.779/82.8
		160.819/116.7	160.819/116.7	160.819/116.7
(MCAA)		DP (V)	-35, -30, -25	-35, -30, -25
(DCAA)		EP (V)	-4, -4, -4	-4, -4, -4
(TCAA)		CE (V)	-16, -14, -10	-16, -14, -10
		CXP (V)	-3, -3, -3	-3, -3, -3
		LOQ (μ g/L)	3, 3, 3	10, 3, 3

3.2.6.2 NDMA and DCAN

NDMA concentrations were determined using LC-MS/MS equipped with a Raptor biphenyl column (150×3.0 mm; $2.7 \mu\text{m}$, Restek, Pennsylvania, USA). Each sample was spiked with $20 \mu\text{g/L}$ of NDMA-d6 as an internal standard. Mobile phases used included A: 0.1% formic acid in ultrapure water and B: acetonitrile. A calibration curve was established using eight concentrations of working standard solutions of NDMA (2, 5, 10, 30, 50, 100, 200, $400 \mu\text{g/L}$) spiked in ultrapure water. The LOQ was $2 \mu\text{g/L}$. The LC-MS/MS operating conditions and MRM parameters are listed in Table 3.9.

DCAN concentrations were also determined using LC-MS/MS equipped with a RP column (Inertsil Ph-3, $3 \mu\text{m}$, 2.1×150 mm, GL Science) with the same mobile phase B as for NDMA, while mobile phase A was ultrapure water. The calibration curve for DCAN measurement contain five points of DCAN standard solutions (5, 10, 30, 100, $300 \mu\text{g/L}$) spiked into ultrapure water. The LOQ of DCAN was $5 \mu\text{g/L}$. The LC-MS/MS operating conditions and MRM parameters are listed in Table 3.10.

3.2.6.3 DOC

DOC was quantified by measuring the TOC concentration in filtered samples. TOC was measured by the combustion catalytic oxidation method at 680°C using a TOC- V_{CSN} (Shimadzu, Kyoto, Japan) analyzer. Since inorganic carbon (IC) accounts for a relatively large proportion of total carbon (TC) in environmental waters, the error of TOC values of the waters, yielded by subtracting TIC from TC, is large. Therefore, the non-purgeable organic carbon (NPOC) method was employed, in which TOC is determined by measuring TC in a sample from which IC is removed. The calibration curve was prepared from five levels of working potassium hydrogen phthalate standard solution (i.e., 0.1, 0.3, 1, and 2 mg C/L).

3.2.6.4 pH

The pH was determined by a pH meter (LAQUA F-53, Horiba, Kyoto, Japan) with a pH electrode (9630-10D, Horiba, Kyoto, Japan).

Table 3.9. LC-MS/MS condition for NDMA.

LC	LC system		Prominence 20A (Shimadzu)
	Column		Raptor biphenyl (3.0 × 150 mm, 2.7 μm, Restek)
	Column temperature (°C)		40
	Sample cool temperature (°C)		4
	Inject volume (μL)		10
	Mobile phase		0.1% aqueous formic acid/acetonitrile
	Flow rate (mL/min)		0.5
	Measurement time (min)		13
MS/MS	Gradient condition of organic solvent (%)		5 (0–2 min) → 75 (6 min) → 95 (6.01–8 min) → 5 (8.01 min)
	MS/MS system		4000 Q TRAP (Sciex)
	Ionization mode		APCI positive
	Curtain gas (psi)		55
	Ion source voltage (V)		5500
	Ion source temperature (°C)		250
	Nebulizer gas pressure (psi)		30
	Turbo gas pressure (psi)		70
	Collision gas (psi)		8
	Q1/Q3		75.1/43.1, 81.1/46.0
	DP (V)		56, 76
	MRM condition		EP (V)
	(NDMA, NDMA- <i>d6</i>)		10, 10
			CE (V)
			23, 27
			CXP (V)
			6.2, 6.0
			LOQ (μg/L)
			2, 2

Table 3.10. LC-MS/MS condition for DCAN.

LC	LC system		Prominence 20A (Shimadzu)
	Column		Inertsil Ph-3 (2.1 × 150 mm, 3 μm, GL science)
	Column temperature (°C)		50
	Sample cool temperature (°C)		4
	Inject volume (μL)		30
	Mobile phase		Ultrapure water/acetonitrile
	Flow rate (mL/min)		0.3
	Measurement time (min)		25
	Gradient condition of organic solvent (%)		10 (0–3 min) → 60 (9 min) → 100 (10 min)
MS/MS	MS/MS system		4000 Q TRAP (Sciex)
	Ionization mode		APCI negative
	Curtain gas (psi)		30
	Ion source voltage (V)		-4500
	Ion source temperature (°C)		500
	Nebulizer gas pressure (psi)		40
	Turbo gas pressure (psi)		0
	Collision gas (psi)		4
	MRM condition	Q1/Q3	107.9/35.0
		DP (V)	-25
		EP (V)	-5
		CE (V)	-23
		CXP (V)	-1
		LOQ (μg/L)	5

3.3 Results and discussions

3.3.1 Properties of the reservoir waters and *G. semen* cultures

Table 3.11 presents the cell populations, DBPFPs, and DOC concentrations of the reservoir waters and *G. semen* cultures. Values for the *G. semen* culture (cultured in AF-6 medium) were shown in range because the cultures were prepared separately on multiple occasions. The DOC of the *G. semen* culture (cultured in Lake Biwa water supplemented with AF-6 nutrients) could not be accurately quantified due to high background DOC concentrations derived from the culture medium. The cell populations in the reservoir waters were 3,200 cells/mL during the bloom in 2017 and 9,000 cells/mL in 2023, respectively. Microscopic examination revealed that most of the identified plankton were raphidophytes, suggesting a pronounced proliferation of this group. In the laboratory cultures of *G. semen*, the cell density ranged from 500 to 2,000 cells/mL when cultured in AF-6 medium alone but was notably higher under a Lake Biwa water-based medium. The influence of environmental water on algal growth during cultivation has been discussed in a separate study.¹⁰³

Among HAAFPs, the MCAAFPs of all samples were not determined because of their low concentrations. Thus, only DCAA and TCAA formation has been discussed in this thesis. The DCAAFPs of the reservoir waters were 98 (2017 sample) and 190 (2023 sample), respectively, while the TCAAFPs were 570 and 1200 $\mu\text{g/L}$. For *G. semen* cultures, DCAAFPs were 95–180 $\mu\text{g/L}$ (AF-6 medium) and 300 $\mu\text{g/L}$ (Lake Biwa water+ AF-6 nutrients), respectively, while the TCAAFPs were 770–1400 and 2100 $\mu\text{g/L}$. TCAAFPs were much higher than DCAAFPs and were dominant among the HAAFPs for all samples. Although the reservoir water exhibited a higher cell density than the *G. semen* cultures, its TCAAFP was lower, likely due to dilution by surrounding water and reduced nutrient availability relative to the culture medium.

Regarding N-DBPs, measurements were conducted using the 2023 reservoir water and the *G. semen* culture (Lake Biwa water+ AF-6 nutrients), which exhibited higher cell density. DCAN formation was confirmed in both samples, with concentrations of 39 $\mu\text{g/L}$ in the reservoir water (below the quantification limit) and 52 $\mu\text{g/L}$ in the culture. Although these values were higher than the regulatory limit (10 $\mu\text{g/L}$) and thus should

not be entirely disregarded, the present study focused primarily on TCAA, which was produced at much higher concentrations (regulatory limit: 30 $\mu\text{g/L}$). NDMA was not detected in any sample.

Compared with the HAA yields from other algae,^{38,40,93} including green algae, blue-green algae, or diatoms, although chlorination conditions were not all the same, the TCAAFPs of the two materials were much higher than those of the others while no large differences were observed for the DCAAFPs. These high TCAAFPs suggested that raphidophytes are unique algae and could specifically metabolize compounds with high TCAAFPs compared to other algae.

Till date, only a few phenols, aromatic amines, aromatic amino acids, and a trichlorinated compound have shown high TCAAFPs and their dominance over HAAFPs.^{101,104} These chemicals or compounds with the same moieties were hypothesized to be HAA precursors metabolized by raphidophytes.

In algae, lipids, proteins, glycans, and humic substances constitute the main biochemical components of DOC.^{38,57} The TCAAFPs of their model compounds (BSA, starch, fish oil, humic acid, and fulvic acid) were less than 40 $\mu\text{g/mg C}$.^{38,57} In addition, except for starches and fish oils with low TCAAFPs ($<5 \mu\text{g/mg C}$), the DCAAFPs were higher than the TCAAFPs.^{38,57} Considering the high TCAAFPs observed in raphidophytes and their dominance over HAAFPs, these biochemicals were not regarded as the HAA precursors from raphidophytes. Activated aromatic amino acids (e.g., tyrosine and tryptophan) meet the relevant criteria,¹⁰⁴ suggesting that the HAA precursors may consist of such aromatic amino acids or peptides/proteins. Additionally, other structures were considered as potential HAA precursors, including lipids, glycans, and humic substances containing numerous reactive phenolic, aromatic amine, or amino acid substructures.

Table 3.11. Cell population, HAAFPs, DCANFPs, NDMAFPs, and DOC of the reservoir water and *G. semen* cultures.

DBPFPs were expressed as average $\pm$ standard deviation from three replicates.

	Cell (/mL)	MCAAFP (μ g/L)	DCAAFP (μ g/L)	TCAAFP (μ g/L)	DCANFP (μ g/L)	NDMAFP (μ g/L)	DOC (mg C/L)
Reservoir water Oct. 2017 sample	3200	N. D. ^a	98 $\pm$ 36 [38 $\pm$ 12] ^b	570 $\pm$ 36 [220 $\pm$ 12] ^b	– ^c	– ^c	2.6
Reservoir water Oct. 2023 sample	9000	N. D. ^a	190 $\pm$ 18 [23 $\pm$ 2.1] ^b	1200 $\pm$ 39 [140 $\pm$ 4.6] ^b	BOL ^d (39 $\pm$ 0.5) [4.6 $\pm$ 0.01] ^b	– ^c	8.4
<i>G. semen</i> culture (AF-6 medium) ^e	550–2200	BOL ^d ($<$ 100)	95–180 [10–17] ^b	770–1400 [82–130] ^b	– ^c	– ^c	6.9–13
<i>G. semen</i> culture (Lake Biwa water+ AF-6 nutrients)	3000 $\pm$ 930	BOL ^d ($<$ 100)	300 $\pm$ 28	2100 $\pm$ 99	52 $\pm$ 1.1	BOL ^d ($<$ 20)	– ^c

^a "N.D." means not detected.

^b Values in brackets indicate normalized concentrations (μ g/mg C).

^c Not measured or quantified.

^d "BQL" means below the quantification limit. The values in parentheses represent quantified concentrations that were BQL while values for samples with no detectable peaks or unquantifiable concentrations are reported as '<BQL value', which also takes into account the dilution factor during the chlorination experiment.

^e Values of the *G. semen* culture (AF-6 medium) were expressed as ranges because the cultures were prepared separately on multiple occasions.

3.3.2 Characterization of the TCAA precursors originating from raphidophytes by fractionation techniques

In this section, characterization experiments were performed using a reservoir water collected in 2017 and *G. semen* cultures grown on AF-6 medium. The HAAFPs are expressed in $\mu\text{g/L}$ because of the difficulty in obtaining the DOC of each fraction. Retention of the HAA precursors in the cartridges, columns, and membranes was confirmed by comparing the HAAFPs of both the samples before and after separation.

3.3.2.1 HPLC fractionation

Figure 3.4 shows the DCAAFPs and TCAAFPs of the 1 min fractions for 10 min in the reservoir water and *G. semen* culture separated by the alkyl RP column. To date, there have been few examples of detailed separation of AOM using HPLC fractionation. The DCAAFPs and TCAAFPs of the 1–2 min fractions of both samples were the highest among all fractions, i.e., 81 and 49 $\mu\text{g/L}$, and 440 and 210 $\mu\text{g/L}$, respectively. The mobile phase A, ultrapure water, was at 100% for 10 min and the HAA precursors in both samples were not retained on the column; this suggests that they were not hydrophobic, which means that hydrophobic humic-like substances are unlikely to be HAA precursors.

After the 1–2 min fractions were collected and concentrated, they were further fractionated using the HILIC column in parallel with the UV absorbance at 254 nm measurements. Figure 3.5 describes the DCAAFPs and TCAAFPs of the 1 min fractions from 17 to 30 min and the UV absorbance at 254 nm profiles in the reservoir water and the *G. semen* culture. The HAA precursors in both samples were sufficiently retained by the HILIC column, suggesting that both HAA precursors were ultra-hydrophilic compounds. Additionally, sharp peaks were observed in both UV absorbance at 254 nm chromatogram. For the reservoir water, the DCAAFPs and TCAAFPs for 24–26 min consecutive fractions were higher than those of the other fractions (Figure 3.5 (a)), and a high intensity of UV absorbance at 254 nm was also observed in the same retention time window (Figure 3.5 (c)). This high intensity of UV absorbance at 254 nm suggested that the HAA precursors from raphidophytes in the reservoir water contained aromatic ring.¹⁰⁵ In addition, the TCAAFPs of the 24–26 min consecutive fractions were higher (320–450 $\mu\text{g/L}$) than

the DCAAFPs (54–67 $\mu\text{g/L}$). Additionally, in previous studies, activated aromatic compounds were shown to form more TCAA than DCAA and compounds without activated aromatic moieties could not form TCAA at the high concentration, except for trichlorinated compound under conditions of sufficient free chlorine concentration and reaction time at neutral pH,^{101,102,104,106,107} which further implied that the HAA precursors contained aromatic ring. However, since the aromatic ring contributes to hydrophobicity, the presence of hydrophilic functional groups is required in the structures of the HAA precursors. Preliminary experiments indicated that phenols and anilines were not retained in the HILIC column, whereas aromatic compounds with sulfo and phospho groups (e.g., phenyl sulfonate, phenyl phosphate) were well retained (24–29 min). Furthermore, the multiple overlapping peaks in the UV absorbance at 254 nm chromatogram suggest the presence of several HAA precursors with similar physicochemical characteristics in the fractions. For the *G. semen* culture, the DCAAFPs and TCAAFPs of four fractions (20–21, 22–23, 23–24, and 26–27 min) were higher than those of others. In particular, the HAAFP in the 23–24 min fraction was the highest. However, the retention time of the high-intensity UV absorbance at 254 nm was observed only at 22–24 min. These results suggest that more than three kinds of HAA precursors are present in the *G. semen* culture and that the major HAA precursors might have aromaticity, while others in 20–21 and 26–27 min fractions did not. Compared to the retention characteristics of the reservoir water, the different retention times of the *G. semen* culture fractions with the high HAAFPs indicated that their major HAA precursors are not the same, although they are both ultra-hydrophilic.

3.3.2.2 SPE fractionation

Figure 3.6 shows the HAAFPs of the samples before and after being passed through two SPE cartridges, Oasis MCX and MAX, which were composed of the hydrophilic-lipophilic balance (HLB) polymeric skeleton with additionally different ionic functional moieties for ion-exchange interactions such as sulfone and quaternary amine functionalities, respectively.¹⁰⁰ No large differences were observed in the retention characteristics between the two samples. The HAAFPs dramatically decreased to a non-detectable level after passing through Oasis MAX cartridges, whereas the decreases for Oasis MCX cartridges were much lower (by 17–30%). The lower retention in the MCX cartridges

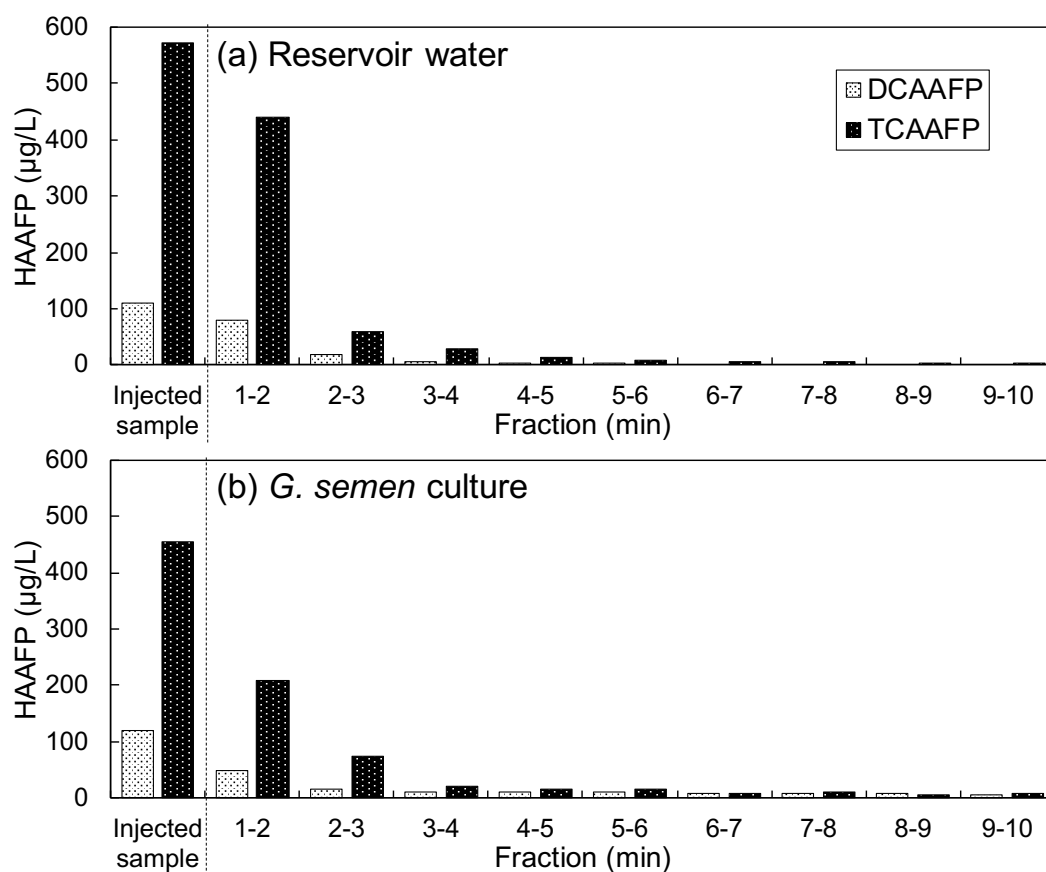


Figure 3.4. HAAFPs of the individual fractions from 1 to 10 min of (a) reservoir water and (b) *G. semen* culture, fractionated by HPLC with a RP column (ZORBAX SB-Aq). *Experimental conditions of the HAAFP test:* pH 7 (5 mM phosphate buffer); Cl₂ dose, 30 (for the fractions of reservoir water) or 60 (for the fractions of *G. semen* culture) mg Cl₂/L; temperature, 20 °C; and reaction time, 24 h in dark.

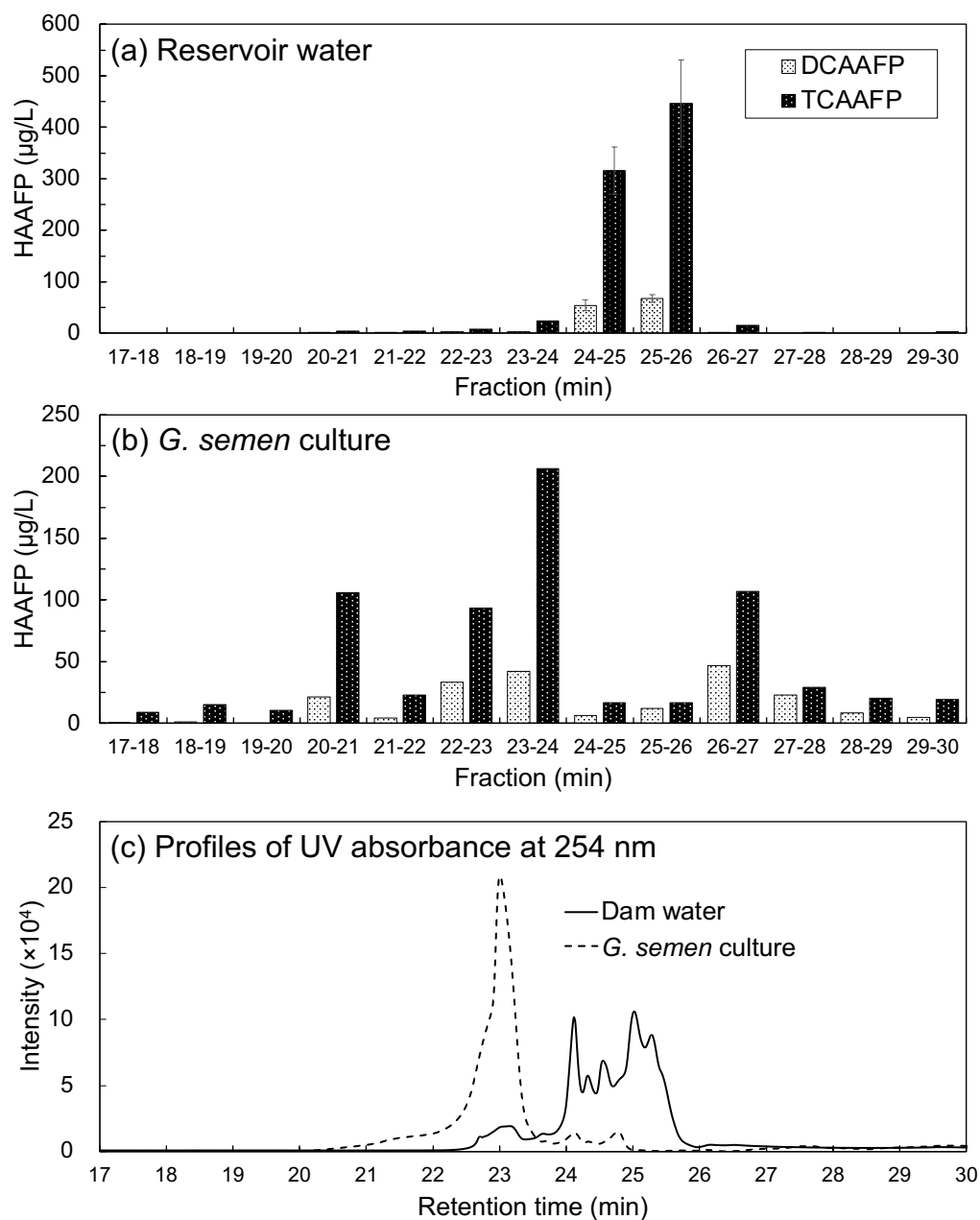


Figure 3.5. HAAFPs of the individual fractions from 17 to 30 min of (a) reservoir water; (b) *G. semen* culture, and (c) profile of UV absorbance at 254 nm from 17 to 30 min, fractionated by HPLC with a HILIC column (InfinityLab Poroshell 120 HILIC-OH5).

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); Cl₂ dose, 30 (for the fractions of reservoir water) or 60 (for the fractions of *G. semen* culture) mg Cl₂/L; temperature, 20 °C; and reaction time, 24 h in dark. Error bars represent the minimum and maximum values from duplicate experiments.

indicated that the HLB and ion-exchange interactions by the sulfone functionality were not suitable for the retention of the HAA precursors, i.e., the HAA precursors were not neutral and were not ionized basic at pH ~ 7 . Basic substances (e.g., amines with high pK_a values) were not HAA precursors. Additionally, the large decreases in the HAAFPs in the MAX cartridges showed that quaternary amine functionalities functioned as adsorbents for the HAA precursors. Because the MAX cartridges retain acidic compounds, the HAA precursors are ionized acidic at pH ~ 7 , suggesting that they have acidic functional groups (e.g., carboxylic, sulfonic, and phosphonic groups).

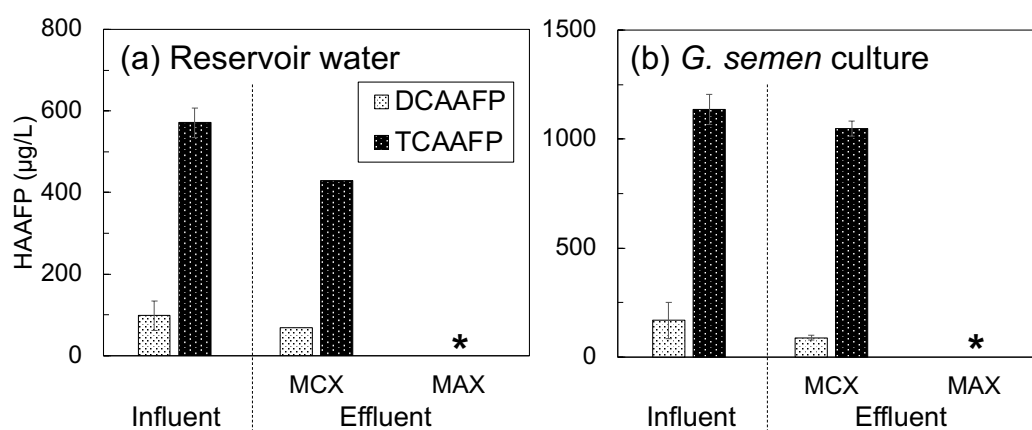


Figure 3.6. HAAFPs of (a) reservoir water and (b) *G. semen* culture before and after passing through SPE cartridges.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); Cl_2 dose, 30 (for reservoir water) and 75–100 (for *G. semen* culture) mg Cl_2/L ; temperature, 20 °C; and reaction time, 24 h in dark. * indicates that HAAs could not be detected in all measurements. Error bars represent the standard deviation of triplicate or quadruplicate measurements (for influent) and the minimum and maximum values from duplicate experiments (for effluent).

3.3.2.3 Fractionation by UF membrane

Molecular weights of the HAA precursors were estimated using centrifugal UF devices with molecular cutoffs of 3, 10, and 30 kDa. Figure 3.7 shows the HAAFPs of the molecular fraction (<3, 3–10, 10–30, and >30 kDa). In both samples, almost all the HAA precursors were >3 kDa. Owing to their extreme hydrophilic nature, they were initially expected to be small molecules but turned out to be relatively large compounds. molecular weight distributions of the HAA precursors were quite different among the samples.

The DCAAFPs and TCAAFPs of the reservoir water of the four molecular fractions (<3, 3–10, 10–30, and >30 kDa) were 6.4, 56, 0, and 50 $\mu\text{g/L}$, and 40, 200, 160, and 180 $\mu\text{g/L}$, respectively. The HAAFP of the molecular fraction <3 kDa was the smallest, and those of the molecular fractions >3 kDa were evenly distributed. For the *G. semen* culture, the DCAAFPs and TCAAFPs of the four molecular fractions (<3, 3–10, 10–30, and >30 kDa) were 15, 8.8, 26, and 69 $\mu\text{g/L}$, and 82, 69, 400, and 570 $\mu\text{g/L}$, respectively. The HAAFPs of the fractions >10 kDa were larger than those of the smaller molecular weight fractions, indicating that the molecular weights of the HAA precursors in the *G. semen* culture were higher than those in the reservoir water. Comparing the molecular weights of the HAA precursors with previously studied algae, the HAA precursors of fresh water raphidophytes were observed to be quite different. Wang et al. reported that the low molecular fraction (<3 kDa) of AOM from a mixture of *Microcystis*, *Oscillatoria*, *Aphanizomenon*, and *Anabaena* accounted for >40% of the total HAAFPs.⁵⁵ Zhou et al. also highlighted the importance of the molecular weight <1 kDa and >100 kDa fractions from the IOM and EOM of *M. aeruginosa* as HAA precursors.¹⁰⁸ The compounds >100 kDa in IOM extracted from *M. aeruginosa* and *Chlorella* sp. contribute 70–75% of their HAAFPs.⁶¹ Majority of the HAA precursors from other algae were either low molecular weight (<3 kDa) or high molecular weight (>100 kDa), whereas the major HAA precursors from raphidophytes had medium molecular weights, suggesting that they are very unique.

3.3.2.4 Molecular weight of TCAA precursors by SEC HPLC fractionation

Majority of HAA precursors from raphidophytes were estimated to be >10 kDa (Section 3.3.2.3). However, the upper limit of the molecular weight is still unknown. Thus, it was determined by HPLC fractionation using an SEC column. SEC HPLC fractionation was performed using the high-molecular-weight fraction of *G. semen* culture isolated with 3kDa UF membrane. Figure 3.8 (a) shows the HAAFPs of the SEC fractions (every 1 min interval) from 8 to 17 min of the high-molecular-weight fraction. Most HAAFPs were present in the range of 12–15 min. The HAAFP of the 13–14 min fraction was the highest (DCAAFP, 73 $\mu\text{g/L}$; TCAAFP, 450 $\mu\text{g/L}$). The wide peak of the HAA precursors over three consecutive minutes indicates that the molecular size of the precursor was dis-

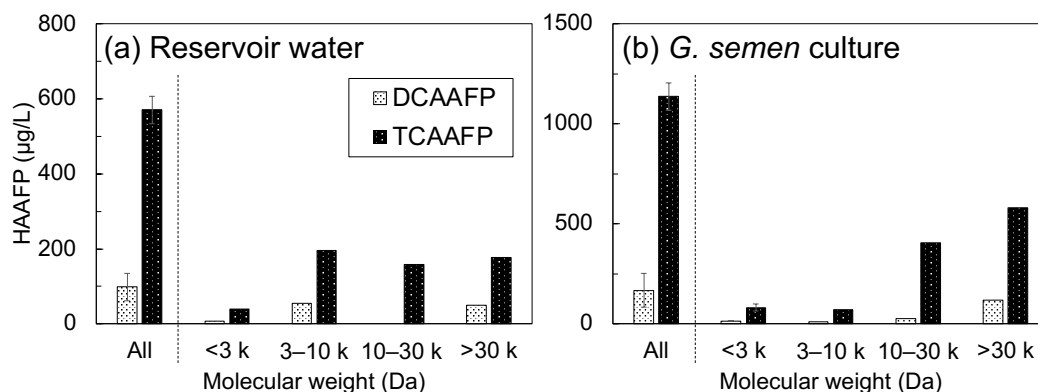


Figure 3.7. HAAFPs of the molecular weight fractions of (a) reservoir water; and (b) *G. semen* culture, separated by ultrafiltrate membranes.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); Cl_2 dose, 30 (for reservoir water) and 100 (for *G. semen* culture) mg Cl_2/L ; temperature, 20 °C; and reaction time, 24 h in dark. Error bars on the values of fractions represent the minimum and maximum values from duplicate experiments, error bars on the samples before the separation represent the standard deviation of triplicate or quadruplicate measurements

tributed over a relatively wide range. From the comparison of the retention times of three reference proteins (i.e., BSA (66.4 kDa at 11.7 min), myoglobin (17 kDa at 12.7 min), and aprotinin (6512 Da at 21.1 min)) with cyanocobalamin (1355 D at 31.0 min) (Figure 3.9) and the results of the UF experiment (Figure 3.7), the molecular weights of the HAA precursors were inferred to be in the range of 10–60 kDa. In parallel with HPLC fractionation, UV absorbance at 254 nm profiles (Figure 3.8 (b)), and fluorescence at 350/450 and 280/330 nm (excitation/emission) (Figure 3.8 (c)) were obtained. These wavelengths are indicators of humic acid-like substances and tyrosine-like proteins, respectively.¹⁰⁹ The fractions with high HAAFPs and the retention times of the peaks with high UV absorbance at 254 nm coincided well, indicating the presence of several aromatic compounds in the fractions with high HAAFPs. Unlike UVA, several fluorescence peaks did not coincide with the fractions with high HAAFPs. Fluorescent substances detected even in fractions without high HAAFP (i.e., 280/330 nm peaks at 8–10 and 15–16 min and a 350/450 nm peak at 15–16 min) showed the presence of several other substances in the high-molecular-weight fraction that did not contribute to HAAFPs. These compounds with low HAAFPs contributed to the DOC of the high-molecular-weight fractions and consumed chlorine, indicating that the real DCAAFP and TCAAFP (per DOC) of the HAA precursors from *G. semen* were higher than those of the fraction and the molar ratio

of the HAAFP to the chlorine consumption were higher. Thus, the HAA precursors may have specific moieties that more efficiently form HAA than those that have been reported so far.

Summarizing the aforementioned fractionation results, the majority of the HAA precursors in the reservoir waters and the *G. semen* cultures were to be ~10–60 kDa and extremely hydrophilic with acidic functionalities. Moreover, based on their high absorbance of UV at 254 nm, it is inferred that they have aromatic rings. Given that HAA precursors are large ultra-hydrophilic biomolecules, proteins, glycans, or their mixtures with acidic and phenolic functional groups were hypothesized to be the main HAA precursors. Additionally, a comparison of the retention properties of both HAA precursors showed that their physicochemical properties were slightly different, and that the molecular weights from the *G. semen* cultures were larger than those of the reservoir waters. However, because they shared several common chemical properties, the differences in their chemical structures might not be very large.

3.3.3 Isolation of TCAA precursors from algal materials in large volumes

In previous studies, AOM has been separated based on its origin (e.g., EOM or IOM), biological composition (e.g., humic substances, proteins, lipids, and carbohydrates), or chemical properties (e.g., molecular weight and hydrophilicity), and the characteristics of each fraction have been analyzed. However, few attempts have been made to extract specific fractions with high DBPFPs to elucidate the structures of particular DBP precursors. In addition, large sample volumes need to be purified for molecular-level structural analyses such as NMR and FT-IR, as well as for treatability experiments targeting TCAA precursors. Therefore, a new method was developed to isolate unique TCAA precursors from raphidophyte samples in large quantities. First, the samples were sonicated to disrupt the algal cells, followed by filtration. The high-molecular-weight fraction was then collected using UF membranes, after which MCX SPE was performed to prepare the sample solutions. The suitable fractionation methods for impurity removal were selected based on the results explained in section 3.3.2 and developed an optimal pretreatment pro-

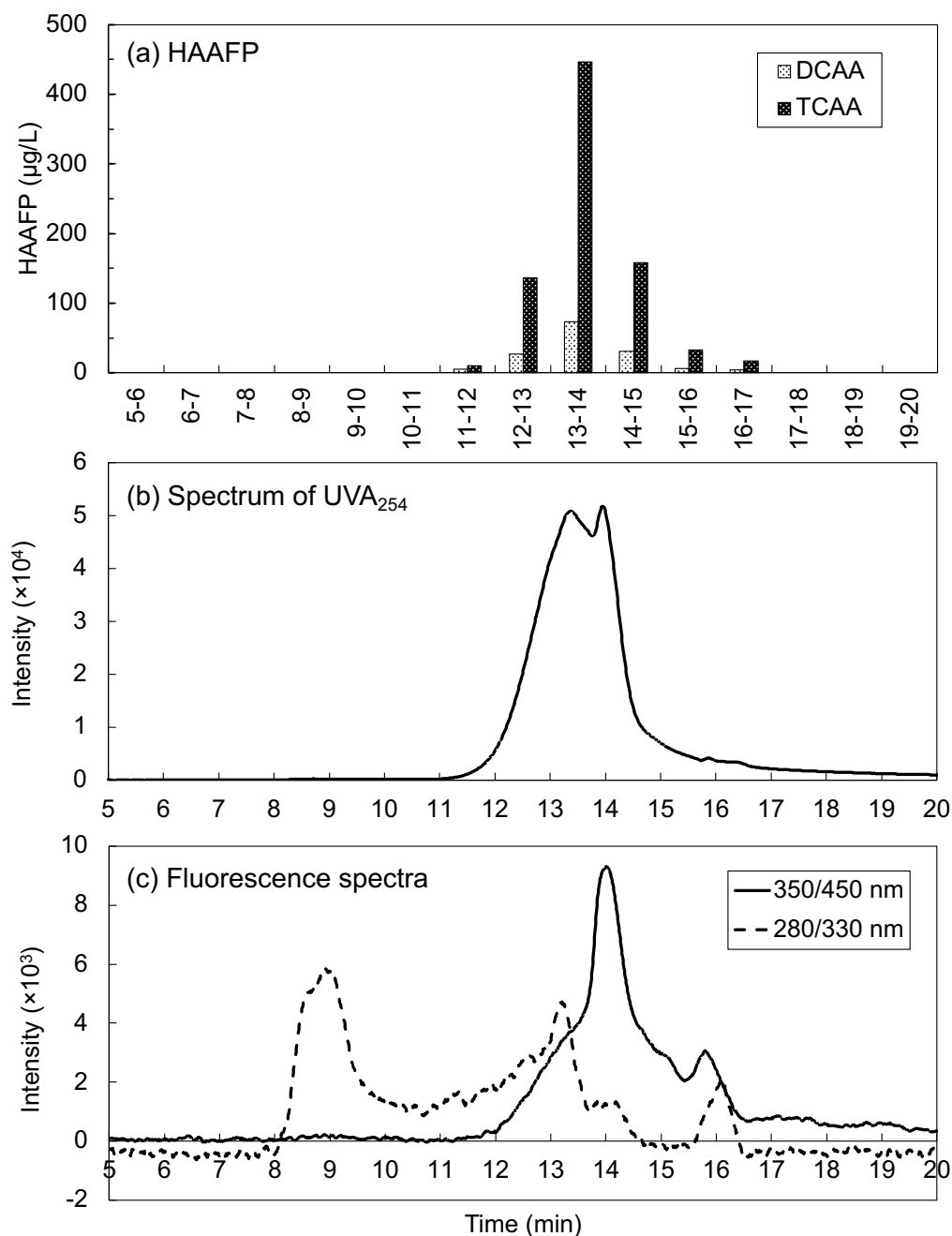


Figure 3.8. (a) HAAFPs of the fractions after HPLC fractionation with a size exclusion column (Ultrasphere 250); (b) profile of UV absorbance at 254 nm; and (c) profiles of fluorescence at 350/450 and 280/330 nm during HPLC fractionation.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer), initial Cl₂ dose, 30 mg/L; temperature, 20 °C; and 24 h in dark. Before the HAAFP test, each fraction was desalted with Amicon Ultra-4 mL (3kDa) and concentrated by 1.2.

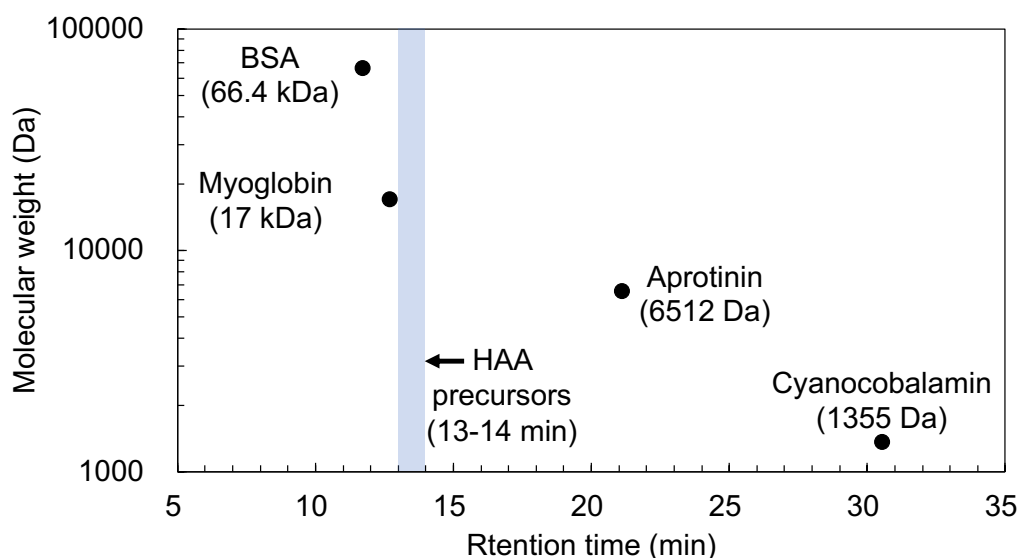


Figure 3.9. Retention times of HAA precursors, BSA, myoglobin, aprotinin, and cyanocobalamin in the HPLC system with the size exclusion column (Ultrasphere 250).

cess by performing the fractionations sequentially on large sample volumes. Extraction methods were also applied using culture medium from *G. semen* cultured in Lake Biwa water (300 mL flask) and environmental water samples collected in 2023.

The TCAAFPs of the algal materials at various stages of the pretreatment process were utilized to evaluate the isolation efficiency of TCAA precursors (Figure 3.10). The TCAAFPs in the original *G. semen* culture and reservoir water were 2,100 and 1,200 $\mu\text{g/L}$, respectively (Table 3.11). Through the pretreatment procedure, the TCAAFPs were reduced to 1,100 and 480 $\mu\text{g/L}$, respectively, indicating that 41–51% of the TCAA precursors were collected. When focusing solely on the dissolved TCAA precursors, the majority were successfully isolated.

During the filtration process, the TCAAFP decreased by 20–21%, suggesting that approximately 20% of the TCAA formation was derived from particulate matter such as cell debris. Subsequent desalting and concentration using UF membranes resulted in an additional 27–31% decrease, likely due to the removal of small molecular compounds. Furthermore, the removal of hydrophobic and basic compounds by mixed-mode MCX cartridges led to a 2.3–6.6% decrease in the TCAAFPs, suggesting that basic compounds are not major TCAA precursors from raphidophytes.

After pretreatment, the TCAAFPs per unit of DOC in the *G. semen* culture and reser-

voir water were 480 and 230 $\mu\text{g}/\text{mg C}$, respectively. The approximately two-fold difference in TCAAFPs between the samples suggests that cultured raphidophytes produce TCAA precursors with higher FPs than wild raphidophytes, despite both belonging to the same genus. The DCAAFPs were one-seventh to one-sixth of the TCAAFPs, with values of 64 and 38 $\mu\text{g}/\text{L}$, respectively. Furthermore, the ratios of TCAAFPs to DCAAFPs remained largely unchanged during pretreatment procedure, suggesting that the TCAA precursors were identical or similar to the DCAA precursors.

For detailed substructure estimation, liquid-state NMR was employed instead of solid-state NMR (see Chapter 4), which is typically utilized for DOM analysis, because liquid-state NMR has a higher resolution and sensitivity with a smaller sample volume, and hydrophilic isolates from raphidophytes were expected to dissolve sufficiently in D_2O .¹¹⁰ However, upon lyophilizing the samples from the *G. semen* culture, they did not sufficiently redissolve, likely owing to the presence of impurities. Therefore, preparative HPLC fractionation with a HILIC column was performed. Figure 3.11 (a) illustrates the HAAFPs of each fraction. Results show that the TCAAFPs were detected in three fractions: 0.33–2.5 min, 11–13 min, and 17–19 min. The 11–13 min fraction exhibited the highest TCAAFP concentration (5,600 $\mu\text{g}/\text{L}$). The lyophilized 11–13 min fraction was redissolved in ultrapure water, and the TCAAFP was nearly equal to the value before the lyophilization. Thus, the fraction obtained for 11–13 min was used for NMR and FT-IR analysis.

As depicted in Figure 3.11 (b), the retention time of the high UV absorbance at 254 nm corresponds to those of the fractions with high TCAAFP values. Focusing on 11–13 min fraction, the average UV absorbance at 254 nm was 11 cm^{-1} , with a maximum of 34 cm^{-1} and a minimum of 1.3 cm^{-1} . Given that the DOC concentration was 6.2 mg C/L (Table 3.12), the estimated specific UV absorbance at 254 nm (SUVA-254), which is calculated as UV absorbance at 254 nm divided by DOC, was 1.8 (ranging from 0.22 to 5.5) $\text{L}\cdot\text{mg C}^{-1}\cdot\text{cm}^{-1}$. This suggests that the percent aromaticity is approximately 5–40%, with an average of around 15%, indicating the presence of aromatic compounds within the structure.¹⁰⁵ In addition, slight absorbance was also observed in the 9–11 min retention time fraction, where no HAAFP was found. This indicates that non-TCAA precursor substances were further removed by HPLC fractionation.

The TCAAFP per unit carbon content in the 11–13 min fraction is listed in Table 3.12. The removal of impurities by fractionation that did not contribute to the formation of TCAA resulted in a higher TCAAFP per unit of DOC in the fraction compared to that before fractionation. No previous research has shown a TCAAFP comparable to 960 $\mu\text{g}/\text{mg C}$ in high-molecular-weight compounds, indicating the specificity of TCAA precursors derived from raphidophytes. Even the most well-known TCAA precursors, phenols and chlorophenols, have TCAAFPs of approximately 400–600 $\mu\text{g}/\text{mg C}$.¹⁰¹

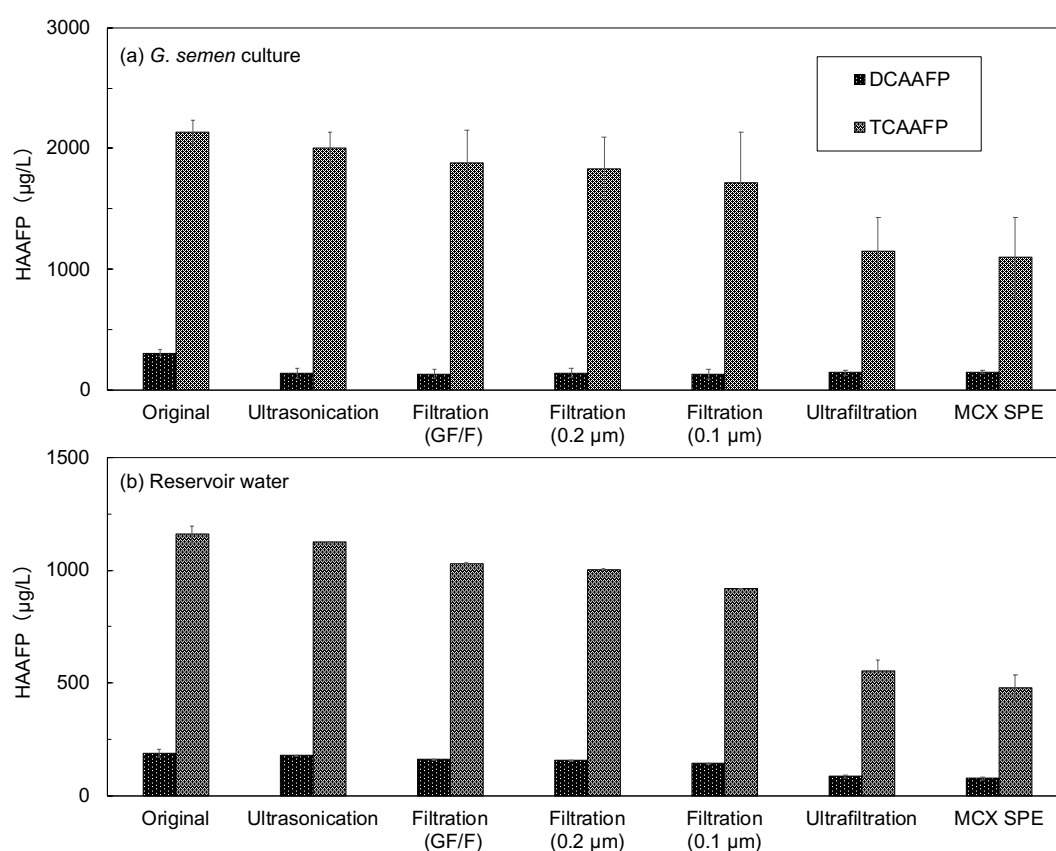


Figure 3.10. HAAFPs of (a) *G. semen* culture and (b) reservoir water during the pretreatment process.

Experimental conditions of the HAAFP test: sample dilution, 10 (for original, ultrasonicated, and filtered samples) and 200 times (for samples after UF and MCX SPE); pH 7 (5 mM phosphate buffer); Cl_2 dose, 30 (for samples after UF and MCX SPE) and 100 (for original, ultrasonicated, and filtered samples) $\text{mg Cl}_2/\text{L}$; temperature, 20 °C; and reaction time, 24 h in dark. The HAAFP values were calculated by considering the concentration and dilution factors. Error bars on the HAAFP values represent the standard deviations from three replicates.

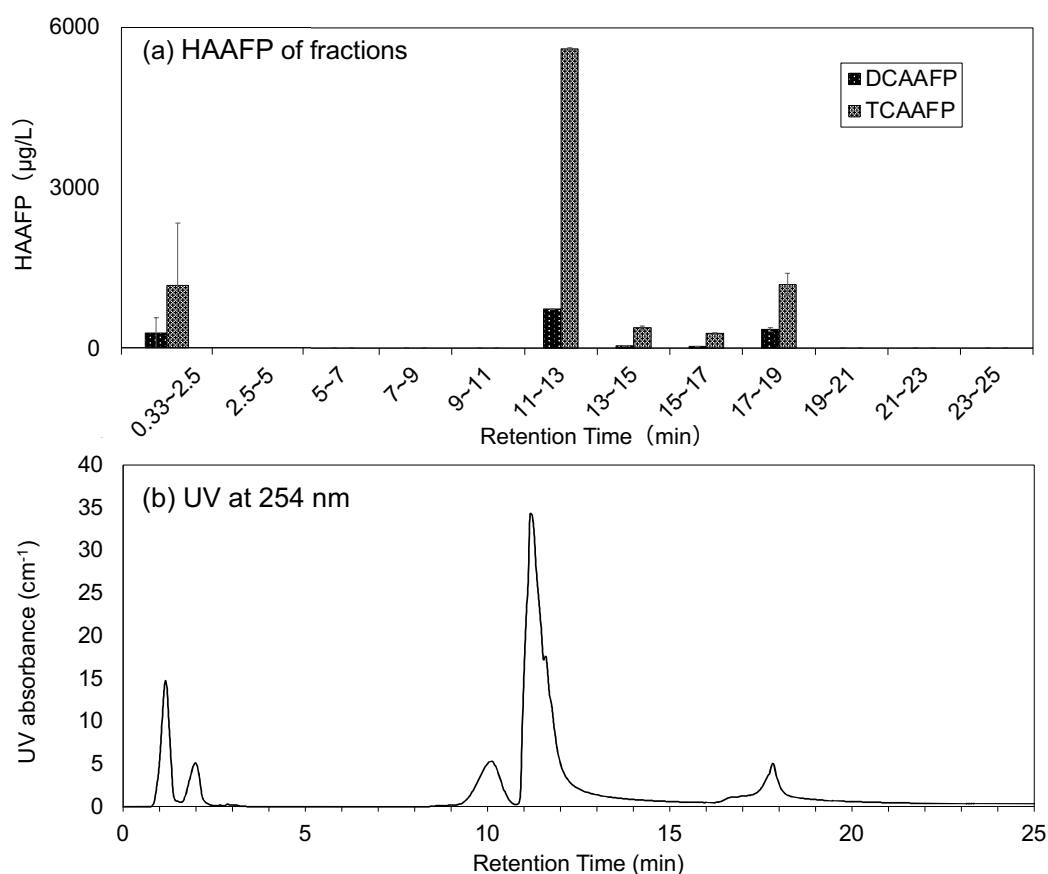


Figure 3.11. (a) HAAFPs of the fractions obtained from HPLC fractionation of pretreated *G. semen* cultures with a hydrophilic interaction liquid chromatography column (Inertsil WP300 Diol, 14 × 150 mm); (b) UV absorbance profile at 254 nm during HPLC fractionation.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); sample dilution, 100 (for 0.33–2.5, 11–13, 17–19 min fractions) and 10 times (for the other fractions); Cl_2 dose, 30 mg Cl_2/L ; temperature, 20 °C; and reaction time, 24 h in dark. The HAAFP values were calculated by considering the dilution factors during HAAFP test. Error bars represent the minimum and maximum values from duplicate experiments. Injected samples were 20 times concentrated load effluent from MCX SPE cartridges.

Table 3.12. TCAAFPs, DCAAFPs, and their ratios of pretreated raphidophyte samples. *Experimental conditions of the HAAFP test:* pH 7 (5 mM phosphate buffer); sample concentration, 1 mg C/L; Cl₂ dose, 30 mg Cl₂/L; temperature, 20 °C; and reaction time, 24 h in dark. FPs were expressed as average $\pm$ standard deviation from three replicates.

Sample name	DOC (mg C/L)	DCAAFP (μ g/mg C)	TCAAFP (μ g/mg C)	TCAAFP/ DCAAFP
After pretreatment with SPE				
<i>G. semen</i> culture	50 $\pm$ 17	63 $\pm$ 22	440 $\pm$ 33	7.5
Reservoir water	41 $\pm$ 1.6	38 $\pm$ 1.6	230 $\pm$ 18	6.1
Fraction from preparative HPLC fractionation (from <i>G. semen</i> culture)				
Retention time of 11 to 13 min	6.2 $\pm$ 2.4	140 $\pm$ 49	960 $\pm$ 300	7.1

3.4 Summary

In this chapter, the DBPFPs of raphidophyte samples were evaluated, revealing high TCAAFPs and low N-DBPFPs. In addition, TCAA precursors derived from raphidophytes were characterized, and their properties were analyzed using multiple fractionation techniques. The results indicated that the TCAA precursors are high-molecular-weight compounds ($\sim$ 10–60 kDa) with extremely high hydrophilicity and acidic functional groups. Although slight variations in properties were observed between TCAA precursors present in *G. semen* cultures and those in environmental water samples, their overall characteristics were largely similar.

Based on these property analyses, methods for preparing highly purified TCAA precursor isolates for subsequent molecular-level analyses and TCAA precursor removal experiments were established. Filtration, UF to isolate high-molecular-weight fractions, and passage through MCX cartridges were suitable for isolating TCAA precursors. However, in the case of *G. semen* cultures, the isolates aggregated during subsequent lyophilization and could not be redissolved. To overcome this, large-scale HPLC fractionation using a HILIC column was performed, yielding further purified isolates of TCAA precursors. Analysis of the HAAFP of the concentrated and purified TCAA precursor solution revealed yields approximately 1.5–2 times higher than those of previously reported major

TCAA precursors, highlighting the unique characteristics of TCAA precursors produced by raphidophytes.

In the following chapter, these TCAA precursors were analyzed at the molecular level, and attempts were made to infer their partial structures.

Chapter 4

Structural Estimation of High-molecular-weight and Ultra-hydrophilic Trichloroacetic Acid Precursors Originating from Raphidophytes

4.1 Introduction

Chapter 3 identified AOM originating from raphidophytes as a significant source of TCAA precursors and described the procedures for their isolation. Building on that foundation, this chapter focuses on elucidating the chemical and structural characteristics of these TCAA precursors to better understand their reactivity during chlorination and to provide insights into their treatability in water treatment processes.

First, TCAA precursors isolated from *G. semen* cultures were analyzed in terms of

The results of the present chapter are based on the following peer-reviewed publication: Tada, Y., Hori, K., Kosaka, K., Echigo, S., and Itoh, S. (2025). Structural estimation of high-molecular-weight and ultra-hydrophilic trichloroacetic acid precursors originated from raphidophytes. *Environmental Science & Technology*, 59(42). <https://doi.org/10.1021/acs.est.5c05108>

their optical properties (UV-visible absorbance and EEM), water quality parameters (e.g., DOC, DON, dissolved organic halogen), HAA formation at different reaction time, pH, and chlorine dose, and the quantification of carbohydrate and protein. Subsequently, the substructures of these TCAA precursors were elucidated using a combination of analytical techniques, including NMR, HRMS, and FT-IR. The interpretation of these spectra was supported by the fundamental physicochemical information obtained from the characterizations presented in the previous and current sections. Finally, model compounds containing the proposed substructures were examined as potential TCAA precursors.

4.2 Materials and methods

This section describes the experimental methods employed in the characterization of TCAA precursors isolated from raphidophytes. The overall workflow, including pretreatment and the samples used for each analysis, is illustrated in Figure 4.1. This section is organized into the following sections: reagents and solutions used in all experiments (4.2.1); algal materials (4.2.2); HAA formation in chlorination under various conditions (4.2.3); quantification of water quality parameters, including UV absorbance, EEM, DOC, DON, dissolved organic halogen, carbohydrates, and proteins (4.2.4); molecular-level analyses using NMR, FT-IR, and HRMS (4.2.5); and quantification of HAAFPs in model compounds containing the proposed substructures (4.2.6).

4.2.1 Reagents and solutions

All reagents and standard solutions were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) unless otherwise stated. Standard stock solutions of HAAs (a mixture of nine brominated and/or chlorinated acetic acids) and 3,4,5-trimethoxyphenol were obtained from the Kanto Chemical Corporation (Tokyo, Japan). Glucose, maltose, and Gomisins K3 (schisanhenol) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Acetosyringone was obtained from Cosmo Bio Co., Ltd. (Tokyo, Japan). All aqueous solutions were prepared using ultrapure water purified by a Milli-Q Plus system (Merck Millipore, Billerica, USA) and stored at 4 °C.

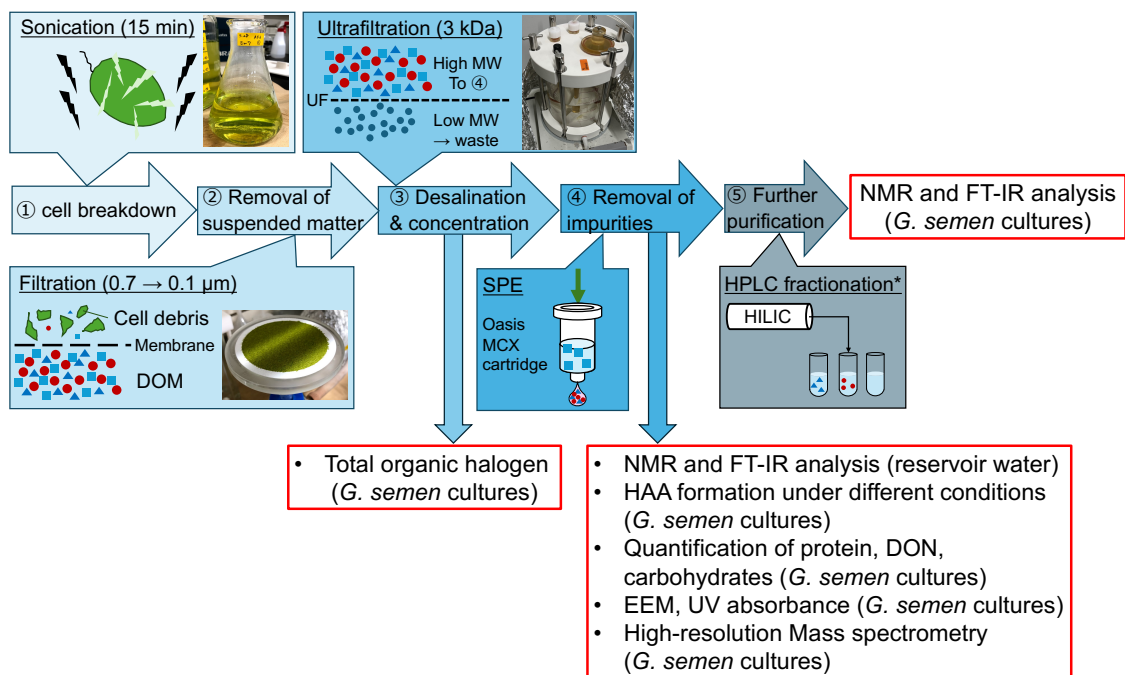


Figure 4.1. Flowchart of the experimental procedure for TCAA precursor analysis.

4.2.2 Algal materials

The algal materials used in this study were the same as those described in Chapter 3, namely, reservoir water collected in 2023 during a raphidophyte bloom and *G. semen* culture media prepared using Lake Biwa water. TCAA precursors were isolated using the method developed in Chapter 3.

4.2.3 HAA formation characteristics in chlorination

The pretreated *G. semen* culture (i.e., the load effluent from the Oasis MCX cartridge) was used. Except for the chlorine dose and sample concentration, the chlorination procedure was the same as that described in Section 3.2.3 of Chapter 3. The sample was diluted with ultrapure water to achieve 1 mg C/L, resulting in a total volume of 4 mL in 10 mL amber glass vials containing a magnetic stir bar. Phosphate buffer (0.04 mL of 500 mM solution; final concentration, 5 mM) was added to adjust the pH to 5, 7, or 9. Sodium hypochlorite solution was then added to achieve chlorine concentrations of 1, 2, 5, 10, 30, or 60 mg Cl₂/L, and the samples were stirred for 20 s. The chlorination reactions were conducted in the dark at 20 °C for 0.5, 1, 2, 5, 10, and 24 h. After measuring the

residual chlorine, the reactions were quenched by adding ammonium chloride to a final concentration of 50 mg/L. The chlorinated samples were transferred to vials and analyzed for HAA concentrations using LC-MS/MS (4000QTRAP, Sciex, MA, USA).

The HAAFP discussed herein refers to the HAA concentration obtained after 24 h of chlorination at pH 7 and a chlorine dose of 30 mg Cl₂/L.

4.2.4 Quantification of water quality parameters

4.2.4.1 EEM and UV-visible absorbance spectrometry

The EEM and UV-visible absorbance spectra of the pretreated *G. semen* culture (i.e., the load effluent from the Oasis MCX cartridge) were measured. The sample concentration was adjusted to 20 mg C/L. EEM spectra were obtained using a spectrofluorophotometer (RF-5300PC, Shimadzu, Kyoto, Japan) with a 0.5 mL four-sided transparent quartz cuvette. The excitation wavelength ranged from 220 to 450 nm, and the emission wavelength ranged from 220 to 600 nm. UV-visible absorbance spectrum was obtained using a UV-visible spectrophotometer (UV-1280, Shimadzu) with a 0.5 mL two-sided transparent quartz cuvette. Scan range was from 190 to 800 nm. Ultrapure water was used as a blank and scanned in advance for background subtraction and noise removal.

4.2.4.2 DOC and DON

DOC of the pretreated *G. semen* culture (i.e., the load effluent from the Oasis MCX cartridge) was measured using the same method described in Section 3.2.6.3. For DON measurement, the combustion catalytic oxidation technique was also used to determine TN at 720 °C with a TOC-*V*_{CSN} analyzer (Shimadzu) coupled with a total nitrogen measuring unit (TNM-1, Shimadzu). A calibration curve was established using five concentrations of nitrate nitrogen standard solution (0.1, 0.3, 1, and 2 mg N/L).

4.2.4.3 Dissolved organic halogen

Dissolved organic chlorine (DOCl) and dissolved organic bromine (DOBr) were measured by combustion ion chromatography. A 50 µL aliquot of the pretreated *G. semen* culture (i.e., the UF retentate containing high-molecular-weight fractions) was placed on a

ceramic boat filled with appropriate amount of quartz wadding and then introduced into a combustion unit (AQF-2100H, Nittoseiko Analytech Co. Ltd., Kanagawa, Japan), where organic halogens were decomposed into inorganic forms (i.e., Cl^- and Br^-) and absorbed into H_2O_2 solution. 100 μL of the solution was then pumped into a suppressor-type ion chromatograph (HIC-20ASP, Shimadzu). The calibration curve was prepared from ten levels of working Cl^- and Br^- solution (i.e., 0, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 mg/L). The specific analytical conditions are described in Table 4.1. Ultrapure water was used as a control sample.

Table 4.1. Combustion ion chromatography conditions for DOCl and DOBr.

	Category	Conditions
Combustion	LC	Prominence 20A (Shimadzu)
	Combustion system	AQF-2100H (Nittoseiko Analytech Co. Ltd.)
	Furnace temperature (inlet)	1000 °C
	Furnace temperature (outlet)	1100 °C
	Carrier gas	Argon gas (200 mL/min) → inlet boat (10 s hold) → switch over to oxygen gas (10 min hold, 400 mL/min)
	Argon gas	200 mL/min
	Oxygen gas	400 mL/min
	Sample volume	50 μL
	Absorption solution	H_2O_2
	Absorption volume	10 mL
Ion chromatography	LC system	HIC-20A-SP (Shimadzu)
	Conductivity detector	CDD-10A (Shimadzu)
	Column	Shim-pack IC-SA2 (250 mm × 4.0 mm, Shimadzu)
	Column temperature	40 °C
	Inject volume	100 μL
	Mobile phase	Sodium hydrogen carbonate: 12 mM + Sodium carbonate: 0.6 mM
	Flow rate	1.0 mL/min

4.2.4.4 Quantification of protein and carbohydrates

The pretreated *G. semen* culture (i.e., the load effluent from the Oasis MCX cartridge) was used for protein and carbohydrates quantification. The sample concentration was set at 40 mg C/L. Protein quantification was performed using the NanoOrange™ Protein Quantitation Kit (Thermo Fisher Scientific, MA, USA). Procedure was followed by their

experimental protocols.¹¹¹ The calibration curve was prepared from four levels of working BSA solution (i.e., 1, 3, 6, and 10 mg/L). The fluorescence values were determined at 485 nm for excitation and 590 nm for emission using a microplate reader (PowerScan HT, Agilent Technologies, CA, USA).

For the quantification of carbohydrates, the Total Carbohydrate Assay Kit (Cell Biolabs, CA, USA) was used. Procedure was followed by their experimental protocols.¹¹² A 180 μ L sample was mixed with 900 μ L of concentrated sulfuric acid in a 1.5 mL microtube. The mixture was then heated at 90 °C for 15 minutes. After cooling for 3 minutes at 4 °C, the absorbance was measured at 490 nm using a UV-visible spectrophotometer (UV-1280, Shimadzu) as a control. Next, 180 μ L of 5% phenol diluent was added to each sample, and the mixture was allowed to react for 5 minutes. After the reaction, the absorbance of each sample at 490 nm was measured. The absorbance of the control was subtracted to calculate the amount of carbohydrates. Standard glucose samples, with concentrations of 0, 0.03125, 0.0625, 0.125, and 0.25 mM, were used to construct the calibration curve.

4.2.5 Molecular-level analyses of TCAA precursors

4.2.5.1 Liquid-state NMR spectrometry

Two milligrams of the lyophilized sample from the *G. semen* culture were dissolved in 300 μ L of deuterium oxide (D₂O, ISOTEC, GA, US) and transferred to a micro-NMR tube (SHIGEMI Co., Ltd., Tokyo, Japan). Additionally, 10 mg of the isolates from the reservoir water were dissolved in 800 μ L of D₂O, and after centrifugation, the supernatant was transferred to a micro-NMR tube. NMR analysis was performed using a JNM-ECZ800R (JEOL Ltd., Tokyo, Japan) with a 5-mm CH/UltraCOOL probe at 25 °C. One-dimensional (1D) ¹H (800.14 MHz) and ¹³C (201.20 MHz) NMR spectra were acquired with 45° and 30° pulse angles, 5 s and 2.35 s relaxation delays, and 128 and 19200–20000 scans, respectively. Sodium 3-(trimethylsilyl) propionate-d₄ was used as an internal standard. The intense signals of H₂O and DHO (4.78 ppm) in the ¹H NMR spectra were mitigated. Additionally, distortionless enhancement by polarization transfer (DEPT) spectra were obtained at a flip angle of 135° (DEPT-135 spectra). The parameters were identical to those

of the ^{13}C NMR spectra but with 4096 scans. Furthermore, two-dimensional (2D) NMR spectra were recorded, for the isolate from the reservoir water, including ^1H - ^1H correlation spectroscopy (COSY), ^1H - ^{13}C heteronuclear multiple quantum correlation (HMQC), and ^1H - ^1H total correlation spectroscopy (TOSCY). The COSY spectrum was acquired using a 1.5 s relaxation delay and 32 scans. The HMQC spectrum was acquired using a 1.5 s relaxation delay and 64 scans, and the TOSCY spectrum was measured using a 1.5 s relaxation delay, 16 scans, and a 50 ms mixing time.

4.2.5.2 FT-IR spectrophotometry

The NMR sample (dissolved in D_2O) for *G. semen* culture was dried again under a gentle stream of nitrogen gas at 40 °C (MG-2000, EYELA, Tokyo, Japan), while the reservoir water sample was prepared using the same process as that used for NMR analysis. The dried samples were mixed with KBr (for IR spectrophotometry). The ratio of the samples to KBr was 0.5% by weight. Spectra were measured using an FT-IR spectrometer (IRAffinity-1, Shimadzu). The IR spectrum of the blank sample (only KBr) was used as the baseline. The wavenumber range was 400–4000 cm^{-1} , and the number of scans was 45.

4.2.5.3 HRMS

The pretreated *G. semen* cultures and reservoir waters (i.e., load effluents from the SPE cartridges), were analyzed using an LC-Orbitrap mass spectrometer (LC-Orbitrap/MS) (UltiMate 3000 UHPLC, Q Exactive Focus, Thermo Fisher Scientific). For following differential analysis, each sample was measured three times. Ionization was performed using both negative and positive modes of atmospheric pressure chemical ionization (APCI) because the inferred substructures were shown to be phenolic hydroxyl groups and glycans (see Section 4.3.2). Although electrospray ionization (ESI) is commonly used for such analyses, these functional groups are often difficult to ionize by ESI due to strong hydration and limited acidic/basic sites, which hinder their ionization and release from liquid droplets.¹¹³ Furthermore, because the analytes were high-molecular-weight compounds and direct analysis by LC-Orbitrap/MS can be challenging, APCI was chosen as a stronger ionization method to improve sensitivity.¹¹³ In addition, in-source fragmentation

in the APCI source was expected to generate smaller, detectable fragments, facilitating structural elucidation of the subunits. No LC column was used, and a 10 μ L sample was injected using an autosampler. The mobile phase was 50% ultrapure water and 50% methanol, with a flow rate of 200 μ L/min. The resolution was set to 70,000, with a scan range of 133.4–2000 m/z . Calibration was performed immediately before each measurement. Additional analytical conditions are provided in Table 4.2.

To identify key peaks related to TCAA precursors, differential analysis was performed using Compound Discoverer 3.1.0.305 (Thermo Fisher Scientific). As controls, an equivalent pretreated medium without raphidophytes was used for the *G. semen* culture, and an equivalent pretreated ultrapure water was used for the reservoir water. The metabolomics mode was selected as workflow, with a minimum peak intensity threshold of 100,000 and an analyzed retention time range of 0.5–3 min. Default parameters were applied for all other settings. Potential TCAA precursor substructures were estimated based on estimated molecular formulas from peaks screened using volcano plots. A volcano plot visually represents statistically significant differences in compounds present in between two compared samples. The horizontal axis indicates the $\log_2$ fold change between the two groups (i.e., sample and control), while the vertical axis represents the $-\log_{10}$ of the p -value. The significance threshold was set at a fold change of 8 or higher ($\log_2 \geq 3$) and a p -value of 0.001 or lower. All peaks within this targeted region were manually checked and false positives were removed.

4.2.6 Quantification of HAAFPs in model compounds with proposed substructures

Figure 4.2 shows the model compounds selected as potential TCAA precursors containing the proposed substructures originated from raphidophytes, based on the results of NMR, FT-IR, and HRMS analyses. Phenol, sugars (glucose and maltose), and phenols with ether linkage (3,4,5-trimethoxyphenol, acetosyringone, and Gomisins K3) were selected as the basic components of the proposed structures. Phenolic glycosides (arbutin, phlorizin, tannic acid, and sennoside B) were selected. Stock solutions of the model compounds were prepared in ultrapure water at approximately 100–1000 mg/L within 2 d before the

Table 4.2. LC-Orbitrap/MS conditions.

System	LC system	UltiMate™ 3000 SD (Thermo Fisher Scientific)
	Orbitrap/MS system	Q Exactive Focus (Thermo Fisher Scientific)
	Ionization method	APCI
Scan parameters	Scan Type	Full MS
	Scan range	133.4 to 2000 (<i>m/z</i>)
	Fragmentation	None
	Resolution	70,000
	Polarity	Negative/Positive
	Microscans	1
	Lock masses	Off
	AGC target	3.0×10^6
	Maximum inject time	100 msec
APCI source	Sheath gas flow rate	20 L/min
	Aux gas flow rate	5 L/min
	Sweep gas flow rate	0 L/min
	Discharge voltage	– (kV)
	Discharge current	20 (μ A)
	Capillary temp.	300 (°C)
	S-lens RF level	50
	Aux gas heater temp	400 (°C)
LC component	Flow	0.2 (mL/min)
	Injection	10 (μ L)
	Mobile phase	50% ultrapure water and 50% methanol
	Mode	Isocratic elution
	temperature	–
	Column	No column
	Measurement time	5 min

HAAFP test. The HAAFP test was performed on the samples diluted to 1 mg C/L, under a chlorine concentration of 30 mg Cl₂/L, at pH 7, with a 24 h reaction time.

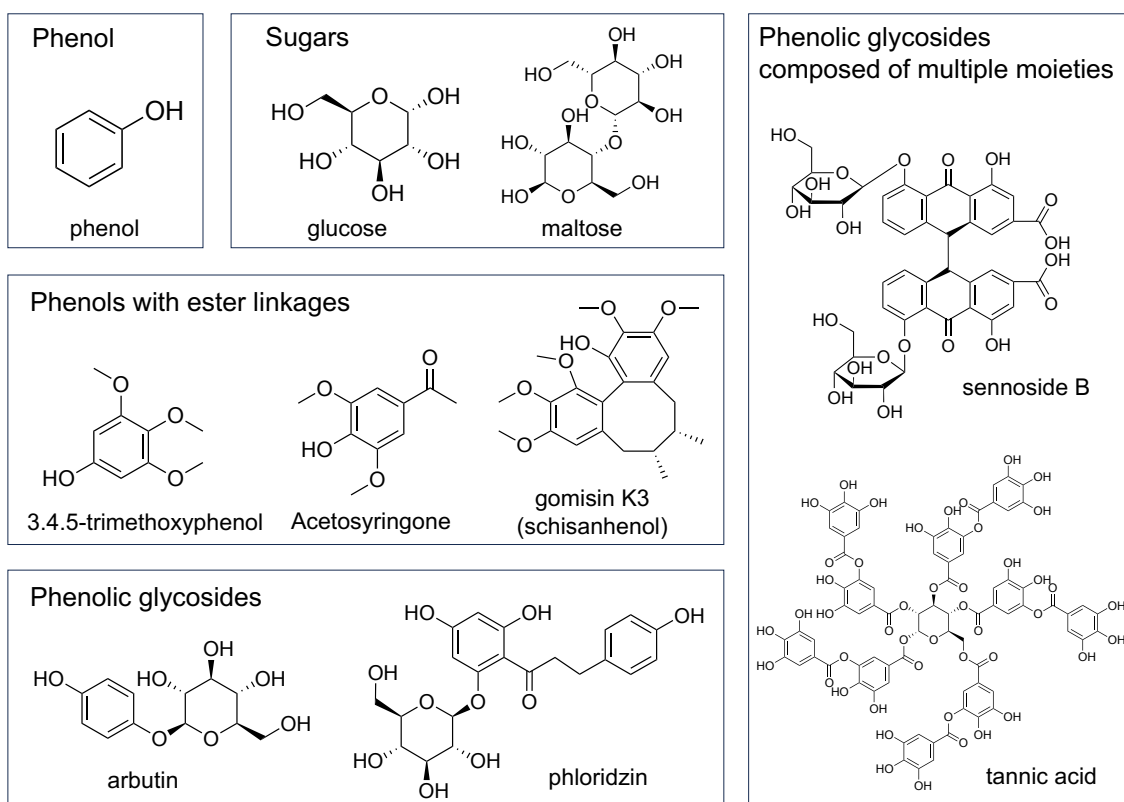


Figure 4.2. Structures of model compounds.

4.3 Results and discussion

4.3.1 Characterization of water quality, optical properties, and HAA formation of TCAA precursors originating raphidophytes

4.3.1.1 HAA formation characteristics

Figure 4.3(a) and (b) shows the temporal changes in HAA formation and chlorine consumption at different reaction time from the pretreated *G. semen* cultures. The sum of TCAA and DCAA concentrations (i.e, referred as HAA total in Figure 4.3(a)) at 30 min after the start of chlorination was 410 $\mu\text{g}/\text{mg C}$, which accounted for more than half of the value observed after 24 h. The concentrations of total HAA and TCAA increased with

reaction time, while DCAA formation increased rapidly within the first hour and then remained almost constant thereafter. These results indicate that, under sufficient chlorine conditions, the reactions between the TCAA precursors from *G. semen* culture and chlorine proceed rapidly and DCAA formation is faster than that of TCAA. The chlorine consumption reached 3.0 mg/mg C at 0.5 h and 4.2 mg/mg C at 24 h, consistent with the observation that HAA formation plateaued over time. These behaviors were similar to that previously reported for phenolic compounds.¹¹⁴

Figure 4.4 illustrates the changes in HAA formation under different chlorine doses. When the chlorine doses were 1 and 2 mg Cl₂/mg C, the TCAA concentrations were lower than those of DCAA, whereas at 5, 10, 30, and 60 Cl₂/mg C, the TCAA concentrations exceeded those of DCAA. This result indicates that the tendency of HAA formation changes depending on the amount of chlorine doses. The molar ratios of TCAA to DCAA at chlorine doses of 1, 2, 5, 10, 30, and 60 Cl₂/mg C were 0.29, 0.54, 2.50, 3.56, 4.86, and 6.11, respectively. These results suggest that, under sufficient chlorine conditions, the precursors are further chlorinated to form TCAA, whereas under chlorine-limited conditions, fewer chlorine atoms are substituted onto the precursors, leading to the preferential formation of DCAA.

Figure 4.5 shows the changes in HAA concentrations at different pH values. TCAA increased under acidic conditions, while DCAA slightly increased under acidic conditions as well. Previous studies have shown that for two phenolic compounds, phenol and 1,3,5-trihydroxybenzene, TCAA formation decreases under basic conditions, whereas aliphatic compounds such as acetonedicarboxylic acid show no pH dependence in HAA formation.^{114,115} Because the precursors also showed a decrease in TCAA under basic conditions, it is suggested that these precursors may contain phenolic structural features.

4.3.1.2 Quantification of DON, DOC, dissolved organic halogen, protein and carbohydrate of pretreated *G. semen* cultures

The DON of the pretreated *G. semen* cultures was 2.1 mg N/L, representing 5.2% of the DOC (40 mg C/L). Since the sample was passed through the mixed-mode MCX cartridge, the presence of basic functional groups, such as amines, was unlikely. Given that the chemicals were of natural origin, it is possible that structures such as amide, pyr-

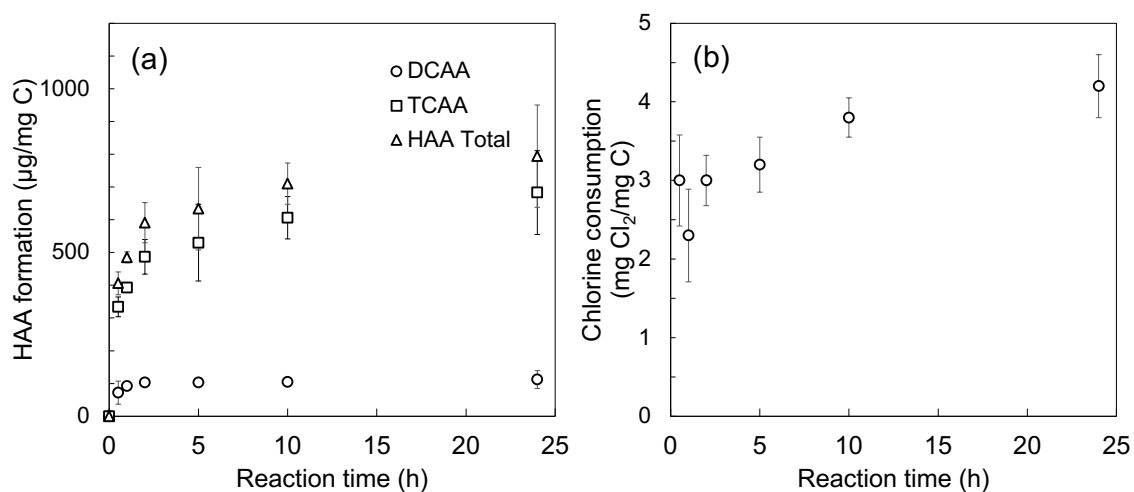


Figure 4.3. (a) HAA formation profile and (b) chlorine consumption of TCAA precursors isolated from *G. semen* cultures during chlorination during chlorination at different reaction times.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); sample concentration, 1 mg C/L; Cl₂ dose, 30 mg Cl₂/L; temperature, 20 °C; and reaction time, 0.5, 1, 2, 5, 10, and 24 h in dark.

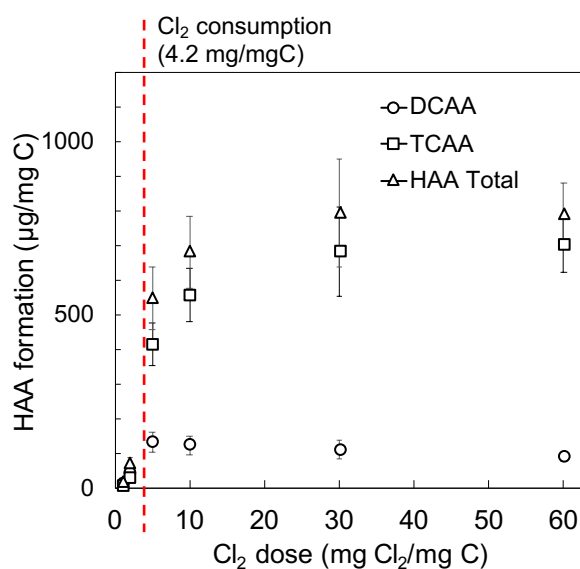


Figure 4.4. HAA formation profile of TCAA precursors isolated from *G. semen* cultures during chlorination at different chlorine dose.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); sample concentration, 1 mg C/L; Cl₂ dose, 1, 2, 5, 10, 30, or 60 mg Cl₂/L; temperature, 20 °C; and reaction time, 24 h in dark.

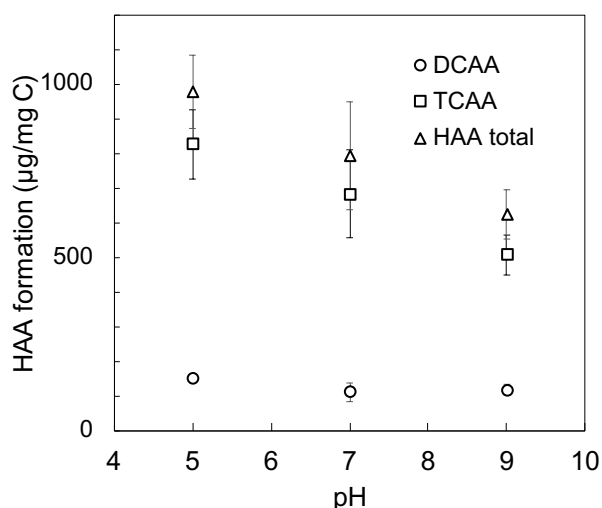


Figure 4.5. HAA formation profile of TCAA precursors isolated from *G. semen* cultures during chlorination at different pH values.

Experimental conditions of the HAAFP test: pH 5, 7, or 9 (5 mM phosphate buffer); sample concentration, 1 mg C/L; Cl_2 dose, 30 mg Cl_2 /L; temperature, 20 °C; and reaction time, 24 h in dark.

role, or nitrile groups were present. In addition, no proteins were detected (<1.0 mg/L), confirming that proteins are not HAA precursors. This was attributed to the removal of nitrogen-containing basic functional groups, such as amines, and hydrophobic substances during SPE. Additionally, the amount of carbohydrate was 10 mg/L in terms of glucose weight, corresponding to 4.2 mg C/L in terms of DOC. This means that the carbohydrate accounted for more than 10% of the total DOC. These results indicate that the TCAA precursors from *G. semen* are not proteins or peptides, but rather glycans as their component. It was also suggested that nitrogen in the sample is not part of a protein or peptide.

Despite the considerably high TCAAFP of the precursor from *G. semen* cultures, its chlorine consumption was only 4.2 mg Cl_2 /mg C. TCAA was formed efficiently with low chlorine consumption. The molar ratio of the TCAAFP to chlorine consumed was 0.045, which was almost twice that of phenol (i.e., 0.022–0.031^{101, 102}) and 2,4,6-trichlorophenol (i.e., 0.030¹⁰¹). Although high TCAAFP with low chlorine consumption indicated that the presence of chlorine atoms in their structures, neither DOCl nor DOBr were detected. This indicates that specific chemical moieties without halogen atoms facilitate the formation of HAA from the precursors. Low chlorine consumption of the sugar-like blocks may be a factor.¹¹⁶

Table 4.3. Basic characteristics of the pretreated *G. semen* cultures.

Parameter	Value
DOC (mg C/L)	40
DON (mg N/L)	2.1
DON/DOC (%)	5.2
DOCl (mg/L)	N.D. (same as blank) ^a
DOBr (mg/L)	N.D. (same as blank) ^a
Protein (mg/L as BSA equivalent)	<LOQ ^b
Carbohydrate (mg/L as glucose equivalent)	10

^a Below detection (same as ultrapure water).

^b LOQ = 1 mg/L.

4.3.1.3 EEM spectrum and UV-visible absorbance profile of TCAA precursors isolated from *G. semen* cultures

Figure 4.6 shows the fluorescence spectrum of the pretreated *G. semen* cultures (i.e., the load effluent from the SPE cartridges). Strong signals were observed at 280 nm/420 nm and 320 nm/430 nm (excitation/emission wavelengths). According to a table of the major signals in the EEM spectrum,¹¹⁷ these peaks correspond to fulvic acids, suggesting that the TCAA precursor molecules are rich in carboxylic acids and hydroxyl groups. Additionally, no fluorescence signals associated with humic acids or proteins containing tyrosine and tryptophan-like structures as their building blocks, were detected.¹¹⁷ This result is consistent with the fact that the fraction isolated was predominantly composed of hydrophilic acids as shown in Chap 3.

Figure 4.7 shows the UV-visible spectra of the sample containing the isolated TCAA precursors. No absorbance was observed at wavelengths above 400 nm, indicating that visible-light-absorbing chromophoric compounds, such as pigments derived from algal chloroplasts, were not present in the isolated fraction.¹¹⁸ A small, broad peak at approximately 280 nm and intensely large peak at 210 nm were observed. Similar peak patterns were observed in the absorption spectra of phenolic acids,¹¹⁹ suggesting the presence of carboxylic acids and aromatics as in the EEM.

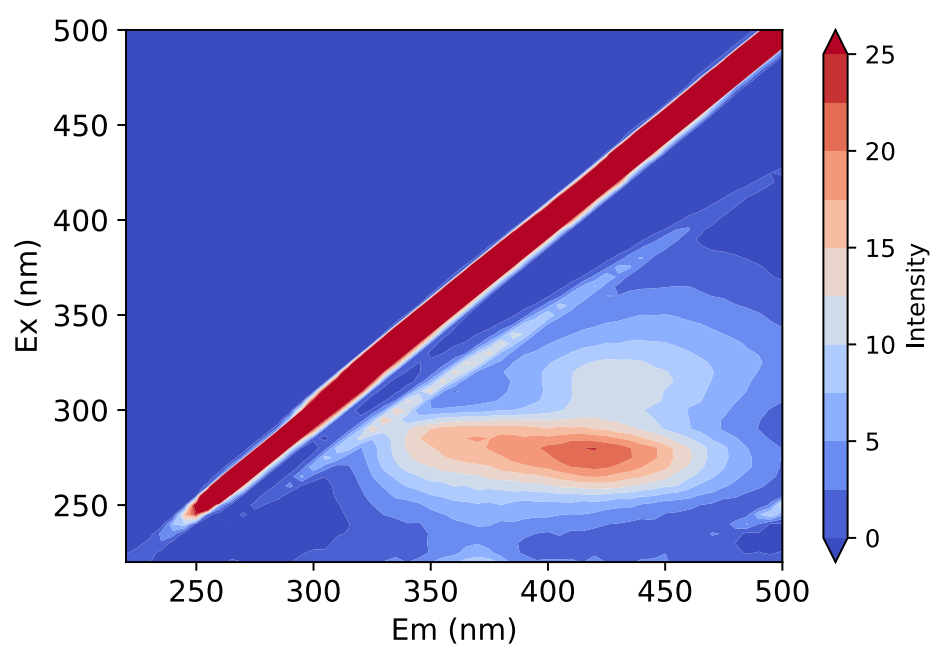


Figure 4.6. EEM spectrum of isolated TCAA precursors from *G. semen* cultures.

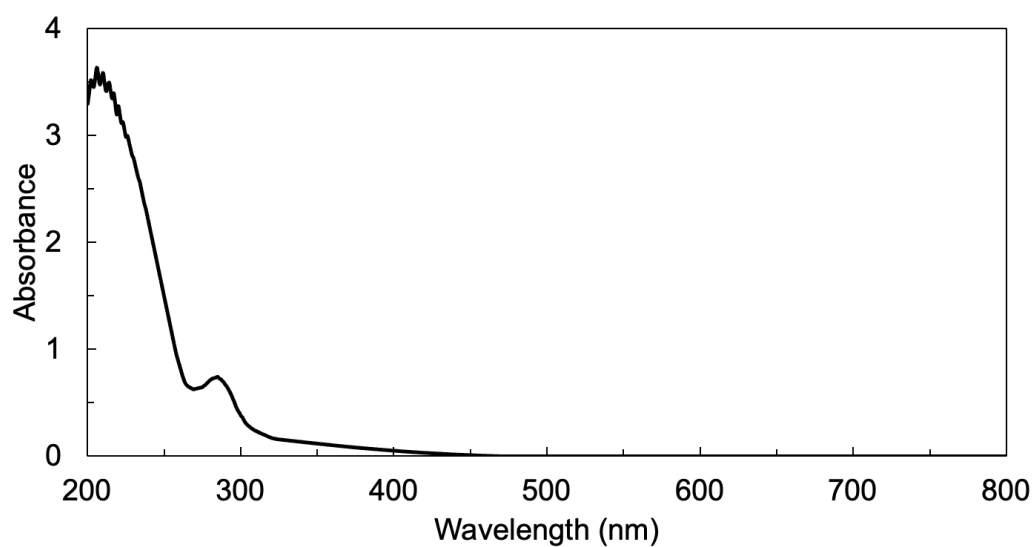


Figure 4.7. UV-visible absorbance spectrum of isolated TCAA precursors from *G. semen* cultures.

4.3.2 Molecular-level characterization of TCAA precursors isolated from raphidophytes

4.3.2.1 NMR spectra of Isolated TCAA precursors from raphidophytes

NMR spectroscopy was employed to analyze the lyophilized HPLC fraction (i.e., 11–13 min fraction) from the *G. semen* culture and the lyophilized load effluent from SPE cartridges of the reservoir water. The substructures were estimated from the 1D ^1H , ^{13}C NMR spectra, and 2D NMR spectra. Carbon, hydrogen, oxygen, and nitrogen atoms were considered as the primary components. Based on the results described in the previous sections, substances with amino functional groups and proteins were excluded as candidates for TCAA precursors, and the presence of glycans was presumed in the samples. Additionally, functional groups such as hydroxy and amide groups were excluded from the interpretation of the ^1H -NMR spectra because the ^1H (proton) in the O-H and N-H bonds was easily exchanged with ^2H (deuterium), making their signal intensities much lower.¹²⁰ Furthermore, halogen atoms, such as Cl and Br, were excluded as they were not detected.

The ^1H -NMR spectrum of *G. semen* was first interpreted and compared with that of the reservoir water. Figure 4.8 (a) shows the ^1H -NMR spectrum of TCAA precursors obtained from the *G. semen* cultures. The low and broad signals at 1.2 and 2.0 ppm, respectively, were attributed to aliphatic protons, such as those in methyl ($-\text{CH}_3$), methylene ($-\text{CH}_2-$), and methine ($-\text{CH}-$) groups. Large and overlapping signals in the range of 3.1–4.0 ppm, resulting from the presence of protons on carbon atoms bonded to ether or hydroxyl groups (e.g., alcohol),¹²⁰ suggested the presence of carbohydrates and methoxy groups within the TCAA precursors.¹¹⁰ The strong signals at 3.40, 3.51, and 3.62 ppm and their peak intensity magnitude ratios were similar to the ^1H -NMR spectra of polysaccharide extracts from diatoms in a previous study.¹²¹ The presence of carbohydrates, as demonstrated in the previous section, was confirmed. Additionally, signals from 5.9 to 6.8 ppm indicated the presence of aromatic compounds substituted with electron-donating functional groups, such as hydroxy and ether groups (due to resonance effects) and/or unsaturated hydrocarbons.¹²⁰ The sharp signal at 8.1 ppm also indicated the presence of aromatics substituted with electron-withdrawing functional groups, such as carboxylic acids and esters.¹²⁰ For the spectra of individual chemicals obtained from PubChem and

SpectraBase (John Wiley & Sons, Inc.), phenol, methoxybenzene, and methoxyphenol exhibits strong signals at 6.3–6.9 ppm, while pyranose-type glucose shows complex signals at 3.1–4.0 ppm, suggesting that these structures may be present as substructures of the precursors.

The ratios of the peak areas in the ^1H NMR spectra represented the abundance of each proton. The integration area of each signal was summed, and the percentage of the presence ratio to the total was calculated. The presence ratio of aliphatics (0–2.0 ppm), carbohydrates (3.0–4.5 ppm), and aromatics and unsaturated hydrocarbons (>5.9 ppm) present were 15%, 33%, and 42%, respectively. A high abundance of aromatics, unsaturated hydrocarbons, and carbohydrates constitutes the basic substructures of the TCAA precursors from *G. semen*, with few aliphatic substructures.

The ^1H -NMR spectrum of the TCAA precursors from the reservoir water is presented in Figure 4.8 (b). The strong signals at 1.95 ppm (acetic acid) and 4.8 ppm (H_2O and DHO) were attributed to impurities. Broad and multiple signals between approximately 0.5 to 2.3 ppm were attributed to aliphatic protons, while the bulk of signals at 3.2–4.4 ppm was considered to originate from glycans. The signals at 6.4, 6.7, 7.0, and 8.1 ppm also originated from aromatic protons and unsaturated hydrocarbons. The signal patterns were similar in raphidophyte samples. Additionally, the percentages of aliphatic compounds (0–2.3 ppm, excluding the large peak at 1.95 ppm), carbohydrates (3.2–4.4 ppm), and aromatics and unsaturated hydrocarbons (>5.9 ppm) were 32%, 52%, and 11%, respectively. The lower proportion of aromatic and unsaturated hydrocarbon moieties may explain the lower HAAFPs observed in the reservoir water compared to the *G. semen* culture.

For ^{13}C -NMR spectra, the spectrum from *G. semen* (Figure 4.9 (a)) was first interpreted using a DEPT-135 spectrum (Figure 4.10) and compared with that of the reservoir water (Figures 4.9 (b) and 4.11). In DEPT-135 spectra, signals from CH and CH_3 groups appear as positive, whereas signals from CH_2 groups appear as negative, and signals from quaternary carbons are absent. Figures 4.9 (a) and 4.10 present the ^{13}C -NMR and corresponding DEPT-135 spectra of the TCAA precursors from *G. semen* culture. The signals at 62.5 and 72.1 ppm were attributed to secondary and tertiary carbons from the DEPT spectrum, respectively, and revealed the existence of alcohols and ethers ($\text{R-CH}_2\text{-O}$ and

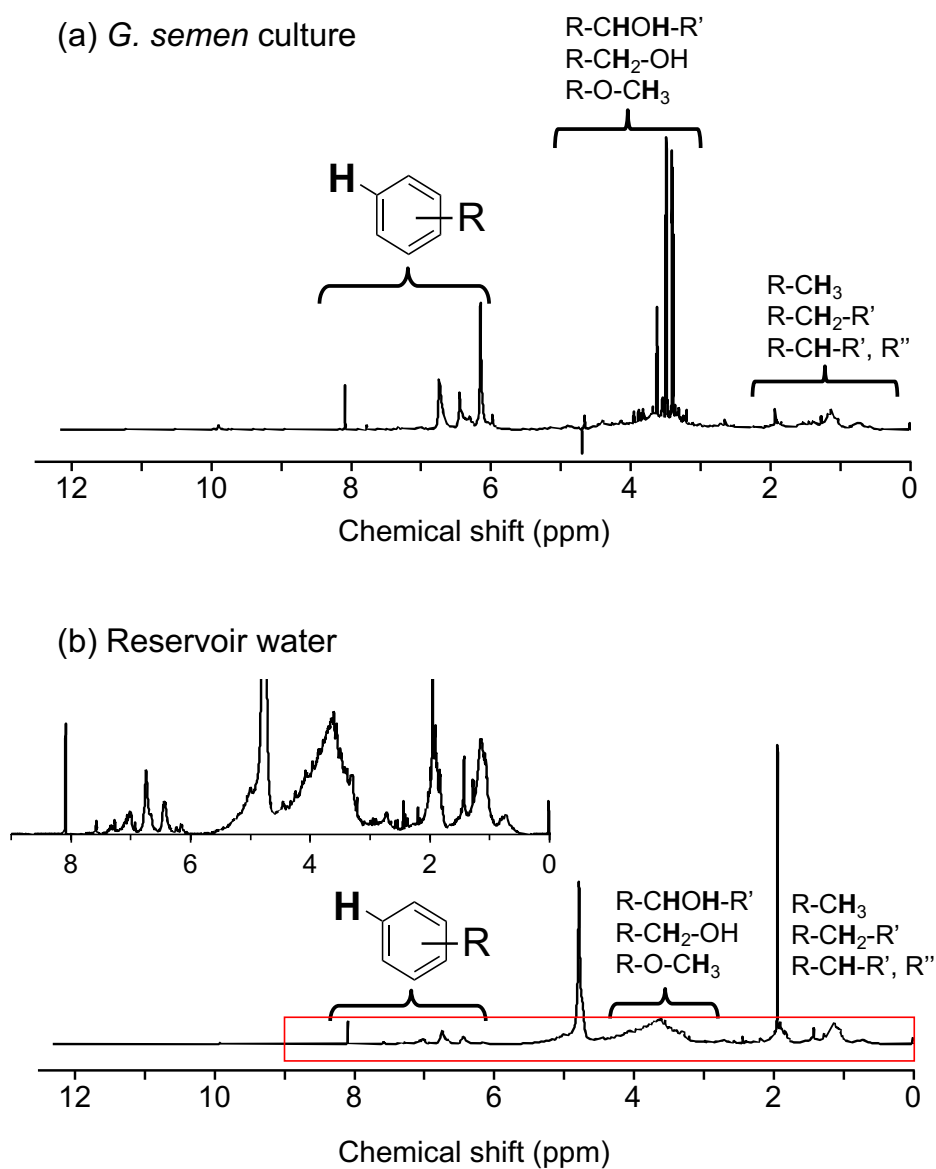


Figure 4.8. ^1H -NMR spectra of TCAA precursors isolated from (a) *G. semen* culture and (b) reservoir water. The inset spectrum within Figure (b) magnifies the region outlined in red.

R-CH-O), indicating the presence of carbohydrates, as shown in ^1H -NMR.¹²⁰ The peaks at 95.4, 99.3, 155.5, and 156.5 ppm were attributed to quaternary carbons owing to the absence of signals in the DEPT spectrum. The peaks at 155.5 and 156.5 ppm originated from carbons on aromatic rings bonded to phenolic hydroxyl groups, revealing the presence of phenolic hydroxyl groups.¹²⁰ Signals at 95.4 and 99.3 ppm indicated the presence of acetals or hemiacetals (containing two ethers or an ether and hydroxy function), aromatics with multiple electron-withdrawing functionalities, or combinations of these substructures.¹²⁰ Although aromatic protons were observed in the ^1H -NMR spectrum, no tertiary carbons from the aromatic rings, which typically exhibit signals between 110–150 ppm, were observed. This absence may be attributed to the small number of aromatic tertiary carbons present, the signals being buried in the background owing to the broadening of signals from tertiary aromatic carbons, interactions within and between the TCAA precursors, or an insufficient sample volume, resulting in inadequate signal detection. This issue must be addressed in future studies.

Figures 4.9 (b) and 4.11 show the ^{13}C -NMR and DEPT-135 spectra of the TCAA precursors from the reservoir water. Signals at 20.4 and 176.7 ppm likely were originated from impurities (acetic acid). Similar to the precursors from *G. semen* culture, the signals at approximately 95.4, 99.2, and 156 ppm indicated the presence of comparable substructures. However, the signals at 62.5 and 72.1 ppm observed in the *G. semen* sample were absent. Instead, a peak at 165.7 ppm was identified, which was suggested to be a quaternary carbon from the DEPT spectrum. This peak was derived from carboxylic acids and their derivatives (e.g., amides).¹²⁰ While it is possible that the carboxylic acid moiety is bonded to an aromatic ring, this cannot be definitively concluded based on the current data alone. These differences between the samples may explain the difference in TCAAFP.

The 2D NMR spectra also provided information on the substructures of the TCAA precursors. Owing to sample availability, only TCAA precursors from the reservoir water were measured. Figures 4.12 and 4.13 present the ^1H - ^1H COSY and ^1H - ^1H TOCSY spectra of the TCAA precursors, showing the signals of hydrogen bonded to adjacent carbons or carbons several positions apart. No signal was detected in the upper left area (X: > 4.8 ppm, Y: within the range of 0–4.8 ppm), indicating that protons with high (0–4.8 ppm) and low (>4.8 ppm) chemical shift were not present on the adjacent carbon

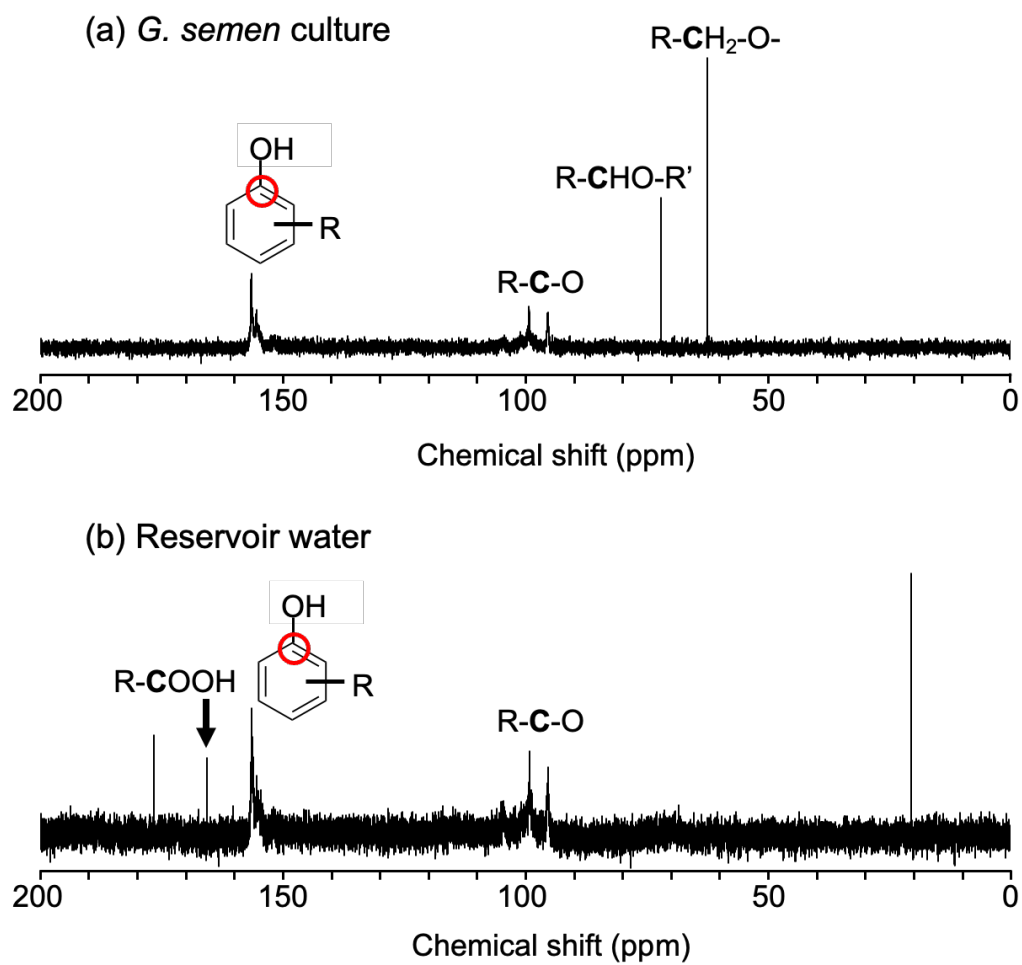


Figure 4.9. ^{13}C -NMR spectra of TCAA precursors isolated from (a) *G. semen* culture and (b) reservoir water.

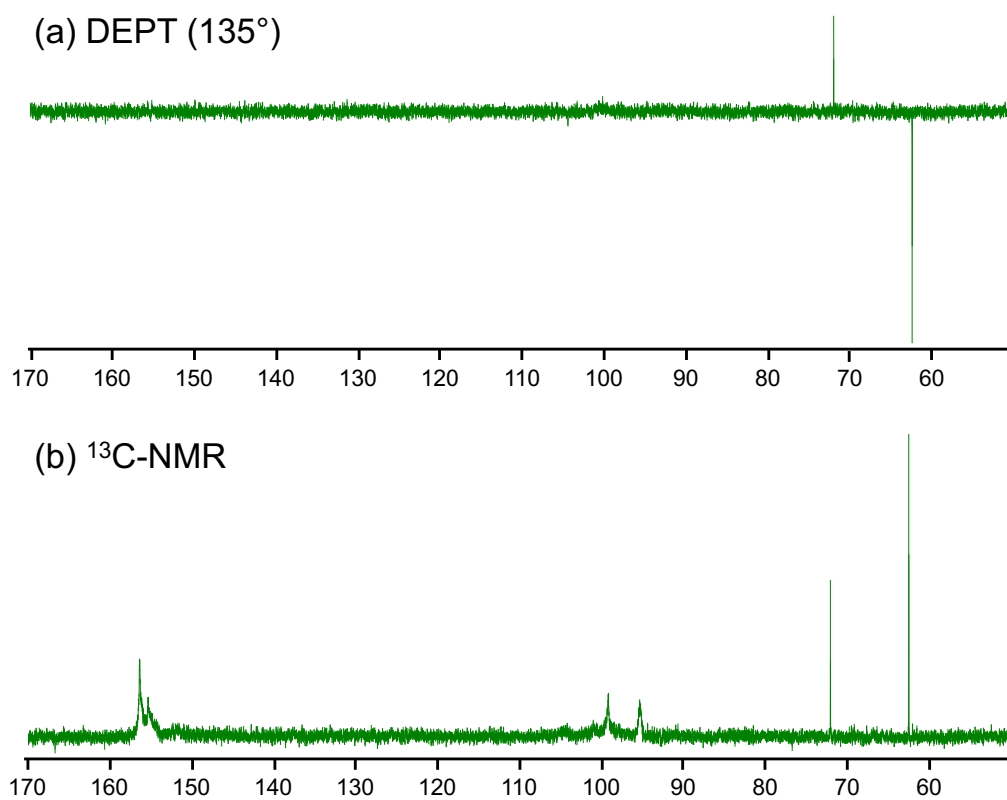
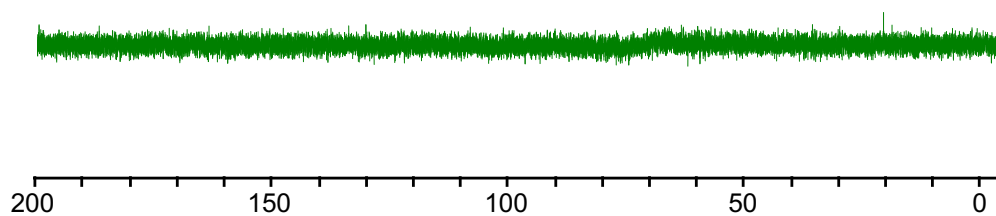


Figure 4.10. (a) DEPT spectrum (135°) and (b) ^{13}C -NMR spectrum of TCAA precursors isolated from *G. semen* culture

(a) DEPT (135°)



(b) ^{13}C -NMR

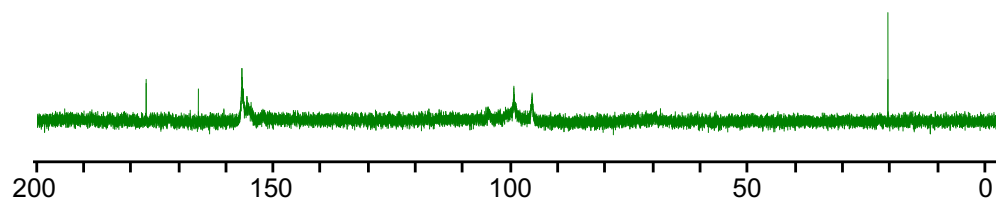


Figure 4.11. (a) DEPT spectrum (135°) and (b) ^{13}C -NMR spectrum of TCAA precursors isolated from reservoir water.

atoms. These results support the interpretation of ^1H NMR spectra that protons with lower chemical shifts (6–8 ppm) are associated with aromatic compounds. Additionally, correlations were detected between aliphatic and glycan structures, suggesting that these structures are interconnected. Furthermore, the only intensified peak at X: 2.2 ppm and Y: 20.4 ppm was detected on the ^1H - ^{13}C HMQC spectrum (Figure 4.14), which illustrated the connections between ^1H and ^{13}C atoms, resulting from methyl functional groups. There were several other peaks with low intensities in the diagram; however, they were not sufficiently sensitive for characterization.

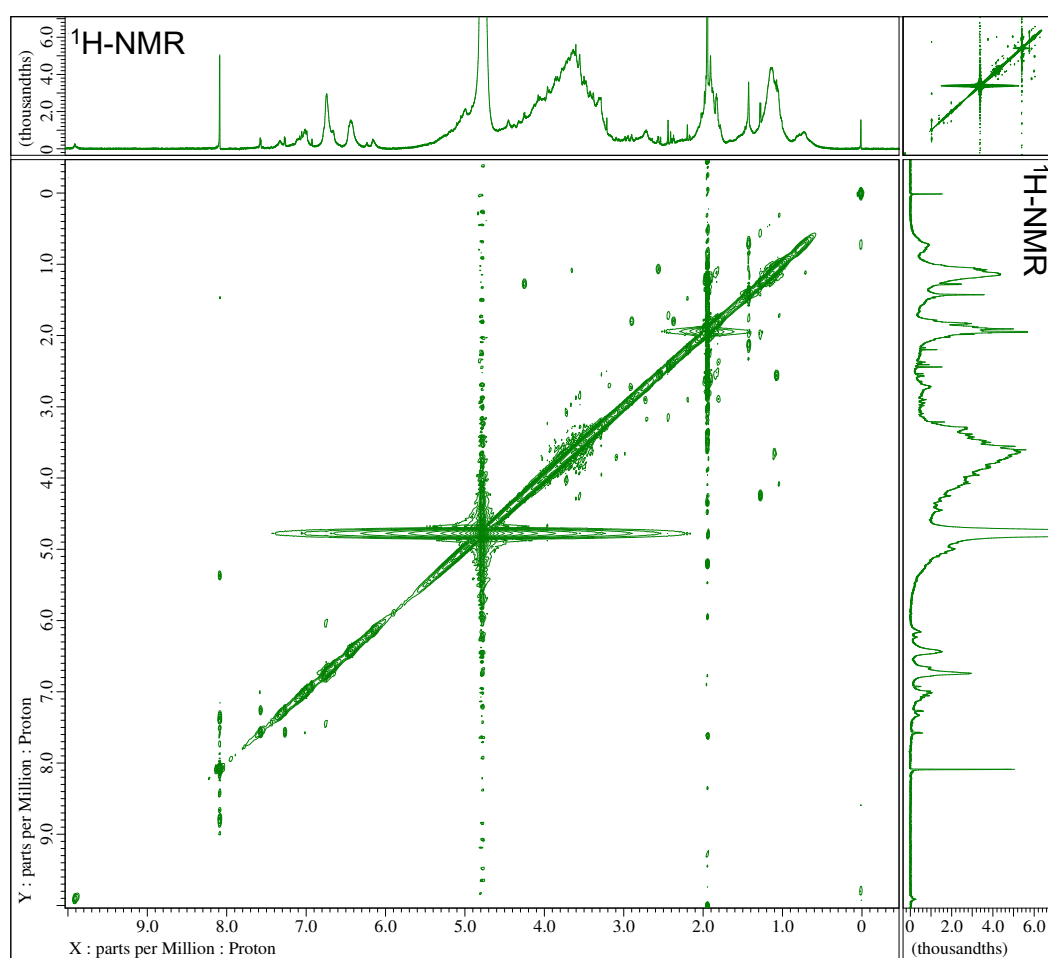


Figure 4.12. ^1H - ^1H COSY spectrum of TCAA precursors isolated from reservoir water.

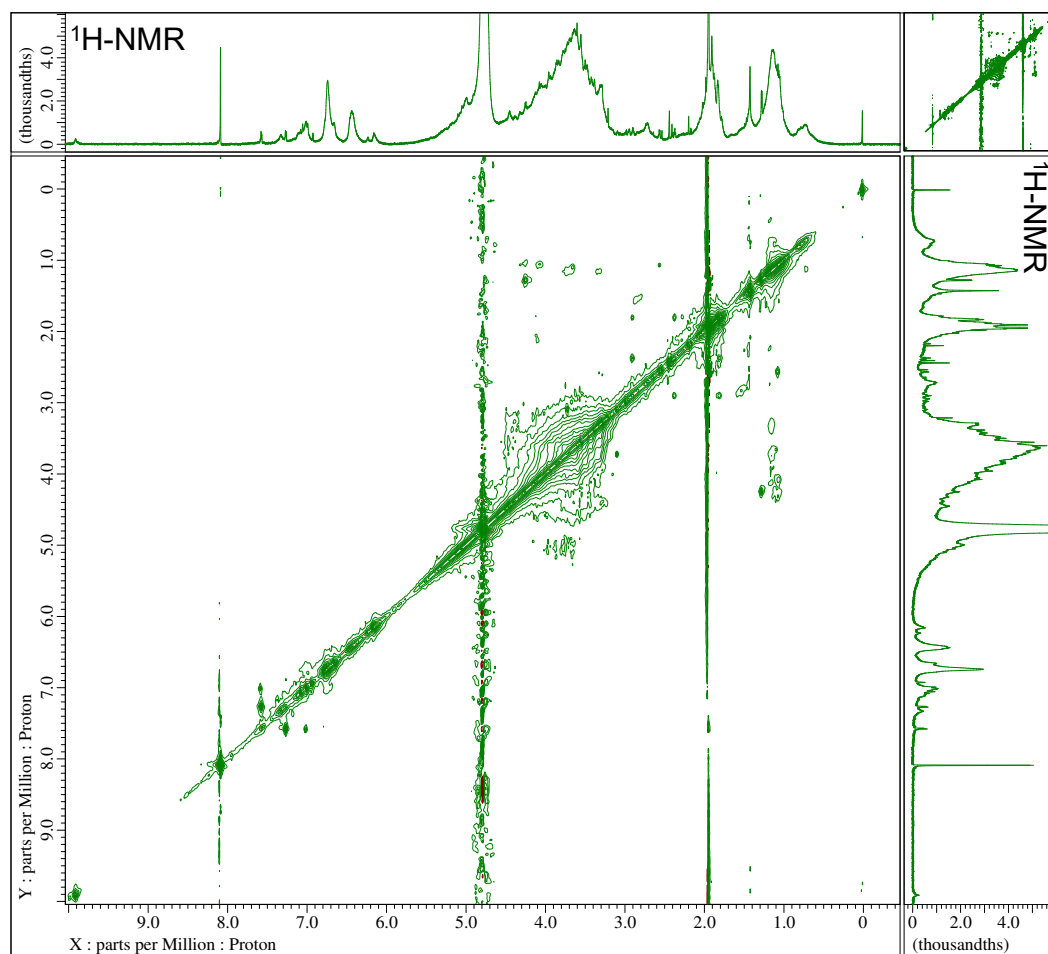


Figure 4.13. ^1H - ^1H TOCSY spectrum of TCAA precursors isolated from reservoir water.

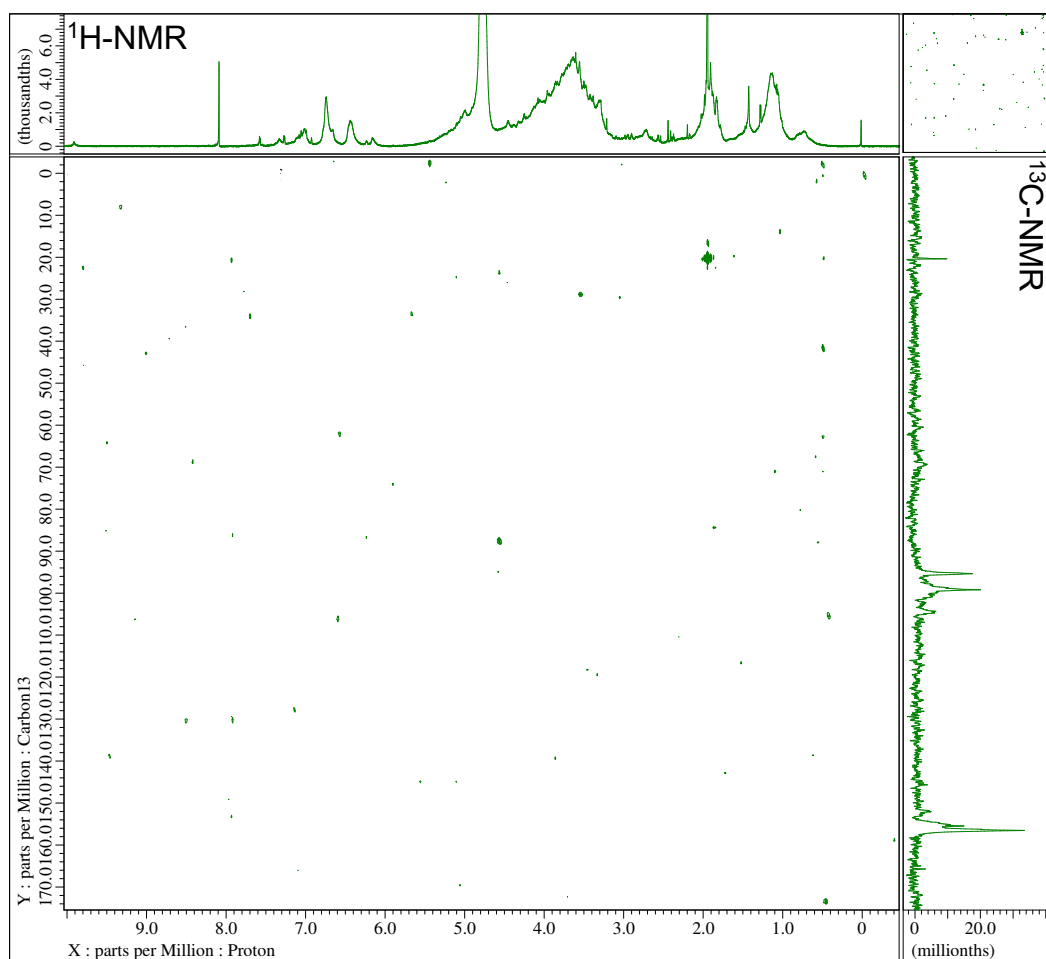


Figure 4.14. ^1H - ^{13}C HMQC spectrum of TCAA precursors isolated from reservoir water.

4.3.2.2 FT-IR spectra of isolated TCAA precursors from raphidophytes

Figure 4.15 shows the IR spectra of TCAA precursors from raphidophytes. The complex spectra suggested the presence of various substructures in the TCAA precursors. Despite the differences in transmittance, the signal wavenumbers of the precursors from *G. semen* culture and wild raphidophytes coincided, indicating that the structures were highly similar. The large and broad bands at approximately $3,200\text{ cm}^{-1}$ in both spectra were derived from the O-H stretching vibration that contributed to inter- and/or intramolecular hydrogen bonds and N-H stretching vibration.^{57, 120, 122} They are considered part of phenolic, glycan substructures, or amide bonds. The small broad peaks around $2,600\text{ cm}^{-1}$ were also derived from O-H stretching vibration.^{57, 120, 122} Additionally, the absorption bands from $1,670$ to $1,540\text{ cm}^{-1}$ were derived from the C=C stretching vibrations of aromatic

moieties, C=O stretching and N–H deformation vibration of primary amides, and/or the C=O stretching vibrations of ketones.^{57, 120} These results support the NMR results, which show the presence of phenol as a substructure. Moreover, compounds with ether bonds and acetals, which are possible substructures shown in the NMR spectrum, exhibited spectra between 1260 and 1000 cm^{-1} due to C–O stretching.¹²⁰ It has been shown that glucose and starch display several signals within these wavenumbers,⁵⁷ which likely reflect these structures. However, at lower wavenumbers (less than 1500 cm^{-1}), various molecular substructures are attributed to the signals, making it difficult to determine the specific substructures.

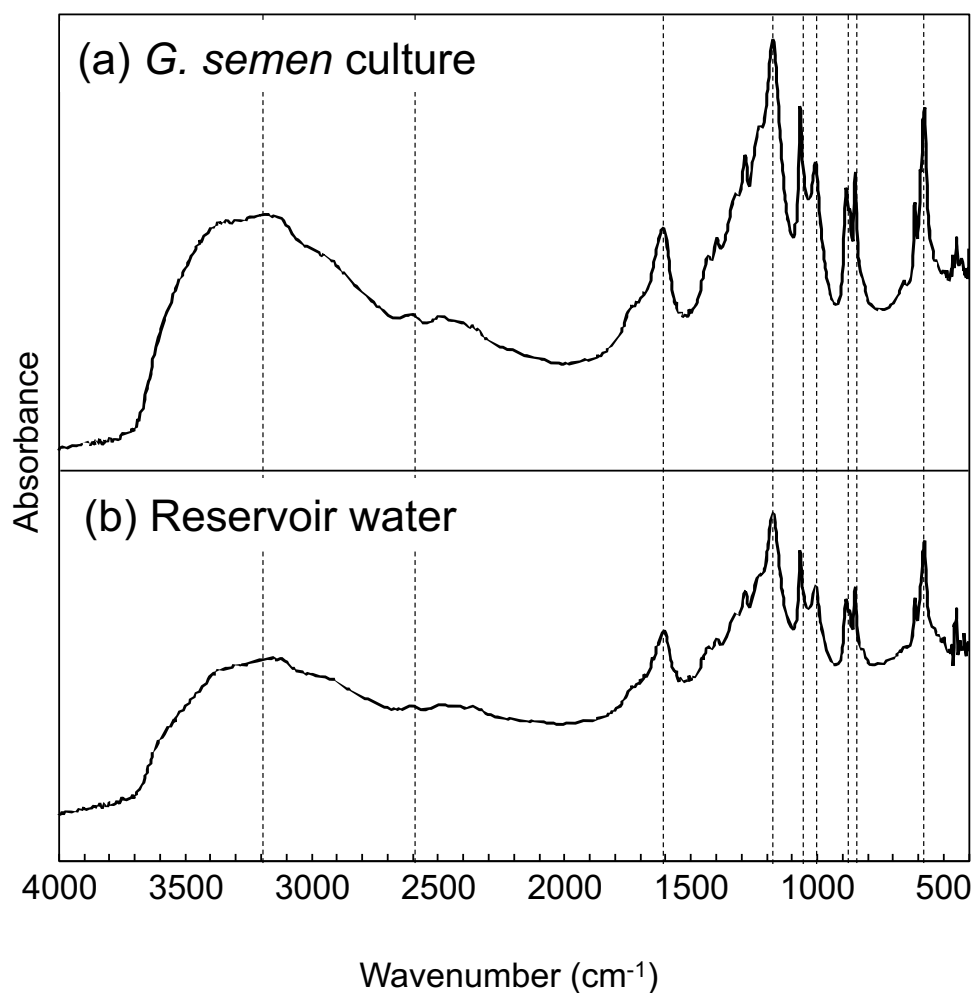


Figure 4.15. FT-IR spectra of TCAA precursors isolated from (a) *G. semen* culture and (b) reservoir water.

4.3.2.3 HRMS analysis of isolated TCAA precursors from raphidophytes

Potential TCAA precursor substructures were estimated based on molecular formulas estimated from peaks screened using volcano plots. Figures 4.16–4.19 present volcano plots with red-circled areas indicating targeted analyzed regions. Additionally, the major estimated molecular formulas of individual peaks in these regions are listed in Table 4.5. Although numerous peaks were detected in the positive mode, none corresponded to realistic molecular formulas; therefore, only the results from the negative mode are presented. TCAA precursors are high-molecular-weight compounds (>10 kDa), yet the estimated molecular formula had relatively smaller molecular weight. This suggests that APCI ionization may have fragmented the polymeric compounds into smaller units or generated multiply charged ions.

The estimated molecular formulas (e.g., $C_{12}H_{18}O_{10}$ and $C_9H_{16}O_9$) primarily consisted of carbon, hydrogen, and oxygen atoms. The high oxygen content indicates that these compounds are likely monosaccharides, oligosaccharides, sugar chains, oxidized sugars, or their derivatives, suggesting that the TCAA precursor incorporates these structures within its substructure. This finding is consistent with NMR results that confirmed the presence of carbohydrate substructures. Additionally, nitrogen-containing compounds were detected, suggesting the possible presence of such as amide bonds. Furthermore, $C_{12}H_{18}O_8$ and $C_{18}H_{24}O_{13}$ were identified as common substructures in both samples.

This study provides direct quantitative and spectroscopic evidence of the presence of these substructures. An example of a TCAA precursor structure is shown in Figure 4.20. Furthermore, despite the differences in signal intensity of FT-IR and NMR spectra, the peak shapes of the TCAA precursors of the cultured and wild raphidophytes were similar, suggesting that they have similar chemical structures.

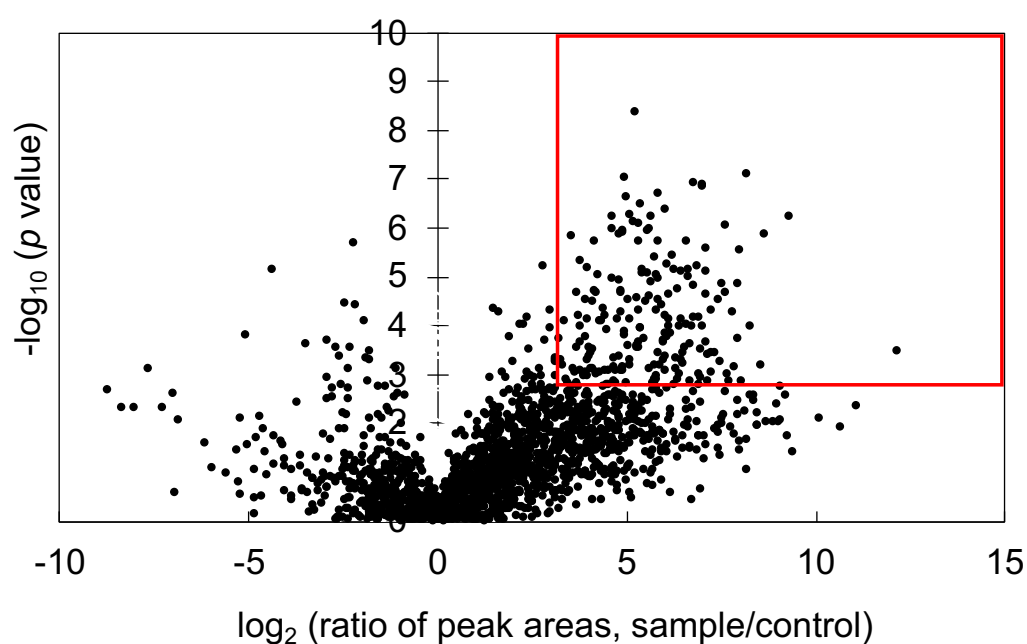


Figure 4.16. Volcano plot of the treated cultured *G. semen* cultures analyzed under APCI negative mode conditions.

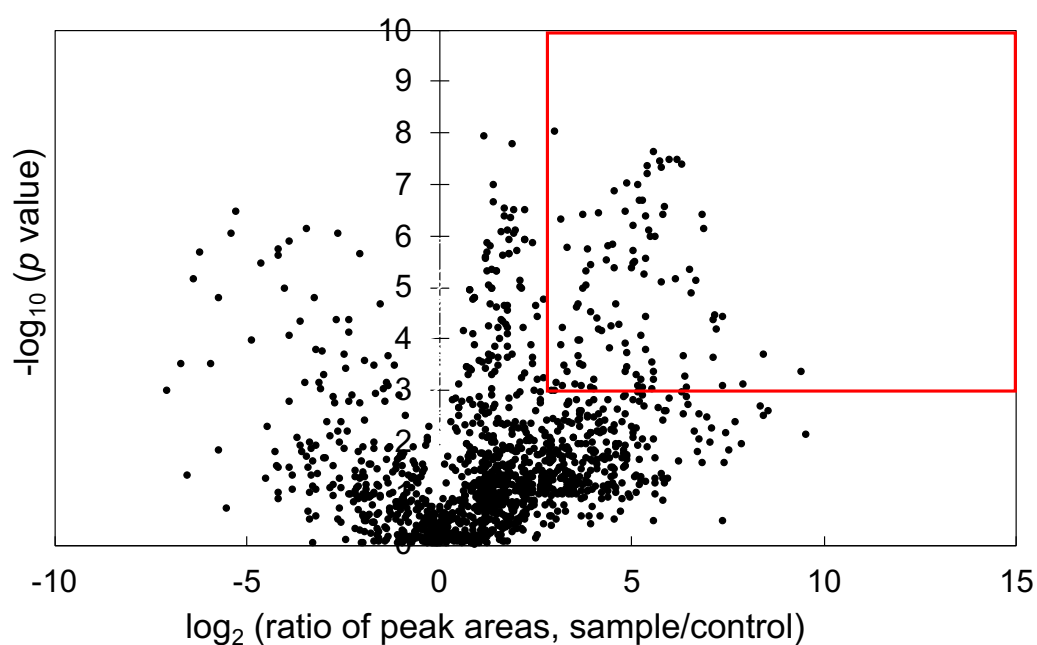


Figure 4.17. Volcano plot of the pretreated *G. semen* cultures analyzed under APCI positive mode conditions.

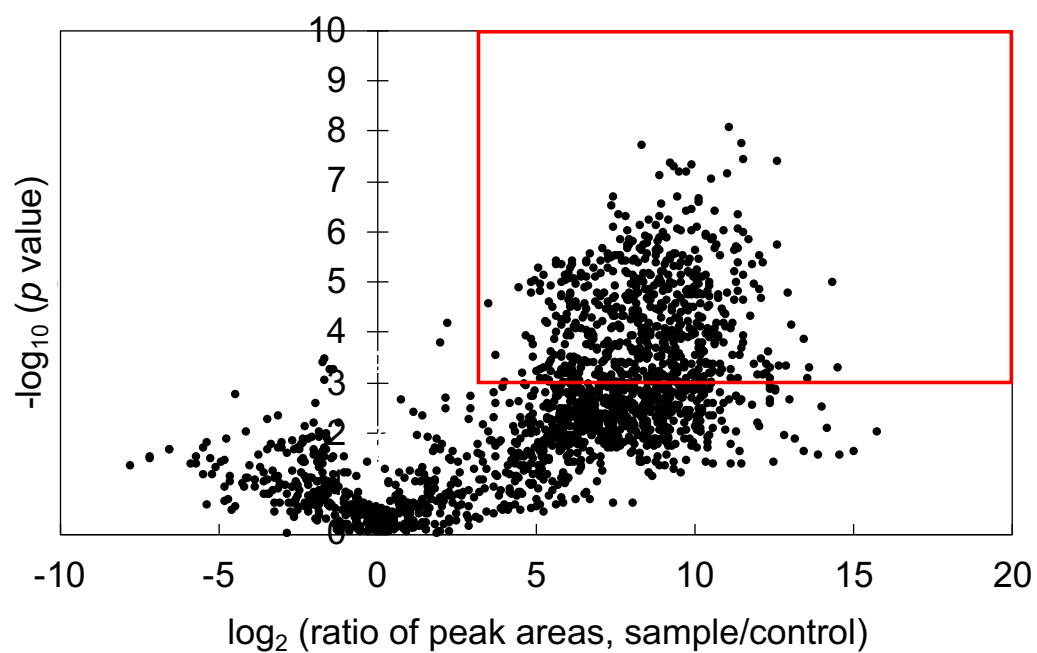


Figure 4.18. Volcano plot of the pretreated reservoir water analyzed under APCI negative mode conditions.

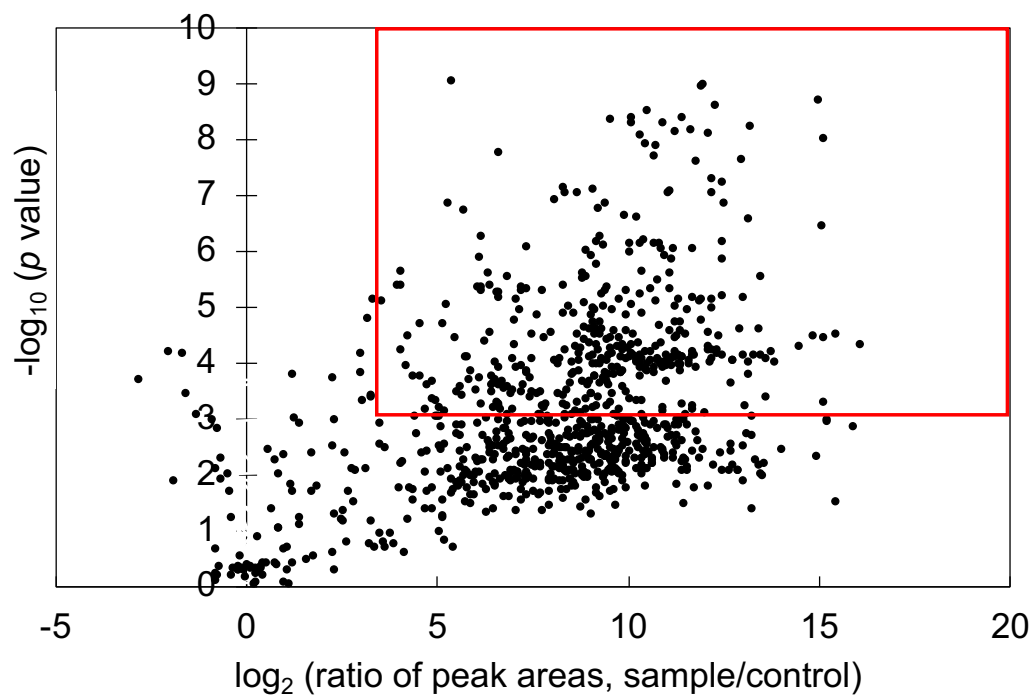


Figure 4.19. Volcano plot of the pretreated reservoir water analyzed under APCI positive mode conditions.

Table 4.4. Predicted molecular formula and m/z of detected fragment peaks from TCAA precursors.

Samples were measured using APCI negative mode.

Predicted molecular formula of substructures	m/z of detected peak
<i>G. semen</i> culture	
$C_{12}H_{18}O_{18}$	337.07809
$C_{12}H_{18}O_{10}$	321.08297
$C_6H_8O_6$	175.02414
$C_{11}H_{18}O_9$	293.08807
$C_9H_{16}O_9$	267.07237
$C_8H_{14}O_7$	221.06645
$C_{12}H_{18}O_8$	271.08257
$C_6H_{10}O_7$	193.03487
$C_{11}H_{18}O_8$	337.11426
$C_{12}H_{16}O_7$	271.08258
$C_{18}H_{24}O_{13}$	447.11486
$C_8H_{13}NO_7$	234.0618
$C_{12}H_{16}NO_{12}$	351.05715
Reservoir water	
$C_{12}H_{18}NO_{11}$	337.078
$C_6H_8NO_6$	175.02412
$C_6H_{10}NO_7$	193.03487
$C_{14}H_{23}NO_{10}$	365.13266
$C_{14}H_{23}NO_{10}$	364.12541
$C_{12}H_{18}O_8$	271.08262
$C_{18}H_{24}O_{13}$	447.11484
$C_{12}H_{14}O_9$	301.05708
$C_{12}H_{14}O_7$	269.06701
$C_{13}H_{16}O_{16}$	375.09381

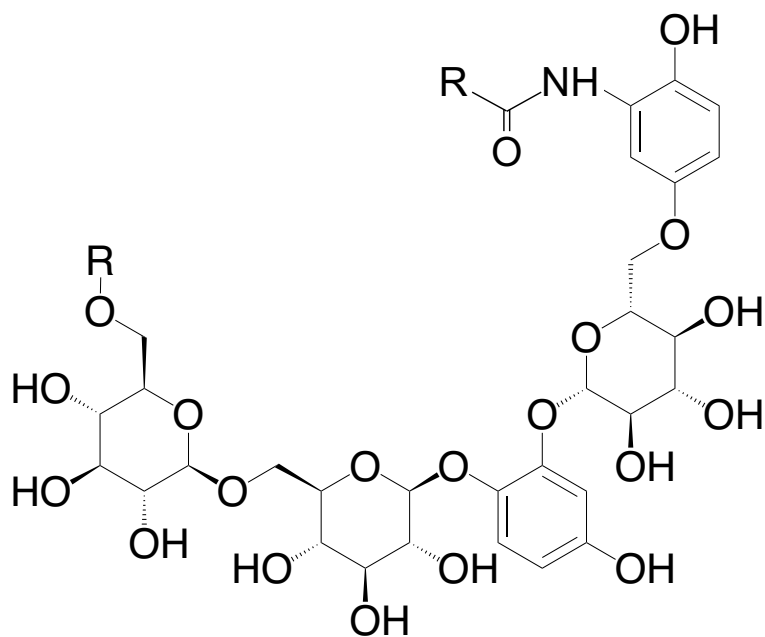


Figure 4.20. One of examples of structures of TCAA precursors originated from raphidophytes.

4.3.3 Comparison of HAAFPs of precursors from raphidophytes with model compounds containing identified substructures

High-molecular-weight TCAA precursors from raphidophytes produced high molar yields, generating up to 210 TCAA molecules per precursor during chlorination (see Table 4.5). These HAA yields were calculated from molecular weights (10–60 kDa), assuming that carbon constitutes 40–60% of the total mass (e.g., approximately 40% for sugars and 77% for phenols). This indicates that a single precursor molecule contains multiple substructures capable of forming TCAA. Furthermore, interpretable NMR spectra suggest the presence of multiple identical or similar substructures at different locations within the molecule. Together, these findings support the hypothesis that macromolecular TCAA precursors are composed of relatively simple subunits arranged in combination. To investigate potential building blocks of these precursors, HAAFP data were compiled from the literature, and commercially available low-molecular-weight compounds containing the identified substructures were tested. The TCAAFPs of these model compounds are shown in Table 4.5, with DCAAFPs and their ratios also provided as supporting information.

Phenol and glucose were selected as basic compounds. High TCAAFPs were observed for phenol but not for glucose, consistent with the results of previous studies.^{101, 116, 123} The TCAAFP of phenol was high (620 $\mu\text{g}/\text{mg C}$), whereas the precursors from *G. semen* cultures had approximately 1.5 times higher TCAAFPs. Additionally, maltose showed no HAAFPs, which is consistent with the lack of HAAFPs for glucose, suggesting that glycan substructures are not directly involved in TCAA formation during chlorination. Moreover, arbutin (glycosylated hydroquinone) showed TCAAFP as gram of carbon (300 $\mu\text{g}/\text{mg C}$) that was approximately half that of phenol, but on a molar basis, its TCAAFP was nearly identical to that of phenol (i.e., 26–27%). This indicates that the glucosic moiety at the para-position of phenol neither contributed to the TCAA formation during chlorination and nor altered the chlorination behavior of the phenolic substructure. Phloridzin had higher TCAAFP (540 $\mu\text{g}/\text{mg C}$) than arbutin. Additionally, the yields of TCAAFP of phloridzin and arbutin are 83% and 26% respectively. The TCAAFP yield of phloridzin was much higher than expected, considering the phenol-to-sugar ratio (i.e., $26\% \times 2 = 52\%$). This was likely due to the benzentriols substructures in the phloretin moiety.¹¹⁶ These results indicate that the HAA formation characteristics of components other than the glycan moieties in the TCAA precursors (e.g., phenolic functional groups) contribute to the overall TCAA formation of the molecule.

According to a database of TCAAFPs for 283 target substances,¹²⁴ only 13 nonhalogenated compounds, mostly methoxyphenols and benzentriols, exhibited higher TCAAFPs than phenols. Multiple electron-donating substituents, such as hydroxy, methoxy, and ether functional groups, are necessary for high TCAAFPs. Thus, model phenolic substances containing these functional groups were investigated. The TCAAFP and its yield of 3,4,5-trimethoxyphenol (890 $\mu\text{g}/\text{mg C}$, 59%) was higher than that of phenol. The TCAAFP was comparable to those of TCAA precursors from *G. semen*. The same trend was observed for DCAAFP, and TCAAFP/DCAAFP ratio was similar to that of the TCAA precursors from raphidophytes. According to Echigo et al.,¹⁰⁴ among methoxyphenols, 2-methoxyphenol and 3,5-dimethoxyphenol exhibited high TCAAFPs at 590 and 1200 $\mu\text{g}/\text{mg C}$, respectively, while the FPs of other forms were relatively lower. This suggests that at least two electron-donating functional groups must be substituted at the meta-position of phenol to achieve a TCAAFP higher than that of phenol. In contrast, ace-

tosyryngone had a low TCAAFP (20 $\mu\text{g}/\text{mg C}$) due to the substitution of chlorine-reactive sites on the phenolic moieties. Similarly, Gomisin K3 had undetectable HAAFPs because its highly substituted phenolic structure prevented interaction with chlorine. Tannic acid, which contains several gallic acids bound to a central sugar, showed a TCAAFP of 50 $\mu\text{g}/\text{mg C}$ and a DCAAFP of 100 $\mu\text{g}/\text{mg C}$, but prolonged reaction time may promote TCAA formation.¹²⁵ Sennoid B showed no HAAFPs because its phenolic groups were bound to electron-withdrawing carboxylic or ketone groups, which hindered the ability of chlorine to interact with the phenolic groups effectively. The presence of electron-donating functional groups, such as a methoxy group at the meta-position of phenol, increased the TCAAFP. In contrast, for phenols with several other electron-withdrawing functional groups, a decrease in TCAAFP was observed. Considering the above, the substructures in the TCAA precursors from raphidophytes, which exhibited a TCAAFP larger than that of phenol, were plausibly phenolic functional group was further substituted with electron-donating functional groups at the proper positions.

Table 4.5. TCAAFPs, DCAAFPs, and their ratios of model compounds with the proposed substructures as TCAA precursors from raphidophytes.

* means less than quantification limit (3 $\mu\text{g/L}$). *Experimental conditions of the HAAFP test:* pH 7 (5 mM phosphate buffer); sample concentration, 1 mg C/L; Cl_2 dose, 30 mg/L; temperature, 20 $^\circ\text{C}$; and reaction time, 24 h in dark.

Name of compounds	TCAAFP ($\mu\text{g/mg C}$)	TCAAFP (% mol-TCAA/ mol-precursor)	DCAAFP ($\mu\text{g/mg C}$)	DCAAFP (% mol-DCAA/ mol-precursor)	TCAAFP/ DCAAFP ($\mu\text{g}/\mu\text{g}$)	TCAAFP/ DCAAFP (mol/mol)
11–13 min fraction from preparative HPLC fractionation from <i>G. semen</i> culture	960	2400–21000	140	430–3900	7.1	5.6
phenol	620	27	15	0.82	43	34
glucose	<LOQ*	–	<LOQ*	–	–	–
maltose	<LOQ*	–	<LOQ*	–	–	–
arbutin	300	26	13	1.4	23	18
phloridzin	540	83	11	2.2	49	37
3,4,5-trimethoxyphenol	890	59	98	8.2	9.1	7.2
acetosyringone	20	1.5	62	5.8	0.32	0.26
Gomisin K3 (schisanhenol)	<LOQ*	–	<LOQ*	–	–	–
tannic acid	50	29	100	74	0.50	0.39
sennoside B	<LOQ*	–	<LOQ*	–	–	–

4.4 Summary

This chapter represents the first attempt to elucidate the chemical structures of unique TCAA precursors in raphidophyte cultures and environmental waters. By combining a newly developed isolation method (see Chapter 3) with measurements of water quality parameters, protein, and carbohydrate, as well as UV absorbance, EEM, NMR, FT-IR, and HRMS analyses, it was revealed that the TCAA precursors contain phenolic and glycan functional groups.

High-molecular-weight TCAA precursors produced exceptionally high molar yields of TCAA, generating up to 210 TCAA molecules per precursor during chlorination, demonstrating their strong DBP-forming potential. In addition, NMR spectra further indicate the presence of multiple identical or similar substructures arranged throughout the molecules, supporting the idea that macromolecular precursors are composed of relatively simple building blocks. Comparison with model compounds suggests that the phenolic groups in these precursors carry electron-donating substituents, such as methoxy groups, which likely contribute to their high ability to form TCAA.

Overall, although the complete molecular structures remain unresolved, the insights gained in this chapter provide a foundation for understanding the formation mechanisms of TCAA from raphidophytes. These findings also set the stage for the next chapter, which investigates the treatability of these precursors and explores optimal strategies for their removal.

Chapter 5

Treatability of High-molecular-weight and Ultra-hydrophilic Trichloroacetic Acid Precursors Originating from Freshwater Raphidophytes in Drinking Water Treatment

5.1 Introduction

This chapter presents the treatability of TCAA precursors originating from raphidophytes. The reduction of TCAAFPs of the precursors in water treatment was evaluated for both lab-cultured raphidophytes and natural samples collected during a raphidophyte bloom to identify effective control methods and their conditions. Four treatments were tested, both individually and in combination: coagulation and sedimentation, powdered activated carbon (PAC) adsorption, pre-chlorination followed by PAC adsorption, and ozonation. Co-

The results of the present chapter are based on the following peer-reviewed publication: Tada, Y., Tsujisaka, Y., Hinneh, K. D., Asada, Y., Kosaka, K., Echigo, S., and Itoh, S. (2025). Treatability of high-molecular-weight and ultra-hydrophilic haloacetic acid precursors from freshwater raphidophytes in water treatment. *Total Environment Engineering*, 2, 100012. <https://doi.org/10.1016/j.teengi.2025.100012>

agulation and sedimentation treatments were included because they are commonly used in water treatment plants that rely on surface water. PAC adsorption was chosen for its proven effectiveness and ease of implementation. Pre-chlorination was selected for its potential to enhance PAC adsorption.¹²⁶ Ozonation was selected due to its frequent use in water treatment and expected ability to remove TCAA precursors as they are likely aromatic compounds (see Chapter 3).

5.2 Materials and methods

5.2.1 Reagents and solutions

Unless otherwise noted, all chemicals used in the experiments were purchased from Fuji-film Wako Pure Chemical Corporation (Osaka, Japan). PACl (PAC250AD) was obtained from Taki Chemical Corporation (Hyogo, Japan). Potassium indigotrisulfonate was purchased from the Tokyo Chemical Industry Corporation (Tokyo, Japan). PACl aqueous stock solution was prepared at 1 g/L (0.6044 g Al₂O₃/L). HAA and DOC working standard solutions were prepared in ultrapure water within one week and two days before the measurements, respectively.

5.2.2 Materials

Raphidophyte (*G. semen*) cultures and a reservoir water sample collected in Muro Dam (Nara Prefecture, Japan), which contained high concentrations of raphidophytes, were also used in this chapter, as in Chapters 3 and 4.

Isolation methods of TCAA precursors from *G. semen* cultures was the same as that developed in Chapter 3. Note that the load effluent from Oasis MCX cartridges was used in this study. The solution isolated from *G. semen* cultures, which contains a high concentration of TCAA precursors, is hereinafter referred to as “TCAA precursor isolates.” The TCAA precursor isolates were diluted with either environmental water (Lake Biwa water) or ultrapure water to increase the DOC concentration by 0.1 or 1 mg C/L. During an abnormal raphidophyte bloom (3,200 cells/mL) observed in the fall of 2017 in Muro Dam, the TCAAFP in raw water increased dramatically from several tens of µg/L to 570 µg/L

(Table 3.11). This increase in TCAAFP corresponds to approximately 1 mg C/L increase in precursor concentration, as calculated. To simulate this abnormal bloom, 1 mg C/L of the pretreated samples was added. In addition, 0.1 mg C/L was applied to evaluate treatability at lower concentrations, representing a smaller algal bloom. Lake Biwa water was used to evaluate the effect of the water matrix on the treatability of TCAA precursors, because it is widely used as a drinking water source for neighboring Kansai areas in Japan, and raphidophyte growth has previously been reported in Lake Biwa.⁹⁶ Lake Biwa water was intermittently collected between 2021 and 2023, immediately filtered through a GF/F filter (Cytiva Whatman, Marlborough, USA), and stored at 4°C under refrigeration. Samples used for coagulation and sedimentation treatments were not filtered. The HAAFP values and DOC of TCAA precursor isolates and Lake Biwa water are summarized in Table 5.1. Note that since different *G. semen* cultures were separately prepared for the treatability experiments, the DCAAFP and TCAAFP values are shown as ranges and differ from those presented in Table 3.12.

The reservoir water used for the coagulation and sedimentation experiments was collected and immediately transported to the laboratory for use without filtration. Waters used for other treatments were filtered immediately after collection and stored in the dark at 4°C. The HAAFP values, cell population, and DOC of the reservoir water are summarized in Table 3.11.

Table 5.1. HAAFPs and DOC of the pretreated *G. semen* cultures.

	MCAAFP ($\mu\text{g}/\text{mg C}$)	DCAAFP ($\mu\text{g}/\text{mg C}$)	TCAAFP ($\mu\text{g}/\text{mg C}$)	DOC (mg C/L)
TCAA precursor isolates	N.D. ^a	79–200	470–640	–
Lake Biwa water	N.D. ^a	11–16	17–22	1.1–1.7

^a “N.D.” means not detected.

5.2.3 HAAFP in chlorination

A 10-mL amber glass vial was used for chlorination to prevent photolysis of dissolved substances in the sample. A magnetic stirring bar, 4 mL of the sample, and phosphate buffer solution (PBS, final concentration: 5 mM, pH 7) were added to the vial. Samples containing TCAA precursors isolates were used without dilution, whereas reservoir water samples were diluted 10-fold because of their high DOC concentrations (Table 3.11). An

excess amount of sodium hypochlorite solution, serving as the chlorine source, was added to the samples at 3 or 30 mg Cl_2/L (Cl_2/DOC weight ratio ≥ 10) to ensure sufficient residual chlorine ($> 1 \text{ mg } \text{Cl}_2/\text{L}$) after the reaction. The mixture was stirred with a magnetic stirrer for 20 s, and the vials were kept in the dark at room temperature (20–25 °C) to allow the reaction to proceed. After 24 h, an ammonium chloride solution (final concentration: 50 mg N/L) was added to quench the chlorine reaction. HAA concentrations were measured using LC-MS/MS. Analytical method was the same as shown in Table 3.8 in Chapter 3. Chlorinated ultrapure water was used as a blank, and HAAFP was calculated by subtracting its HAA value from that of the samples.

5.2.4 Treatment of TCAA precursors from raphidophytes

Initially, the treatability of TCAA precursors from *G. semen* cultures with and without real environmental water was evaluated using four treatments: coagulation and sedimentation, PAC adsorption, ozonation, and pre-chlorination followed by PAC adsorption. These methods were then applied to reservoir water containing wild raphidophytes. Finally, the treatability of the TCAA precursors in the reservoir water was compared across three continuous treatment scenarios. Each operation was repeated at least twice to ensure reproducibility. In the blank test, where ultrapure water underwent the same treatment as the sample, no chlorine consumption or HAAFP were detected in the post-treatment water. This confirmed that the change in the HAAFP of the samples resulted from the removal of the TCAA precursors by the treatment operation.

5.2.4.1 Coagulation and sedimentation

PACl was used as a coagulant because it is commonly used in drinking water treatment plants worldwide. A jar test was conducted using a jar tester (JMD-6E, Miyamoto Riken Industries Co., Ltd., Osaka, Japan). A 1 L sample was transferred to a 1-L glass beaker and placed in the jar tester. PACl was added while stirring at 150 rpm. Following the operational guidelines of the JWWA,¹²⁷ the sequence of operational conditions was set as follows: rapid stirring at 150 rpm for 5 min, slow stirring at 40 rpm for 30 min, and settling for 30 min. The PACl dosages were 3.0, 6.0, and 12 mg $\text{Al}_2\text{O}_3/\text{L}$ for the cul-

ture samples, and 15, 30, and 45 mg Al₂O₃/L for the reservoir water to ensure sufficient turbidity reduction. No pH adjustment was made because the pH remained stable at approximately 7 before and after treatment. The supernatant was filtered through a 0.2 µm HPTFE membrane (Advantec, Tokyo, Japan) and tested for HAAFP. Turbidity and pH were measured before and after treatment.

5.2.4.2 PAC adsorption

Two types of wood-based PAC were used, 5LPD (Swing Corporation, Tokyo, Japan) and Norit SA Super (Norit, Amersfoort, The Netherlands). The properties and pore-size distributions of both PACs are listed in Table 5.2. 5LPD was selected because it has been implemented in several water treatment plants in Japan. It is characterized by a high proportion of small pores, with 83% microporosity (0.3–2 nm). Norit SA super, on the other hand, was chosen for its higher percentage of mesopores (2–50 nm), comprising 53% of the total pore volume, making it suitable for adsorbing both low- and high-molecular-weight compounds. It was surmised to be particularly effective in removing high-molecular-weight TCAA precursors from raphidophytes. PAC was dried at 110 °C for more than 3 h and stored in a desiccator until use. First, treatability under prolonged exposure and high PAC dose conditions was investigated using TCAA precursor isolates from *G. semen* cultures to select the PAC suitable for removal of TCAA precursors. All adsorption experiments were conducted in batches, with 100 mL of the sample added to a 100-mL beaker. The operating conditions were as follows: pH, 7 (0.2 mM PBS); PAC dose, 100 mg/L; temperature, 25 °C; contact time, 24 h; in the dark with gentle stirring. After treatment, the samples were filtered through a 0.2 µm HPTFE membrane and tested for HAAFP.

As only Norit SA Super reduced HAAFP, subsequent experiments were conducted using this PAC at doses of 0, 5, 10, 15, 30, and 100 mg/L with a contact time of 1 h. Direct and accurate weighing of PAC at doses between 5.0 and 30 mg/L was challenging. Therefore, a PAC slurry in ultrapure water was prepared at approximately 1 g/L in a 100-mL beaker and the required amount of PAC was extracted from the slurry while stirring.

Table 5.2. Properties of 5LPD and Norit SA Super.

	Material	Particle size (μm)	Micropore (0.3–2 nm) (cm^3/g)	Mesopore (2–50 nm) (cm^3/g)	Macropore (> 50 nm) (cm^3/g)	BET specific surface area (m^2/g)
5LPD	Wood-based	15	0.47 (83%)	0.08 (14%)	0.02 (3%)	1163
Norit SA Super	Wood-based	9	0.4 (35%)	0.6 (53%)	0.14 (12%)	955

5.2.4.3 Pre-chlorination followed by PAC adsorption

To determine whether the pre-chlorination treatment enhanced PAC adsorption, pre-chlorination and PAC adsorption treatments were performed sequentially. Samples (100 mL) containing 0.1 mg C/L of TCAA precursor isolates from *G. semen* cultures were transferred into 200 mL conical flasks, and the pH was adjusted to 7 using 0.2 mM PBS. Pre-chlorination was performed by adding a sodium hypochlorite solution. Chlorine doses were set to 0.50, 1.0, 2.0, and 3.0 mg Cl_2/L , based on practical feasibility in water treatment operations and to avoid excessive DBP formation. As a reduction in HAAFP was observed when the chlorine dose was equal to or exceeded the chlorine consumption of TCAA precursor isolates, the chlorine dose for reservoir water was increased to 17, 18, 19, and 20 mg Cl_2/L (chlorine consumption: 16.3 mg Cl_2/L). The reaction time was set to 1 h. After pre-oxidation, 15 mg/L 5LPD or Norit SA Super was immediately added, and the mixture was stirred in the dark for 1 h. The samples were then filtered through a 0.2 μm HPTFE membrane, and the HAAFP test was conducted.

5.2.4.4 Ozonation

Owing to differences in sample volumes, batch ozonation was used for *G. semen* culture, whereas semi-batch ozonation was applied to the reservoir water samples. The conditions for *G. semen* culture samples were as follows: sample volume, 20 mL; pH, 7 (5 mM PBS); initial ozone dose, 0.50, 1.0, and 2.0 mg O_3/L ; reaction time, 24 h. Ozone injection was performed by adding an ozone stock solution (10–20 mg O_3/L). The stock solution was prepared immediately before the treatment by bubbling ozone gas (approximately 40 mg O_3/L in gas phase) into ultrapure water for more than 60 min at the gas flow rate of 1.0 L/min. Ozone gas was generated from high-purity oxygen gas (99.995% or higher, Kyoto

Teisan Co., Ltd, Kyoto, Japan) by an ozone generator (AZH-3S, Hamamatsu Vegetable, Shizuoka, Japan). The concentration of the dissolved ozone in the stock solution was determined using the indigo method.⁹⁹.

For the reservoir water, 200 mL of the sample was transferred into a gas-washing bottle with plate filter (pore size; 100–120 μm). PBS (5 mM) was added to adjust the pH to 7. Ozone was continuously injected using ozone gas generated by an ozone generator, in accordance with the dosing method employed in actual water treatment plants. The injection time was fixed at 10 min, and the ozone gas flow rate and concentration in the gas phase were adjusted to achieve the target ozone dose. The ozone doses were calculated as the product of the ozone gas flow rate, the ozone concentration in the gas phase, and the injection time, divided by the sample volume. The amount of ozone dose was 0.50, 1.0, 2.0, 4.2, 8.3, and 16.6 mg O_3/L (i.e., 0.06, 0.12, 0.24, 0.50, 1.0, and 2.0 mg/mg DOC). After ozonation, the samples were kept in the dark at room temperature (20–25 $^{\circ}\text{C}$) for 24 h to completely decompose the residual ozone. Subsequently, HAAFP test was performed.

5.2.4.5 Combination of multiple unit operations

Three scenarios were compared to evaluate the treatability of TCAA precursors using a combination of multiple unit operations. The first scenario involved pre-chlorination, followed by PAC adsorption (Norit SA Super), coagulation, sedimentation, and ozonation. The second scenario involved PAC adsorption, followed by coagulation, sedimentation, and ozonation. The third scenario involved coagulation, sedimentation, and ozonation. In the first scenario, 1 L of reservoir water was transferred into a 1-L beaker, sodium hypochlorite solution was added at 19 mg Cl_2/L . The mixture was then stirred in the dark for 1 h. Next, Norit SA Super was added at 15 mg/L, and the beaker was kept in the dark with continuous stirring for another hour. The PAC dose and contact time were determined based on realistic conditions. The jar test was initiated by the addition of 15 mg $\text{Al}_2\text{O}_3/\text{L}$ PACl, followed by rapid stirring at 150 rpm for 5 min, slow stirring at 40 rpm for 30 min, and settling for 30 min. The PACl dose was set at 15 mg/L to achieve sufficient turbidity reduction (<1.0 turbidity units (TU)). Finally, the supernatant was subjected to ozonation in the semi-batch mode at a dose of 0.50 mg/L, based on realistic dosages. The

samples were incubated in the dark at room temperature (20–25 °C) for 24 h. The HAAFP test was conducted on the raw water and samples collected at each treatment stage. All other scenarios were tested using the same procedures.

5.2.5 Analytical methods

HAAs, DOC, pH method was the same as Chapters 3 and 4. Turbidity was measured using a turbidity meter (TR-55, Kasahara Chemical Instruments Corp., Saitama, Japan) with polystyrene standards, following the standard method outlined by the JWWA.⁹⁹ The turbidity determined by the formazin standard was calculated using the relationship shown in Figure 5.1.

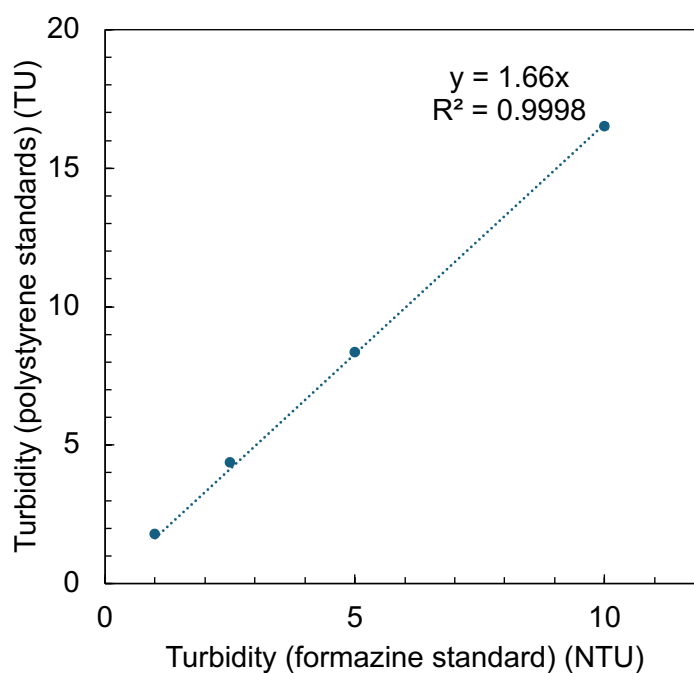


Figure 5.1. Relationship of turbidity between different standards.

5.3 Results and discussion

5.3.1 Treatability of TCAA precursors isolated from *G. semen* cultures by individual unit operations

5.3.1.1 Coagulation and sedimentation

Table 5.4 presents the TCAAFPs, DCAAFPs, pH, and turbidity of the samples before and after coagulation and sedimentation treatments. In Japanese water quality standards, turbidity is quantified using polystyrene standard solutions (expressed in turbidity units, TU), whereas the USEPA surface water treatment rules and the WHO guidelines use formazin standard solutions (expressed in nephelometric turbidity units, NTU). Turbidity data are presented in both units to readers from different regions. Experiments were conducted on different days using samples containing 0.1 or 1 mg C/L of TCAA precursor isolates from *G. semen* cultures. Consequently, the data for the raw water samples (Lake Biwa water) are presented separately. Significant reductions in turbidity and HAAFP were observed after treatment under conditions typically used by a water treatment plant purifying Lake Biwa water (i.e., no pH adjustment and a PACl dose of 3 mg Al₂O₃/L). Using these conditions as a reference, coagulation and sedimentation treatments were performed with varying PACl doses to evaluate the treatability of TCAA precursors spiked into environmental water.

The pH remained close to neutral under all conditions. Although turbidity decreased in all samples after treatment, the reduction was less pronounced than that in raw water. In the sample containing 0.1 mg C/L of TCAA precursor isolates, simulating a smaller algal occurrence compared to the previous bloom in Japan,^{12,13} turbidity was reduced to 0.87 TU (0.52 NTU) at a PACl dose of 3 mg Al₂O₃/L, slightly higher than that in the raw water, suggesting that TCAA precursors inhibited flocculation. Increasing the dose to 6 mg Al₂O₃/L and 12 mg Al₂O₃/L further reduced turbidity to 0.24 (0.14) and 0.17 TU (0.10 NTU), respectively. In the sample with 1 mg C/L TCAA precursor isolates, PACl doses of 3 and 6 mg Al₂O₃/L did not result in sufficient floc formation, which led to inadequate treatment. However, a dose of 12 mg/L achieved turbidity reduction to 0.09 TU (0.05 NTU) comparable to that of the treated raw water. Takaara et al. demonstrated that

Table 5.3. TCAAFP, DCAAFP, pH, and turbidity of the samples before and after coagulation and sedimentation treatment.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); Cl₂ dose, 3 or 30 mg Cl₂/L; temperature, 20 °C; and reaction time, 24 h in dark.

(A)	PAC dose (mg/L)	TCAAFP (μg/L)	DCAAFP (μg/L)	pH	Turbidity (polystyrene standard) (TU)	Turbidity (formazine standard) (NTU)
Raw water	before treatment	22	15	7.7	3.8	2.3
	3	12	8.8	7.3	0.14	0.08
Raw water + 0.1 mg/L of TCAA precursor isolates	before treatment	50	16	7.1	4.3	2.6
	3	17	2.5	7.0	0.87	0.52
	6	16	1.1	7.0	0.24	0.14
	12	9.7	< LOQ	7.1	0.17	0.10

(B)	PAC dose (mg/L)	TCAAFP (μg/L)	DCAAFP (μg/L)	pH	Turbidity (polystyrene standard) (TU)	Turbidity (formazine standard) (NTU)
Raw water	before treatment	18	11	7.7	7.8	4.7
	3	12	8.8	7.3	0.21	0.13
Raw water + 1 mg/L of TCAA precursor isolates	before treatment	550	87	7.3	8.9	5.4
	3	77	18	7.2	4.6	2.8
	6	20	11	6.6	5.4	3.3
	12	8.2	8.5	6.6	0.09	0.05

negatively charged hydrophilic substances from cyanobacteria, such as liposaccharides, inhibited floc formation by interacting with PACl, which prevents its hydrolysis.¹²⁸ The TCAA precursors, which contains acidic functional groups and glycan moieties, might have similarly inhibited aggregation.

The HAAFPs decreased under all conditions after treatment, with greater reductions observed as the PACl doses increased. In the sample containing 0.1 mg C/L of TCAA precursor isolates, HAAFPs fell below the Japanese tap water standards for TCAA and DCAA (30 $\mu\text{g/L}$ each) and the USEPA MCL for HAA5 (60 $\mu\text{g/L}$, defined here as the sum of DCAA and TCAA) at a PACl dose of 3 mg $\text{Al}_2\text{O}_3/\text{L}$. In the sample with 1 mg C/L of TCAA precursors, although turbidity was not sufficiently reduced at PACl doses below 6 mg $\text{Al}_2\text{O}_3/\text{L}$, a significant reduction in TCAAFP was observed. At a PACl dose of 3 mg $\text{Al}_2\text{O}_3/\text{L}$ TCAAFP decreased by 86%, from 550 to 77 $\mu\text{g/L}$, which is below the WHO guideline value for TCAA (200 $\mu\text{g/L}$). At 6 mg $\text{Al}_2\text{O}_3/\text{L}$, TCAAFP was further reduced by 96%, from 550 to 20 $\mu\text{g/L}$, which is below both the Japanese and USEPA drinking water standards. The efficient removal of TCAA precursors, even when the PACl doses were insufficient to reduce turbidity, suggested that PACl preferentially removed TCAA precursors. This indicates that sufficient control of turbidity in water treatment plants can simultaneously ensure the effective reduction of TCAA precursors derived from raphidophytes.

The positively charged PACl likely facilitated charge neutralization, leading to the aggregation of the negatively charged TCAA precursors. These results align with the fundamental principles of water treatment, which state that organic compounds with molecular weights above 1,500 Da are typically removable by coagulation.¹²⁹ Since the TCAA precursors in this study were identified as high-molecular-weight substances ($> 10 \text{ kDa}$), they were effectively aggregated even under low PACl doses.

5.3.1.2 PAC adsorption

Figure 5.2 shows the HAAFPs after PAC adsorption at a high dose (100 mg/L) for an extended contact time (24 h). Adsorption by 5 LPD reduced HAAFPs by only 2–12%, whereas Norit SA Super achieved a reduction of 43–53%. The higher efficacy of Norit SA Super was attributed to its larger mesopore volume (Table 5.2), which is more ef-

fective in adsorbing high-molecular-weight TCAA precursors from raphidophytes. In addition, the surface chemical properties of Norit SA Super may be more favorable for electrostatic adsorption. Since both the surface of the Norit SA Super and the TCAA precursors are negatively charged at neutral pH,¹³⁰ electrostatic adsorption between them is unlikely. However, numerous phenolic hydroxyl groups are present on the surface of Norit SA Super,¹³⁰ suggesting that the aromatic moieties of the TCAA precursors may adsorb onto PAC via π - π interactions. Future studies should focus on elucidating the surface properties and mechanisms underlying adsorption.

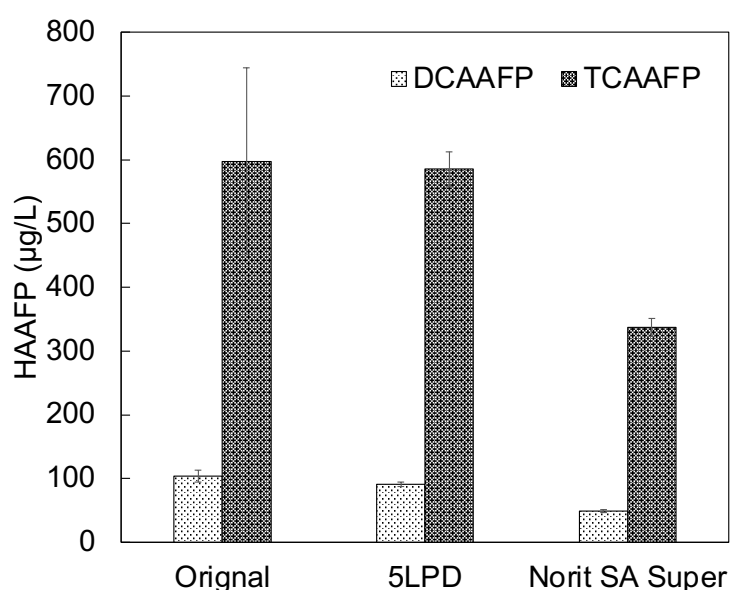


Figure 5.2. HAAFPs before and after PAC adsorption of samples containing 1 mg/L of TCAA precursors isolates.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); Cl_2 dose, 30 mg Cl_2/L ; temperature, 20 °C; and reaction time, 24 h in dark. Error bars represent standard deviation after triplet measurements.

Subsequent experiments to evaluate the treatability of TCAA precursors were conducted exclusively using Norit SA Super. Figure 5.3 (A) shows HAAFPs of samples containing 0.1 mg C/L of TCAA precursors diluted in ultrapure water after adsorption by Norit SA Super adsorption at PAC doses ranging from 0 to 100 mg/L for 1 h. Both TCAAFP and DCAAFP decreased linearly with increasing PAC doses. Linear approximation indicated that DCAAFP decreased by 0.093 $\mu\text{g/L}$ (0.65%) and TCAAFP by 0.33 $\mu\text{g/L}$ (0.65%) per unit of PAC dose (i.e., 1 mg/L). Although the absolute reduction amounts differed, the removal percentages (i.e, the ratios of FPs before and after

treatment) of DCAAFP and TCAAFP were similar. A PAC dose of approximately 55 mg/L was estimated to reduce TCAAFP to less than 30 $\mu\text{g/L}$, ensuring compliance with the Japanese standard. Additionally, it was estimated that HAA5 would remain below its MCL with the addition of approximately 30 mg/L PAC.

As shown in Figure 5.3 (B), the HAAFPs in the samples diluted with environmental water decreased linearly with increasing PAC dose. The trend lines indicated greater reductions in DCAAFP and TCAAFP, with reductions of 0.13 $\mu\text{g/L}$ (0.82%) and 0.41 $\mu\text{g/L}$ (0.85%) per unit PAC dose, respectively, compared to the samples diluted with ultrapure water. Two possible explanations for this enhanced adsorption are: (i) the adsorption of TCAA precursors onto NOM adsorbed on PAC was more effective than direct adsorption on the PAC surface, as NOM provides additional adsorption sites or modifies the surface characteristics of PAC, and (ii) the slightly increased ionic strength due to the addition of environmental water reduced electrostatic repulsion, thereby promoting adsorption. Reduced repulsion allows charged TCAA precursors to approach the adsorbent surface more closely, enhancing adsorption.¹³¹ The influence of increased ionic strength on adsorption characteristics will be investigated in future studies.

Figure 5.3 (C) and (D) show the HAAFPs of the samples containing 1 mg/L of the isolated TCAA precursors diluted in ultrapure or environmental water. In both cases, HAAFPs decreased linearly with increasing PAC dose, as well as in the samples with low concentrations of TCAA precursor isolates. The amount of TCAA precursors adsorbed increased with higher precursor concentrations, although the reduction rates were lower than those at lower concentrations. Additionally, the adsorption increased with the presence of coexisting materials. Similarly, the water matrix may have enhanced the adsorption of the TCAA precursors onto PAC.

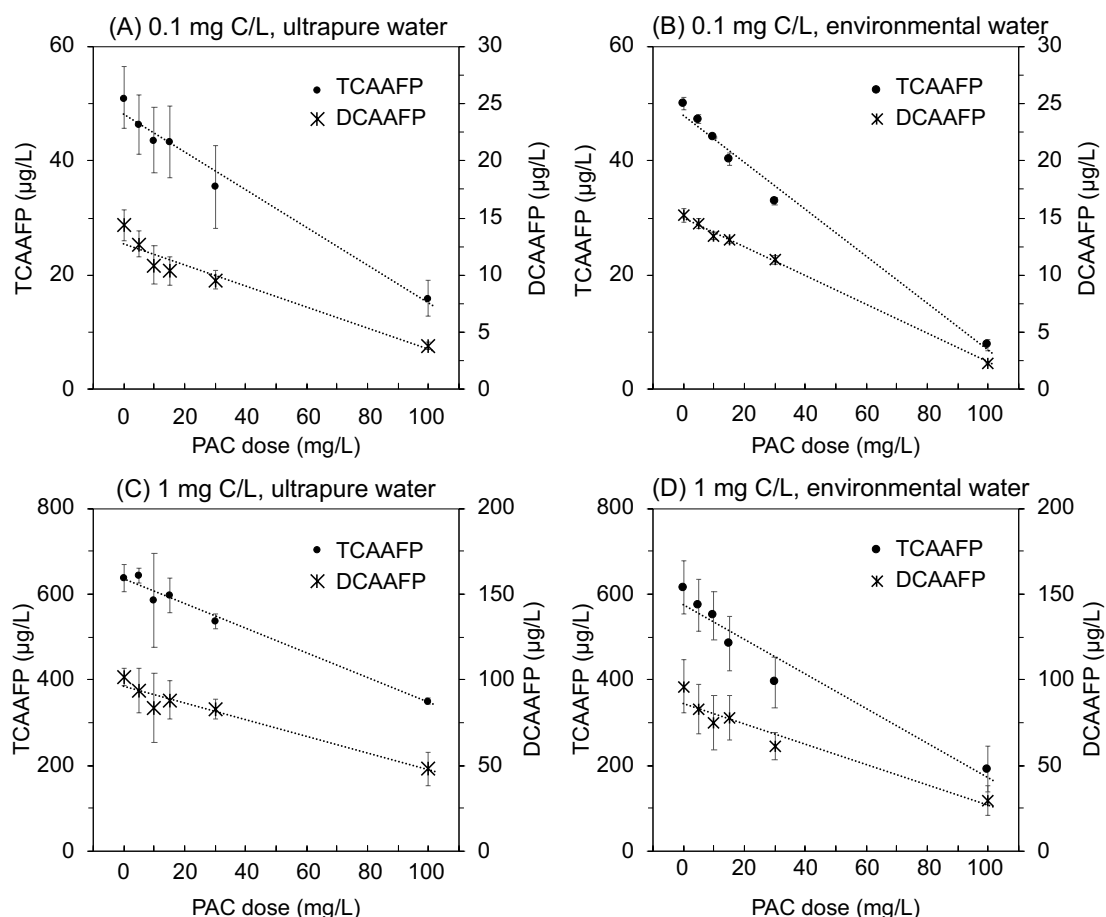


Figure 5.3. HAAFPs after PAC adsorption of samples containing (A) 0.1 mg C/L of TCAA precursor isolates, (B) 0.1 mg C/L of TCAA precursor isolates with environmental water, (C) 1 mg C/L of TCAA precursor isolates, and (D) 1 mg C/L of TCAA precursor isolates with environmental water.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); Cl_2 dose, 3 or 30 mg Cl_2/L ; temperature, 20 °C; and reaction time, 24 h in dark. Error bars represent the standard deviation of triplicate measurements or the minimum and maximum values from duplicate experiments. Trendlines were obtained by the least-squares method.

5.3.1.3 Pre-chlorination followed by PAC adsorption

Chlorine pre-oxidation is commonly used to prevent algae growth in water treatment plants. However, during a raphidophyte bloom in the source water, pre-chlorination was halted because of the risk of increased HAAs in the tap water.¹² Despite this, pre-chlorination may still be effective in the removal of TCAA precursors from raphidophytes. Jiang et al.¹²⁶ demonstrated that combining pre-chlorination with AC adsorption reduces DBP formation more efficiently than AC adsorption alone because aromatic intermedi-

ates formed by pre-chlorination are more adsorbed on AC than DBP precursors. Based on these findings, the effectiveness of combining pre-chlorination and PAC adsorption was investigated.

Figure 5.4 shows the HAAFPs of the TCAA precursor isolates, diluted in ultrapure or environmental water, after PAC adsorption (15 mg/L for 1 h) following pre-chlorination at doses of 0.5, 1.0, 2.0, and 3.0 mg Cl₂/L. HAAFPs includes HAAs produced during pre-chlorination as well as HAA produced during post-treatment chlorination. As shown in Figure 5.4 (A) (diluted in ultrapure, Norit SA Super), TCAAFP of samples after PAC adsorption following pre-chlorination at 0.5 mg Cl₂/L decreased by more than 62% compared to PAC adsorption alone, indicating that pre-chlorination enhanced the effectiveness of subsequent PAC adsorption. However, increasing the chlorine dose above 0.5 mg Cl₂/L resulted in higher TCAAFP levels. A similar trend was observed for 5LPD (Figure 5.4 (B)), where TCAAFP was minimized at 1.0 mg/L chlorine. Given that the chlorine consumption of the sample was 0.42 mg Cl₂/L, this suggests that a chlorine dose equal to or slightly higher than the chlorine consumption enhances PAC adsorption. However, excessive chlorine led to a greater formation of TCAA, as TCAA intermediates decomposed into TCAA, which could not be removed by PAC adsorption.

On the other hand, DCAAFP remained constant regardless of the chlorine dose. The different trends in DCAA and TCAA may be due to their different formation mechanisms. Pre-chlorination transforms the high-molecular-weight TCAA precursors from raphidophytes into lower-molecular-weight compounds, potentially making them more easily adsorbed by PAC. Intermediates, such as chlorophenols, which play a role in the conversion of TCAA precursors to TCAA, have been shown to be adsorbed.¹²⁶ However, when chlorine is added in excess, these intermediates are likely fully converted to TCAA, which is highly hydrophilic and thus less prone to PAC adsorption. Additionally, the main precursors of DCAA are aliphatic compounds,¹⁰⁶ which differ from those of TCAA in their formation characteristics. In fact, the DCAAFP of chlorophenols is low ($\leq 13 \mu\text{g}/\text{mg C}$).¹⁰¹ The concentrations of DCAA-producing intermediates adsorbed were likely unchanged from the original DCAA precursors before pre-chlorination.

Figures 5.4 (C) and (D) show the HAAFPs of precursor isolates diluted in environmental water. As noted in the previous section, environmental water enhanced the TCAA

precursor adsorption on PAC. Pre-chlorination increased the adsorption of TCAA precursors on PAC, although the reduction in TCAAFPs was smaller than that in the samples diluted in ultrapure water. However, the DCAAFPs after the combination of pre-chlorination and PAC adsorption were higher than those after PAC adsorption alone. This was observed only in samples diluted in environmental water, indicating that the DCAA precursors in the environmental water were responsible. It was possibly because other TCAA precursors present in environmental water reacted with chlorine, and the formed intermediates were not efficiently adsorbed on PAC. Although the optimal chlorine dose for reducing TCAAFP was found to potentially increase DCAAFP, this effect was not significant with the TCAA precursor concentration in this experiment. While several considerations should be considered when determining the optimal pre-chlorination conditions, the combination of pre-chlorination and PAC adsorption has been shown to be an effective treatment option for the suppression of HAA formation from raphidophytes.

5.3.1.4 Ozonation

Figure 5.5 (A) shows DCAAFPs and TCAAFPs of samples containing 0.1 mg C/L of TCAA precursor isolates diluted in ultrapure waters after ozonation. DCAAFP decreased to below the LOQ ($<3 \mu\text{g/L}$) at all ozone dose (i.e., 0.5–2 mg/L or 5–20 mg/mg C), and TCAAFP decreased from 51 to 16 $\mu\text{g/L}$ at an ozone dose of 0.50 mg/L, which were below the HAA standard values set by the US and Japan for drinking water. At an ozone dose of 1.0 mg/L, the concentration further decreased to 3.1 $\mu\text{g/L}$, with no additional reduction observed at higher doses. The TCAA precursors contain phenolic moieties attached to a glycan backbone. The phenolic moieties have been shown the primary contributors to TCAA formation,¹⁰¹ while DCAA formation may originate from other functional groups, such as β -diketone substructures. Ozone reacts rapidly with unsaturated bonds which leads to the cleavage of aromatic moieties,¹³² and such degradation of phenolic moieties likely caused the significant decrease in TCAAFP. Additionally, aliphatic compounds, particularly those with a β -diketone substructure, a key precursor of DCAA, have been also reported to be highly reactive with ozone.¹³³ The breakdown of these substructures in TCAA precursors likely contributed to the substantial reduction in DCAAFP.

Figure 5.5 (B) illustrates HAAFPs after ozonation of samples containing 0.1 mg C/L

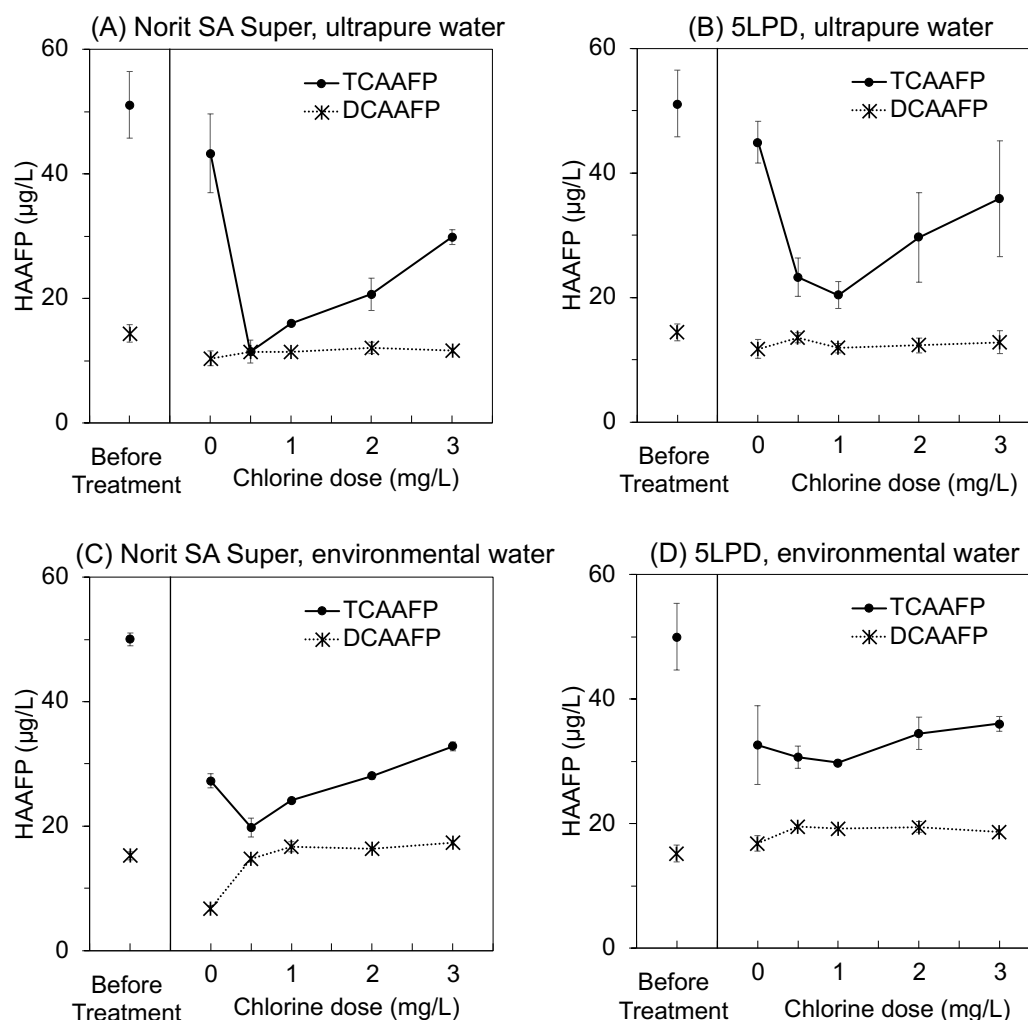


Figure 5.4. HAAFPs after pre-chlorination followed by PAC adsorption of samples containing 0.1 mg C/L of TCAA precursor isolates. (A) Norit SA Super and (B) 5LPD adsorption was conducted samples diluted in ultrapure water, and (C) Norit SA Super and (D) 5LPD was conducted samples diluted in environmental water.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); Cl_2 dose, 3 or 30 mg Cl_2/L ; temperature, 20 °C; and reaction time, 24 h in dark. Error bars represent the standard deviation of triplicate measurements or the minimum and maximum values from duplicate experiments.

of TCAA precursor isolates diluted in environmental water. DCAAFP decreased significantly from 15 to 9.0 $\mu\text{g/L}$ at an ozone dose of 0.50 mg/L and further decreased at higher ozone doses. TCAAFP followed a similar trend, dropping sharply from 50 to 20 $\mu\text{g/L}$ at 0.50 mg/L, then decreasing further to 13 and 8.8 $\mu\text{g/L}$ at 1.0 and 2.0 mg/L, respectively. The reduction in HAAFP was less pronounced in environmental water compared to ultrapure water. The reductions in the TCAAFPs of the samples diluted in ultrapure and environmental water after ozonation at an ozone dose of 1 mg/L were 94 and 74%, respectively. This difference was attributed to competition between TCAA precursors and coexisting substances for ozone, which reduced the amount of ozone available to react directly with the precursors. Assuming that all the ozone reacted with the solute, based on the differences in FP reductions, it was estimated that approximately 79% (i.e., $74/94 (\%/%) \times 100$) of the injected ozone reacted with the TCAA precursors, whereas 21% reacted with the coexisting substances. These findings suggest that TCAA precursors are more reactive with ozone than other compounds present in environmental water.

Figure 5.5 (C) and (D) show the HAAFP after ozonation at a sample concentrations of 1 mg C/L. At high sample concentrations, both DCAAFP and TCAAFP decreased linearly with increasing ozone doses, up to 2.0 mg/L. Similar to lower concentrations, competition with coexisting substances in environmental water reduced the effectiveness of TCAA precursor treatment. Notably, HAAFP could not be completely eliminated within a realistic ozone dose range, highlighting the need for additional treatment processes.

In summary, HAAFP reduction was observed across all treatments, including coagulation and sedimentation, PAC adsorption, pre-chlorination followed by PAC adsorption, and ozonation. The effectiveness of each treatment varied based on the initial TCAA precursor concentration and the presence of coexisting substances. Among them, coagulation-sedimentation achieved the highest HAAFP reduction (Table 5.4). However, at an optimal PACl dose for HAAFP reduction, turbidity removal may be inadequate, emphasizing the need to optimize conditions beforehand to achieve both HAAFP and turbidity reductions. While increasing ozone and PAC doses was expected to enhance treatability (Figures 5.3 and 5.5), their impact remained limited under realistic conditions. Additionally, pre-chlorination showed potential for improving PAC adsorption (Figure 5.4).

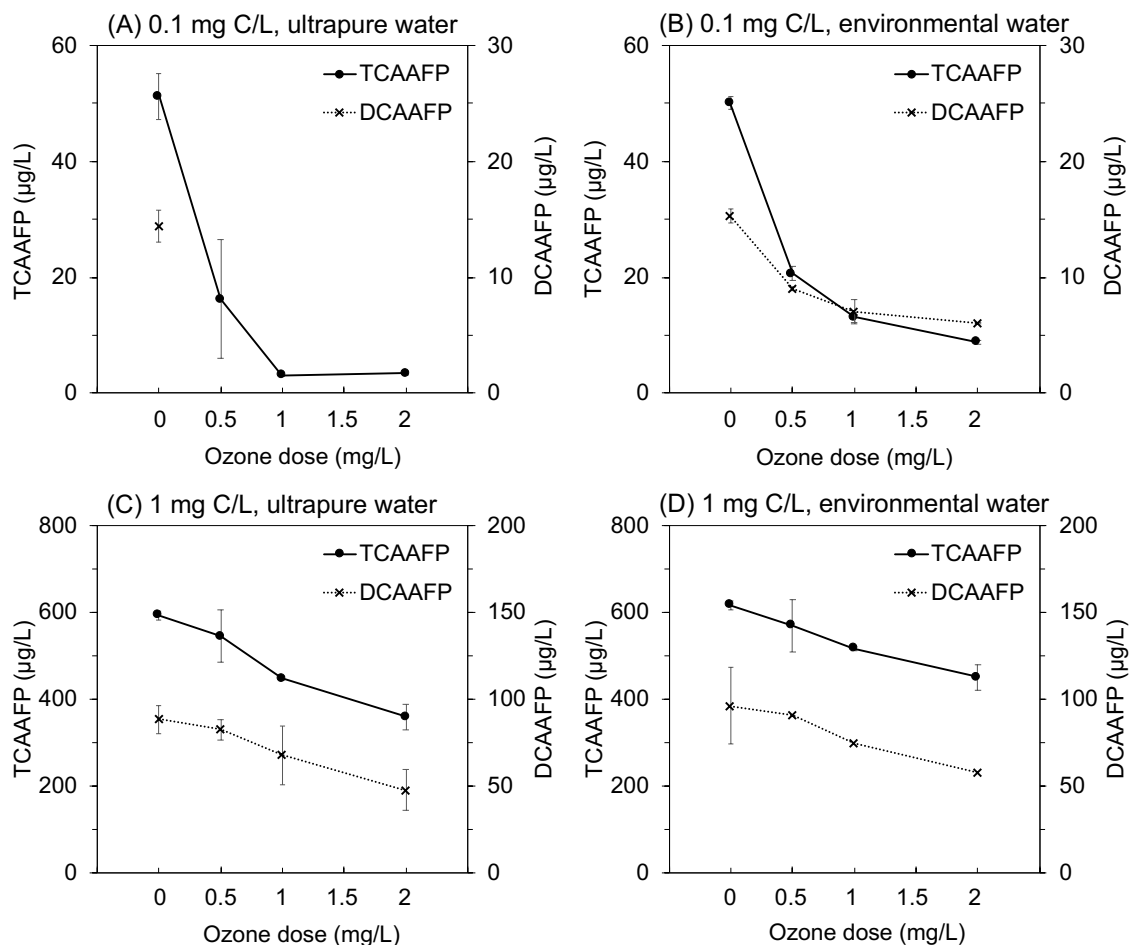


Figure 5.5. HAAFPs after ozonation of samples containing (A) 0.1 mg C/L of TCAA precursor isolates, (B) 0.1 mg C/L of TCAA precursor isolates with environmental water, (C) 1 mg C/L of TCAA precursor isolates, and (D) 0.1 mg C/L of TCAA precursor isolates with environmental water.

Experimental conditions of the HAAFP test: pH 7 (5 mM phosphate buffer); Cl_2 dose, 3 or 30 mg Cl_2/L ; temperature, 20 °C; and reaction time, 24 h in dark. Error bars represent the standard deviation of triplicate measurements or the minimum and maximum values from duplicate experiments. As the DCAAFPs in Figure (A) at ozone doses greater than 0.5 mg/L are below the LOQ, they are not plotted.

5.3.2 Treatability of TCAA precursors from wild raphidophytes in the reservoir water

5.3.2.1 Removal of TCAA precursors by the individual unit operations

Reservoir water collected during a bloom of raphidophytes (*Gonyostomum* spp.) in the Muro Dam, a drinking water source, in the fall of 2023, was treated directly. Except for the coagulation and sedimentation treatments, the treatability of the dissolved TCAA

precursors was evaluated using the filtered samples. Figure 5.6 shows the HAAFP ratios before and after the individual unit operations, such as coagulation and sedimentation, PAC adsorption, pre-chlorination followed by PAC adsorption, and ozonation.

The removal of TCAA precursors from raphidophytes through coagulation and sedimentation was highly effective. At PACl dose of 15 mg $\text{Al}_2\text{O}_3/\text{L}$, DCAAFP decreased by 83% (from 260 to 43 $\mu\text{g/L}$), while TCAAFP decreased by 94% (from 1300 to 79 $\mu\text{g/L}$) (Table S7). The resulting turbidity was 1.7 TU (1.0 NTU) after the treatment, which met the WHO guideline (5 NTU) and the Japanese drinking water standards (2 TU). However, it slightly exceeded Japan's reference value for comfort (1 TU) and was close to the USEPA and WHO recommendations (1 NTU), indicating potential for further improvement. Increasing the PACl dose to 30 and 45 mg $\text{Al}_2\text{O}_3/\text{L}$ further enhanced the HAAFP and turbidity reductions. At 45 mg $\text{Al}_2\text{O}_3/\text{L}$, DCAAFP decreased by 88% (from 260 to 33 $\mu\text{g/L}$), TCAAFP by 97% (from 1,300 to 43 $\mu\text{g/L}$), and turbidity to 0.12 TU (0.07 NTU). Despite these reductions, both DCAAFP and TCAAFP remained above 30 $\mu\text{g/L}$, with their combined concentration exceeding 60 $\mu\text{g/L}$, potentially surpassing USEPA and Japanese HAA standards. This highlights the need for additional treatment methods beyond coagulation and sedimentation, despite their high effectiveness.

Regarding PAC adsorption, HAAFP decreased with increasing PAC dose. DCAAFP decreased by 0.79 $\mu\text{g/L}$ (0.50%) and TCAAFP by 4.9 $\mu\text{g/L}$ (0.52%) for each 1 mg/L increase in Norit SA Super concentration. A PAC dose of 30 mg/L resulted in a 20% reduction in TCAAFP and a 23% reduction in DCAAFP. Even at an excess 100 mg/L PAC dose, the reduction reached only 52–53%, demonstrating that PAC adsorption was only partially effective compared to coagulation and sedimentation.

In the combination of pre-chlorination and PAC adsorption, DCAAFP and TCAAFP at pre-chlorine doses of 0.5 to 3.0 mg Cl_2/L were slightly higher than those observed with PAC adsorption alone. This was likely because the coexisting organic matter preferentially reacted with chlorine to form HAAs. In the experiments using TCAA precursor isolates from *G. semen* cultures, the highest reduction in HAAFP was achieved when a small amount of residual chlorine remained after pre-chlorination. Given the reservoir water's chlorine demand of 16.3 mg/L, a chlorine dose of 17 mg Cl_2/L or higher was expected to further reduce HAAFP.

Reservoir water was pre-chlorinated at pre-chlorine doses of 17, 18, 19, and 20 mg Cl_2/L and then treated with 15 mg/L of Norit SA Super for 1 h. TCAAFP decreased at pre-chlorine doses of 17 mg/L or higher compared to PAC adsorption alone. However, DCAAFP increased by 10–30%, indicating that DCAAFP should also be monitored although TCAAFP, which is much higher in algal blooms, remains a primary concern. The greatest reduction (24%) in TCAAFP compared with PAC adsorption alone was observed at a chlorine concentration of 19 mg Cl_2/L , whereas higher chlorine doses negatively impacted TCAA precursor removal. These findings indicate that although pre-chlorination effectively removed TCAA precursors from wild raphidophytes, an excess chlorine promotes HAA formation, requiring careful dose control.

For ozonation, HAAFP decreased under all tested conditions. At the highest ozone dose (2.0 mg/mg C or 16.6 mg/L), DCAAFP was reduced by 59%. However, at lower doses (≤ 1.0 mg/mg C or 8.3 mg/L), the reduction was less pronounced, indicating that ozone primarily reacted with other dissolved solids in the water, unlike in the HAA isolates experiment. This underscores the importance of removing competing dissolved materials before ozonation to enhance its effectiveness.

Among the treatments, coagulation and sedimentation achieved the highest removal efficiency (83–97%). PAC adsorption and ozonation also contributed to HAAFP reduction (3–59%). These findings suggest that optimizing coagulation and sedimentation should be the primary approach for removing TCAA precursors from raphidophytes, with PAC adsorption and ozonation considered as supplementary treatments when coagulation and sedimentation alone are insufficient.

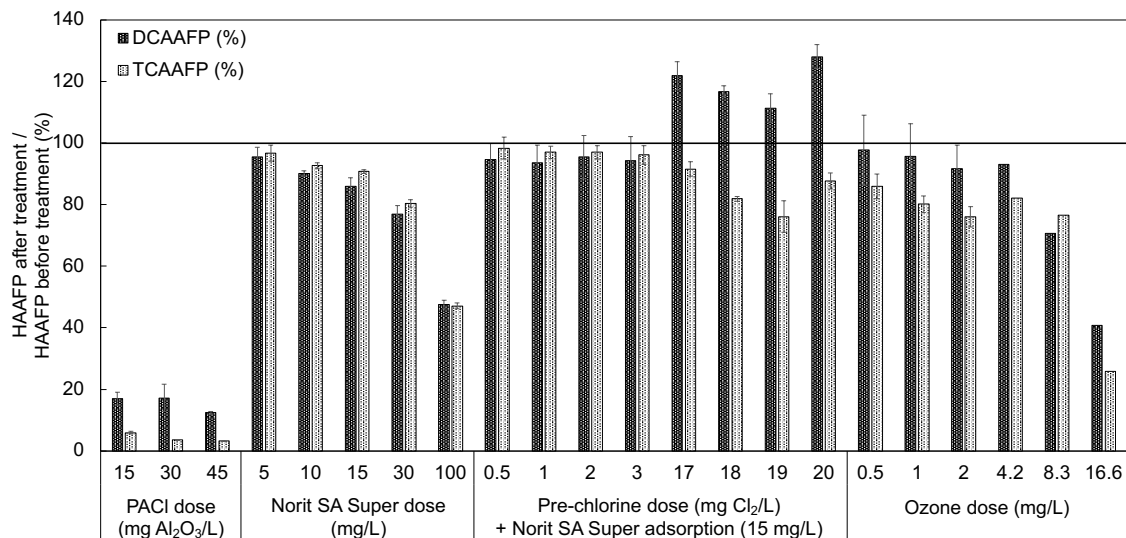


Figure 5.6. Removal efficiency of TCAA precursors in reservoir water by individual treatments.

Experimental conditions of the HAAFP test: 10 times-dilution; pH 7 (5 mM phosphate buffer); Cl₂ dose, 30 mg Cl₂/L; temperature, 20 °C; and reaction time, 24 h in dark. Error bars represent the standard deviation of triplicate measurements or the minimum and maximum values from duplicate experiments.

Table 5.4. TCAAFP, DCAAFP, pH, and turbidity of the reservoir water before and after coagulation and sedimentation treatment.

Experimental conditions of the HAAFP test: 10 times-dilution; pH 7 (5 mM phosphate buffer); Cl₂ dose, 30 mg Cl₂/L; temperature, 20 °C; and reaction time, 24 h in dark.

PAC dose (mg/L)	TCAAFP (μg/L)	DCAAFP (μg/L)	pH	Turbidity (polystyrene standard) (TU)	Turbidity (formazine standard) (NTU)
before treatment	260	1300	7.2	9.7	5.8
15	44	79	7.1	1.7	1.0
30	41	46	7.1	0.26	0.16
45	33	43	7.1	0.12	0.07

5.3.2.2 Removal of TCAA precursors by the combination of multiple unit operations

Individual unit operations demonstrated that coagulation and sedimentation treatments were the most effective at removing TCAA precursors. In this section, various treatment scenarios are investigated, combining coagulation and sedimentation operations with pre-PAC adsorption following pre-chlorination and post-ozonation, to remove substances that

cannot be effectively treated by coagulation and sedimentation alone. The effect of each treatment was evaluated. Figure 5.7 summarizes the removability of TCAA precursors across the three different scenarios.

In Scenario 1, DCAAFP and TCAAFP was initially 200 and 1,200 $\mu\text{g/L}$. After pre-chlorination and PAC adsorption, DCAAFP increased to 240 $\mu\text{g/L}$, but subsequent coagulation, sedimentation, and ozonation reduced them to 130 $\mu\text{g/L}$ (DCAAFP) and 420 $\mu\text{g/L}$ (TCAAFP). In Scenario 2, without pre-chlorination, PAC adsorption led to higher HAAFP levels, but coagulation and sedimentation removed over 96% of TCAAFP, with final levels of 22 $\mu\text{g/L}$ (DCAAFP) and 38 $\mu\text{g/L}$ (TCAAFP). In Scenario 3, without PAC adsorption, final DCAAFP and TCAAFP were slightly higher at 24 (DCAAFP) and 39 $\mu\text{g/L}$ (DCAAFP).

The trends of increased DCAAFP and decreased TCAAFP after pre-chlorination and PAC adsorption were consistent with the results observed in the unit operation experiments. Both PAC adsorption and coagulation and sedimentation effectively reduced DCAAFP and TCAAFP, with the latter showing particularly significant reductions. Ozonation, applied at the commonly used dose of 0.5 mg/L in water treatment plants, resulted in only a slight decrease in the HAAFP. Additionally, pre-chlorination was found to reduce the effectiveness of subsequent coagulation and sedimentation treatments. The treatability of the DCAA precursors decreased from 88 to 31%, and that of TCAA decreased from 96 to 65%. The increased ionic strength owing to the addition of chlorine may have inhibited intermolecular aggregation.

PAC adsorption led to a decrease in HAAFP. However, HAAFP after coagulation and sedimentation in both Scenario 2 (with PAC) and Scenario 3 (without PAC) changed only slightly, suggesting that the TCAA precursors removed by PAC and coagulation and sedimentation are similar substances. Additionally, there was no change in DCAAFP after ozonation, with only a decrease in TCAAFP, similar to the results obtained with the unit ozonation treatment (Figure 5.6). This suggests that the coagulation and sedimentation treatment in the previous stage has little influence on ozonation.

Given that pre-chlorination at high chlorine dose to enhance PAC adsorption significantly reduces the effectiveness of coagulation and sedimentation operations, this approach should be avoided when raphidophytes are present in the source water. In-

stead, PAC adsorption before coagulation and post-ozonation after sedimentation showed slightly better reductions in the HAAFP of the treated water. For the environmental water sample containing 9,000 cells/mL of raphidophytes used in this experiment, the final TCAAFP was 38 $\mu\text{g/L}$ even in Scenario 2, the most effective treatment scenario. Since the evaluation based on FPs indicated that the TCAA formation after all treatments might exceed 30 $\mu\text{g/L}$ (the standard value in Japan) and the sum of TCAA and DCAA formation might exceed 60 $\mu\text{g/L}$ (the standard value in USEPA), careful attention should be paid to operating conditions (e.g., coagulant and chlorine doses). The need for process optimization and monitoring strategies to minimize the risk of exceeding HAA regulatory limits is therefore emphasized.

HAAPs originating from raphidophytes fluctuate based on their cell populations and the concentration of released TCAA precursors. Therefore, the optimal treatment conditions and process should be determined by assessing factors, such as the extent of TOC increase compared to normal conditions. This study provides valuable information on countermeasures to manage elevated HAA concentrations caused by raphidophytes.

In this study, laboratory-scale treatment experiments were conducted. The characteristics of environmental water where raphidophytes grow vary significantly depending on location and time. Although coagulation and sedimentation are commonly used in many treatment plants, ozonation may not be practical due to the high cost of implementation. Furthermore, water treatment must address not only TCAA precursors derived from raphidophytes but also other contaminants, making it difficult to always achieve optimal conditions that balance HAA control and overall treatment efficiency. For application in full-scale water treatment plants, site-specific optimization is necessary, and the potential challenges must be considered.

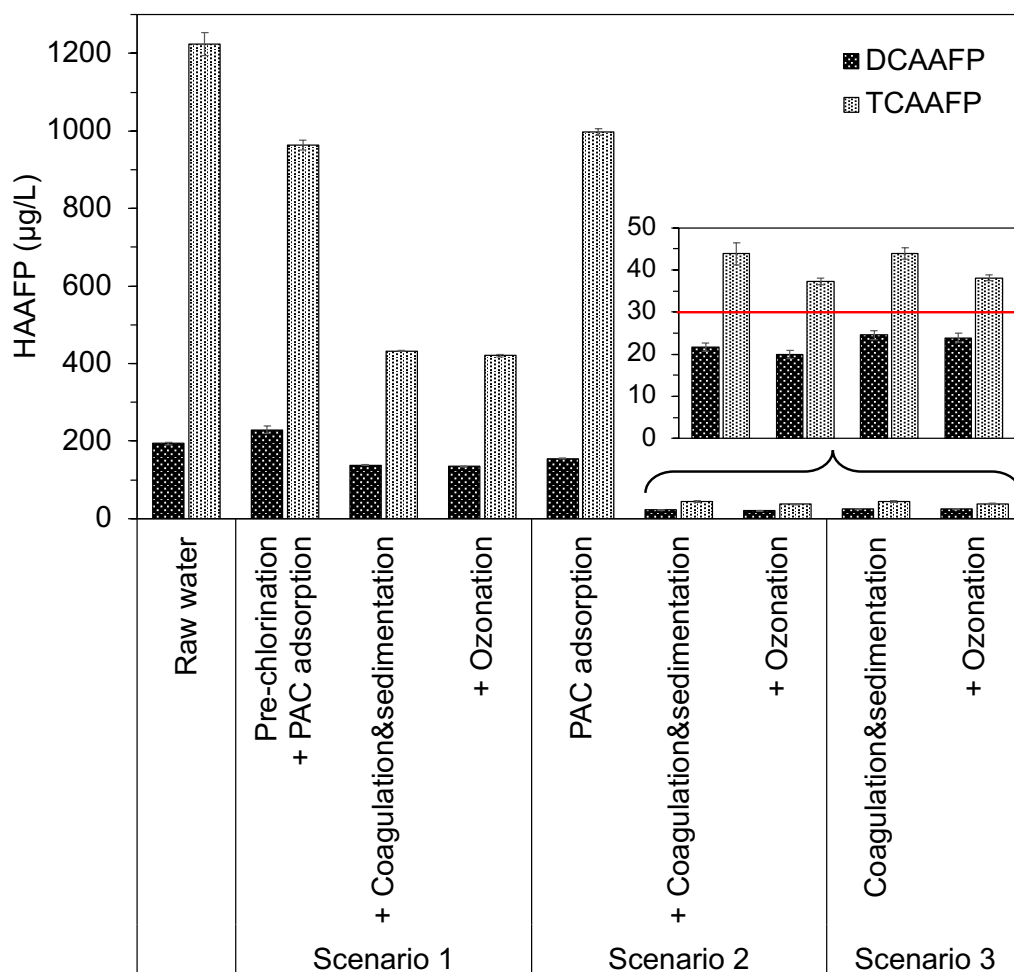


Figure 5.7. HAAFPs after continuous treatment of a reservoir water.

Experimental conditions of the HAAFP test: 10 times-dilution; pH 7 (5 mM phosphate buffer); Cl_2 dose, 30 mg Cl_2/L ; temperature, 20 °C; and reaction time, 24 h in dark. Error bars represent the standard deviation of triplicate measurements or the minimum and maximum values from duplicate experiments. A line is drawn at 30 µg/L of HAAFP, which corresponds to the drinking water quality standards for DCAA and TCAA in Japan.

5.4 Summary

This study evaluated the treatability of ultra-hydrophilic and high-molecular-weight TCAA precursors from cultured and wild raphidophytes to identify effective treatment methods. The findings indicate that coagulation and sedimentation are the most effective treatment methods for HAAFP reduction. Within practical operational conditions, PAC adsorption and ozonation should be used as supplementary methods. Key findings are:

- Coagulation and sedimentation: Achieved up to 90% HAAFP reduction when tur-

bidity was well controlled, regardless of precursor concentration. However, samples containing TCAA precursors exhibited lower turbidity removal efficiency. Early detection of raphidophyte blooms and immediate optimization of treatment conditions (e.g., PACl dosage) are crucial.

- PAC adsorption: Norit SA Super, with a high mesopore content, effectively adsorbed TCAA precursors, whereas PAC (5LPD), which has a lower mesopore and higher macropore content, was ineffective. HAAFP removal improved with increasing Norit SA Super concentrations, making it a valuable option during raphidophyte blooms.
- Pre-chlorination and PAC adsorption: More effective in reducing TCAAFP than PAC adsorption alone. However, chlorine dosage must be carefully controlled to match the water's chlorine demand, as excessive chlorine may increase HAA levels. Additionally, pre-chlorination may reduce the treatability of TCAA precursors in subsequent coagulation and sedimentation processes.
- Ozonation: Effective at reducing HAAFP at higher ozone doses, though its impact was limited within typical operational dose ranges. Ozonation is particularly useful for treating TCAA precursors.

Chapter 6

Conclusions

6.1 Major findings

This study comprehensively investigated TCAA precursors derived from raphidophytes, integrating analyses of their formation, structural characteristics, and treatability to inform effective water treatment strategies. The key findings are summarized as follows:

- **High TCAA formation:** Isolated TCAA precursors from raphidophyte samples (i.e., *G. semen* cultures and reservoir water) exhibited extremely high TCAAFPs, comparable to or exceeding those of phenol and chlorophenol, which are known to possess high TCAAFPs. These results demonstrate that raphidophytes uniquely produce potent TCAA precursors.
- **Structural characteristics of TCAA precursors:** Based on fractionation, optical properties, and water quality parameters, the TCAA precursors were identified as high-molecular-weight (approximately 10–60 kDa), non-protein-like, hydrophilic, and acidic carbohydrate-containing compounds lacking halogen atoms. NMR, FT-IR, and HRMS analyses indicated the presence of glycan and phenolic moieties with repetitive or similar substructures, likely composed of simple phenolic and glycan building blocks. These structural features explain their exceptionally high TCAA yields upon chlorination (up to 2,500 TCAA molecules per precursor).
- **Treatability and removal strategies:** Evaluation of treatment methods demonstrated that coagulation and sedimentation process are most effective in reducing

TCAAFP, achieving up to 90% removal when turbidity is well controlled. Note that care should be taken with the amount of flocculant added, as flocculation inhibition may occur. Supplementary methods such as PAC adsorption with high mesopore content and ozonation further enhance removal. Pre-chlorination combined with PAC adsorption improves TCAA precursor reduction, though careful control of chlorine dosage is necessary to prevent increased TCAA formation and maintain downstream treatability.

Overall, this work provides a comprehensive framework connecting DBP precursor chemistry, and water treatment approaches, offering both fundamental understanding and practical guidance for managing DBP formation in freshwater systems. Moreover, the methodological framework established in this study can be extended to investigate DBP precursor formation from other algae, providing an approach for future research on algae-derived DBPs.

6.2 Future works

This study has highlighted the challenges posed by ultra-hydrophilic, high-molecular-weight TCAA precursors originating from raphidophytes and demonstrated that appropriate treatment conditions can effectively prevent TCAA increases in finished water. To achieve this, early detection of algal blooms and rapid optimization of treatment processes are critical. The development of sensitive and rapid monitoring techniques, such as EEM-based detection and image analysis, will be essential for the early identification of algal blooms in water sources.

Further research is needed to elucidate the environmental and biological factors that promote the growth of raphidophytes and the release of TCAA precursors. The biochemical or ecological reasons why these algae produce such precursors, and whether similar mechanisms exist in other algal species, remain largely unexplored. Integrating biological and water chemistry approaches will be essential to address these questions.

Currently, raphidophyte blooms have been observed at three locations in the Kansai region. Considering recent climate change and ecosystem shifts, such blooms are expected to occur more frequently or may have already spread to other regions. Comprehensive

surveys using environmental DNA analysis should be conducted to assess the nationwide distribution of raphidophytes in drinking water sources and to evaluate the potential risk of TCAA formation across Japan's water supply systems, enabling proactive management and mitigation measures.

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