ABSTRACT

Title of Dissertation: ALGAL TOXICITY AND FORMATION OF HALOGENATED ORGANIC COMPOUNDS IN BALLAST WATER AFTER OXIDATIVE TREATMENT

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Ballast water plays a vital role in the stabilization and operations of modern ships, and it is estimated that 3 to 5 billion tons of ballast water are transferred around the world each year. However, the discharge of ballast water has led to the release of non-indigenous species, and costly and ecologically damaging biological invasions. To combat this serious problem, ballast water discharge is now regulated and ballast water management systems (BWMS) have been developed to meet required discharge limits for the release of live organisms. The most common BWMS rely on chlorination of ballast water to kill planktonic organisms but also result in the formation of disinfection by-products (DBPs) and the potential for aquatic toxicity. The research in this thesis was conducted to advance the understanding of treated ballast water toxicity, and to document the formation of higher molecular weight DBPs using ultrahigh resolution mass spectrometry. Research was conducted with commercial BWMS that were based on either direct chlorination (Ch. 2 & 3) or *in-situ* electrochlorination (Ch. 2 & 4). Ballast water treatment was conducted in

estuarine waters of the Port of Baltimore (Patapsco River, Maryland). In Chapter 2, I tested the algal toxicity of discharged ballast water from four BWMS at the time of discharge and monthly thereafter, showing the longevity of the toxic effect of treated water on micro algae. In Chapters 3 and 4, I used ultrahigh resolution mass spectrometry to identify the molecular composition of dissolved organic matter (DOM) and halogenated DBPs after oxidative treatment of ballast water. By comparing samples before and after direct chlorination, I was able to document the changes in dissolved organic matter and the formation of numerous halogenated DBPs (Ch. 3). In Chapter 4, I was able to document the change in brominated DBPs after a period of 92 days, showing the relative persistence of dibrominated compounds. This work together demonstrates that use of traditional water treatment to solve one environmental problem may, in fact, cause other unintended consequences to aquatic ecosystems.

ALGAL TOXICITY AND FORMATION OF HALOGENATED ORGANIC COMPOUNDS IN BALLAST WATER AFTER OXIDATIVE TREATMENT

by

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List of Abbreviations

Abbreviation	Definition
AOM	Algal organic matter
AOX	Adsorbable organic halogen
Br-DBP	Brominated disinfection by-product
BRAC	3,5-Dibromo-4-hydroxybenzoic acid
BWMS	Ballast water management system
BWWG	Ballast water working group
CHO	Compounds containing carbon, hydrogen and oxygen only
CHOBr	Compounds containing carbon, hydrogen, oxygen and bromine only
CHOBrCl	Compounds containing carbon, hydrogen, oxygen, bromine and chlorine only
CHOCl	Compounds containing carbon, hydrogen, oxygen and chlorine only
CHON	Compounds containing carbon, hydrogen, oxygen and nitrogen only
CHONBr	Compounds containing carbon, hydrogen, oxygen, nitrogen and bromine only
CHONS	Compounds containing carbon, hydrogen, oxygen, nitrogen and sulfur only
CHOS	Compounds containing carbon, hydrogen, oxygen and sulfur only
Cl-DBP	Chlorinated disinfection by-product
DBAA	Dibromoacetic acid
DBP	Disinfection by-product
DICD	Dichloroisocyanurate dihydrate
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DPD	N, N-Diethyl-p-phenylenediamine
EC_{50}	Effective concentration, 50% (median effective concentration)

EPA	Environmental Protection Agency (US)
EOX	Extractable Organic Halogen
ESI^-	Negative ion mode electrospray ionization
ESI-tqMS	Electrospray ionization-triple quadrupole mass spectrometry
ETV	Environmental technology verification (program)
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
GC	Gas chromatography
GC-ECD	Gas chromatography electron capture detector
GC-MS	Gas chromatography mass spectrometry
HAA	Haloacetic acid
HAN	Haloacetonitrile
HCD	Hydroxy-cyclopentene-dione
HMW	High molecular weight
HOCl	Hypochlorous acid
HOBr	Hypobromous acid
HP	Halophenol
HPLC	High-performance liquid chromatography
K_{ow}	n-Octanol/water partition coefficient
IMO	International Maritime Organization
Invasive species	Nonindigenous species that cause environmental, ecological or economic harm
k(H)	Hydrolysis rate constant
kDa	Kilodalton
KMD	Kendrick mass defect
KMD/*z**	Homologous series identifier (CH_2 spacing)
LC	Liquid chromatography
LC_{50}	Concentration estimated to cause 50% lethality

LogP	Partition coefficient between aqueous and lipophilic phases
MAMPEC-BW	Marine Antifoulant Model for PEC calculation with ballast water features
MCD	methoxycyclopent-4-ene-1,3-dione
MDL	Method detection limit
MERC	Maritime Environmental Resource Center
MS	Mass spectrometry
m/z	Mass to charge ratio
NaDDC	Sodium dichloroisocyanurate dihydrate
NDIR	Non-dispersive infrared detector
NIS	Non-indigenous species: A species not historically found in a geographic region
NOM	Natural organic Mater
NA	Not applicable or not available
O/C	Oxygen to carbon ratio of molecular formula
PEC	Predicted environmental concentration
PPL	Solid phase extraction cartridge with styrene-divinylbenzene polymer
ppm	Part per million
PIS	Precursor ion scan
PNEC	Predicted no effect concentration
POX	Purgeable organic halogen
Propagule pressure	Quality, quantity and frequency of propagule introduction
S-DBP	Sulfonated disinfection by-product
SDVB	Styrene-divinylbenzene
SIR	Selected ion recording
SPE	Solid phase extraction
SUVA	Specific UV absorbance (UV_{254}/DOC)
TBP	Tribromophenol

THM	Trihalomethane
TOBr	Total organic bromine
TOC	Total organic carbon
TOCl	Total organic chlorine
TOX	Total organic halogen
tqMS	Triple quadruple mass spectrometry
TRO	Total residual oxidant
TSS	Total suspended solids
UHPLC	Ultrahigh-performance liquid chromatography
UHRMS	Ultrahigh resolution mass spectrometry
UV	Ultraviolet
UVT	Ultraviolet transmittance
Vector	How a non-native species is transported
USCG	U.S. Coast Guard
U.S. EPA	United States Environment Protection Agency
UPLC	Ultra-performance liquid chromatography
UVA	Ultraviolet Absorbance
WREC	Wye Research and Education Center

Note to Readers:

This Dissertation is organized and formatted to follow The University of Maryland, Electronic Thesis and Dissertation (ETD) Style Guide. Chapter 1 is a general introduction into invasive species, ballast water management, toxicity of treated ballast water and the formation disinfection by-products (DBPs) with ballast water treatment. Chapters 2, 3 and 4 of this dissertation are organized as manuscripts for publication in scientific journals. For consistency, all chapters of this dissertation have been similarly formatted. Appendices associated with each research chapter are located at the end of the dissertation. Chapter 5 contains overall conclusions and future research discussion.

Chapter 1

Introduction

1.1 Invasive Species and Global Shipping

The effects of invasive species in many areas of the world have been devastating and are causing enormous damage to local biodiversity and natural resources. Data show that the rate of bioinvasions is increasing in both land and aquatic habitats and that new areas are being invaded with increased frequency, resulting in instances of extensive economic, human health and ecological impacts (Carlton, 1999; Gollasch, 2006; Vila et al., 2010). The process of biological invasions can be broken into four primary stages: transport, introduction, establishment and spread (Ruiz and Carlton, 2003; Lockwood et al., 2007; Blackburn et al., 2011). Ultimately a species is considered invasive after out-competing native species and spreading in the new location, resulting in harmful economic or ecological impacts.

Ballast water is used by modern ships to maintain balance and maneuverability at sea and is a necessary element of modern cargo shipping operations throughout the world. Although ballast water is required for the safe operation of ships, discharged ballast water is recognized as one of the predominant vectors for aquatic non-indigenous species (NIS) transportation and introductions (Carlton, 1985; Ruiz et al., 1997; 2000; Pimentel et al., 2005; Drake et al., 2007; Lovell and Drake, 2009). The ecological and economic impacts of ballast water release arise from the survival of transferred species that may have the ability to establish a reproductive population in the new coastal environment (Carlton, 1999). Introduced aquatic organisms can be extremely diverse, with invading organisms ranging over 15 different animal phyla with a relatively high prevalence of some types of

organisms such as mollusks, crustaceans and worms (NRC, 2011). Many environmental factors and species specific traits can affect the ability of a species to become invasive, all of which can change depending on seasonal influences. The establishment of an introduced species can also vary in different locations depending on biotic interactions (e.g. competition, predation, parasitism and pathogens) and abiotic (i.e. environmental) characteristics (NRC, 2011). Although theories have been developed for predicting successful establishment of an introduced species (Elton, 1958; Kolar and Lodge, 2001; Freestone et al., 2013), the complexity of the biotic and abiotic factors along with species specific traits makes predicting the success of an introduced species challenging.

Multiple vectors can often be identified for the introduction of NIS in a given geographic location (Hulme, 2009). Aquatic invasive species vectors that have been identified include the bait industry, live seafood, ornamental species trade, aquaculture, and marine debris. However, it is clear that commercial shipping (responsible for an estimated 90 percent of global trade) is the largest source of aquatic invasions, through ballast water release and vessel biofouling (Albert et al., 2013). The potential risk from both of these vectors has increased with increased transport speed of modern ships, allowing transported organisms to arrive in healthier condition due to the reduced transit time (Holme, 2009). In recent years, larger ships with larger ballast tanks have also increased the number of organisms released per discharge (propagule size), while the increased number of ships has resulted in an increase in discharge events, both of which contribute to overall increase in the amount of ballast water discharge. In fact, the global input of discharged ballast water has been estimated in several publications at 3 to 10 billion tonnes (Endresen et al., 2004; Tsolaki and Diamadopoulos, 2010; David, 2015;

IMO, n.d.). Although the greatest number of studies have focused on biotic, abiotic and species specific factors of invasive organisms, empirical evidence has established that propagule pressure (i.e. propagule number and concentration) may be the most important factor in bioinvasions (Simberloff, 2009). A reduction in propagule pressure by decreasing organism concentrations and total numbers in ballast water should result in a decrease in the probability of NIS establishment (Lockwood et al., 2005; Albert et al., 2013), although bioinvasions are a stochastic process and the shape of the risk-release (or dose-response) relationship can vary depending on the circumstances (Ruiz and Carlton, 2003; NRC, 2011; Wonham et al., 2013).

The establishment of aquatic invasive species is clearly a complex issue, which limits our current ability to accuratly predict invasions (Williams et al., 2013). For this reason, recent strategies to prevent invasions resulting from discharged ballast water have focused on reducing the number of viable or living organisms delivered by a major vector (e.g., ballast water, Miller et al., 2011; vessel biofouling, Davidson et al., 2017), rather than adopting species specific approaches to limit successful introductions of NIS.

1.2 Ballast Water Regulations

Implementation of vessel ballast water management strategies to address invasive species, such as ballast water exchange (BWE) or treatment, have had some documented success in limiting the transfer and release of non-indigenous species. In particular, the United Nations' International Maritime Organization (IMO) and the U.S. Coast Guard have implemented ballast water regulations (Casas-Monroy et al., 2015). These organizations have set numeric discharge standards based on the predicted association between the concentrations of organisms in discharged ballast water (i.e. inoculum

density) and the risk of NIS establishment. The *International Convention for the Control and Management of Ships' Ballast Water and Sediments* (BWM Convention) of the IMO (IMO, 2004), and national regulations (e.g. USCG, 2012; NZMPI, 2016), require ships to treat their ballast water with certified ballast water management systems (BWMS) to meet numeric limits for viable organisms and bacteria. The BWM Convention requires ships to conduct ballast water management (BWM) to limit the introduction of potentially invasive species (IMO, 2004). In the short-term, some ships can still comply with regulations by conducting BWE with at least a 95% volumetric exchange of ballast water (D-1 Standard) or meet discharge standard concentrations for viable organisms depending on size class or taxonomic category (D-2 Standard). However, ultimately all commercial vessels will need comply with the D-2 standard, which sets concentrations for classes of organisms related to their minimum dimensions: less than 10 viable organisms ≥ 50 μm per m^3 and less than 10 organisms ≥ 10 μm and < 50 μm per ml. In addition, the discharge of indicator organisms (i.e. *Vibrio cholerae*, *Escherichia coli*, intestinal *Enterococci*) cannot exceed specified concentrations (Table 1.1). As of September 8, 2017 (IMO, 2016c) new build ships (keel-laid) have to install ballast water treatment systems, but existing ships are required to install a BWMS at the time of their next International Oil Pollution Prevention (IOPP) survey.

Table 1.1 The IMO D-2 standard for discharged ballast water.

Organism Category	**Regulation**
Plankton, >50μm in minimum dimensions	<10 cells/m^3
Plankton, 10-50 μm	<10 cells/ml
Toxigenic Vibrio cholera	<1 colony forming unit (cfu)/100 ml
Escherichia coli	<250 cfu/100 ml
Intestinal Enterococci	<100 cfu/100 ml

In accordance with the IMO BWM Convention, all BWMS need to go through a Type Approval Certification process in accordance with guidelines set out by IMO (IMO, 2008a). Testing of the efficacy of BWMS, initially a Guideline (G8), has now been replaced by the more enforceable BWMS Code (IMO, 2017a). Like the IMO, the US Coast Guard (USCG) and US Environmental Protection Agency (USEPA) had implemented requirements for certification testing BWMS (USEPA, 2010), with similar testing requirements and discharge standards to those previously adopted by the IMO. Recently, with the passing of the Vessel Incidental Discharge Act (VIDA), the US has modified ballast water treatment and discharge policies to increase consistency domestically, and modified the BWMS approval policy to more closely align with the IMO.

1.3 Ballast Water Management Systems

The requirement for the treatment of ballast water to minimize the release of non-indigenous species has led to the development of a variety of technologies, many of which use chemical biocides (i.e., active substances). Many of the technologies and methods have an origin in water purification or wastewater treatment including chemical processes; flocculation, electrochlorination and ultraviolet light (UV); and physical separation techniques such as filtration and hydrocyclone separation. Some of these methods have been adapted for use in ballast water treatment, in addition to a few novel methods specific to ballast water treatment. Ballast water treatment technologies can be broken into primary treatment of physical separation that is followed by secondary mechanical or chemical treatment of ballast water either at uptake or during a ship's voyage (Tsolaki and Diamadopoulos, 2010; Ren, 2018). Filtration is the most common

form of physical separation which is usually combined with other methods such as UV or oxidant biocide treatment. Filtration is typically based on screen or disk filters with pore sizes commonly in the range of 30-50 μm.

1.3.1 Mechanical Treatment

Mechanical treatment methods include ultraviolet radiation (UV), cavitation, de-oxygenation, ultrasound and magnetic fields. UV is the by far most common mechanical treatment method and is used to damage normal cellular functions, including their DNA, so that they are unable to reproduce (Kolkman et al., 2015). The effectiveness of UV treatment is dependent on transmittance so that high turbidity can be problematic. For this reason most UV-based systems are combined with some form of physical separation (e.g. filtration, cyclonic separation).

1.3.2 Chemical Treatment

Chemical treatment methods used in ballast water disinfection are diverse with the use of chemicals that are stored and prepared onboard, as well *in-situ* production of the biocide as in the case of electrochlorination. Active substances are chemicals that are used for disinfection, while other chemicals can also be found in treated water as part of the active substance preparation, or as by-products of the disinfection process. The most common BWMS treatment is chlorination with dose measured as total residual oxidant (TRO) with less frequent use of ozone, chlorine dioxide, naphthoquinones (menadione and juglone), peracetic acid and hydrogen peroxide. This dissertation is based on data collected on discharge water collected after treatment by BWMS with total residual oxidant (TRO) as the active substance either produced by electrochlorination or a solution of sodium dichloroisocyanurate dihydrate (DICD) that is prepared on-board and

injected back into the ballast water. In BWMS using electrolysis, the process can occur directly in the main ballast water line (i.e. full flow) or in a side stream that produces concentrated TRO which is then injected into the main ballast water line. When fresh water needs to be treated, a BWMS using electrochlorination will rely on reserve tanks of brine or previously collected sea water.

1.4 Approval of Ballast Water Management Systems

In addition to the IMO BWMS Code regulating efficacy of BWMS, there is a companion regulation (Procedure G9) which is invoked to evaluate the safety of BWMS employing active substances (IMO, 2008b). In this context an active substance is defined as a substance that has a general or specific action (chemical or biological) on or against harmful aquatic organisms and pathogens. When active substances are part of the treatment process the possibility of residual chemical release into the environment is taken into consideration. Procedure (G9) "*Procedures for the Approval of Ballast Water Management Systems That Make Use of Active Substances*" (IMO, 2004), calls for an overall safety review and risk assessment during the development of the BWMS. This includes safety of the environment, human health and ships. Under IMO Procedure (G9), evaluations are conducted following the "*Methodology for information gathering and conduct of work of the GESAMP-Ballast Water Working Group (BWWG)*", specifically designed for evaluating BWMS (IMO, 2012). Reviews of chemical-based ballast water treatments are also conducted in the USA by the US Environmental Protection Agency (USEPA), with approval of specific compounds for application to ballast water based on Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) registration certificate from the USEPA. However, no comprehensive framework for evaluating the environmental

acceptability of biocide treated and discharged ballast water has been implemented by any United States agency. As of January, 2019 the GESAMP-BWWG recommended Final Approval (FA) for 42 BWMS using active substances (IMO, 2018a). Approximately 26% of the IMO approved BWMS use some form of chlorination (e.g. electrochlorination, dichloroisocyanurate dihydrate, sodium hypochlorite) resulting in the production of disinfection by-products (DBPs). At present, approximately half of the BWMS that are Type Approved for use on ships use an active substance (IMO, 2015) and approximately 70% of installed BWMS use active substances (ABS, 2019).

Although the IMO Ballast Water Convention (BWC) has entered into force making ballast water treatment mandatory on most vessels, the number of ships that will install oxidant based BWMS is unknown. Considering the number of ships that will require ballast water treatment, the discharge of oxidant treated ballast water with associated DBPs has become a global concern. However, because BWMS installation requirements are based on an IMO timeline, it may take many years to assess the total number of ships, and total volume of oxidant treated water that will be discharged into the environment.

1.5 Assessing the Environmental Impact of Discharged Treated Ballast Water

The environmental acceptability of treated and discharged ballast water has been analyzed in few peer-reviewed published articles (Werschkun et al., 2012; Delacroix et al., 2013). In addition to this limited amount of published data, there is a substantial quantity of treated ballast water information available from BWMS evaluations under IMO Procedure G9, as conducted by the GESAMP-BWWG (Bowmer and Linders, 2010). Evaluations of BWMS have identified a list of the 43 chemicals most commonly associated with treated ballast water, the majority of which are DBPs produced after

treatment with strong oxidants. Two of these treatment methods were employed by the BWMS studied in this dissertation, namely electrochlorination and dichloroisocyanurate dihydrate (DICD) injection.

The list of the 43 most common ballast water chemicals is compiled in Appendix 1. The majority of chemicals are small DBPs that are analyzed by GC-MS or LC-MS. The largest DBP in the database is a tribrominated halophenol (HP), 2,4,6-Tribromophenol (IMO, 2017b). As part of this dissertation, an extensive search was conducted to identify the lowest ecotoxicological values for each of the 43 substances. These ecotoxicity values were extracted from published research, research that was evaluated by national and international organizations (USEPA, OECD, WHO, etc.), as well as data acquired from quantitative structure activity relationship (QSAR) databases when no ecototoxicity data was available. Predicted no effects concentrations (PNECs), based on the lowest ecotoxicity value identified for organisms in 3 trophic levels (plant, invertebrate and vertebrate), and an assessment factor (chosen based on the quality and quantity ecotoxicity data) were also calculated for each of the 43 chemicals. This data is available as an online database (GESAMP-BWWG-*Database of Chemicals Most Commonly Associated with Treated Ballast Water*) at the IMO Global Integrated Ship Information System (GISIS) website (IMO, 2017b). The BWWG-Database, also includes physico-chemical properties and toxicological information on the 43 chemicals, where available.

The BWWG-Database is useful for an overview of potential environmental concerns by comparing the PNECs to model based predicted environmental concentrations (PECs) for individual chemicals. However, a comparison of measured DBP discharge concentrations and algal ecotoxicity data is also useful to ascertain the cause of toxicity

observed in algal toxicity testing of oxidant treated ballast water. In Chapter 3 of this dissertation, a comparison of the highest DBP concentrations and available algae-specific ecotoxicity data is presented for ballast water from the four tested BWMS. This comparison assures that effects are relevant to algae, although typically microalgae are the most sensitive trophic level with regard to DBPs, and algae usually accounts for the lowest ecotoxicity value used in calculating PNECs.

The predicted environmental concentration (PEC) of all DBPs in a harbor situation can be estimated using the discharged ballast water concentration and a ballast water model of a commercial harbor, MAMPEC 3.1.0.3 (Van Hattum et al., 2016), with the assumption that all ships will use a particular ballast water treatment system (Zipperle et al., 2011). MAMPEC is a mathematical model originally developed for antifouling paint biocides, which has been adapted for use in ballast water discharge situations. The MAMPEC modeling has been performed extensively by the BWWG in a Port of Rotterdam scenario, and has also been used to calculate PECs of DBPs in other port scenarios (David et al., 2018; IMO, 2018b). The ratio of the PEC and PNEC is calculated to characterize the risk of a given DBP in the environment when discharged at a given concentration. If the PEC/PNEC is greater than 1, the possibility of environmental impact cannot be ruled out for that particular chemical (IMO, 2017c).

In addition to PEC/PNEC ratios, the toxicity of discharged ballast water after BWMS treatment is evaluated during IMO Procedure G9. A review of data from G9 submissions collected since research on this dissertation began shows that discharged ballast water from oxidant-based BWMS is frequently toxic to microalgae, while typically no toxicity is revealed in vertebrate and invertebrate testing.

1.6 Chemistry of Chlorination Based Treatment Methods

Chlorination has been used by the majority of BWMS using active substances and a large proportion of certified BWMS overall. Chlorine has been used routinely in the treatment of drinking water and wastewater for decades, resulting in water chlorination technologies that are mature compared to other potential biocides for use in ballast water treatment. The biocidal action of chlorine, and other oxidants, is explained by multiple mechanisms including protein denaturation, oxidation, attack and modification of the cell membrane, and hydrolysis (Hawkins et al., 2003). Chlorine-produced oxidants are also effective against the majority of ballast water organisms, although oxidants may not be as effective against eggs and resting stages of some organisms (e.g. cysts), or aquatic viruses. (Hallegraeff and Bolch, 1991; Blackburn and Parker, 2005; Rubino et al., 2013). This is particularly relevant as manufacturers face increasing pressure to develop BWMS that are effective against all life stages of organisms (Hess-Erga et al., 2019), potentially leading to increased doses of oxidants in ballast water treatment. Chlorination of ballast water is characterized by either the addition of active substance or the electrolysis of seawater resulting in oxidants consisting of active chlorine (primarily HOCl and OCl^-) and active bromine (primarily HOBr and OBr^-). Because the chemicals comprising the pool of oxidants are extremely fast reacting, the exact speciation of chemicals is difficult to resolve so the oxidant concentration is quantified as total residual oxidant (TRO) expressed as Cl_2 equivalents (i.e. mg TRO l^- as Cl_2). The dose of TRO in most BWMS is measured in samples taken from the ballast water line by either DPD colorimetric meters or amperometric based analyzers, although ORP analyzers have also been used in a few BWMS for adjusting the oxidant dose.

This dissertation research was conducted on two types of BWMS based on either electrochlorination or direct chlorination by injection of a DICD solution. In fresh water, a rapid hydrolysis of molecular chlorine takes place in ballast water to form hypochlorous acid (HOCl) which rapidly equilibrates with OCl^- as shown in reactions 1 and 2 below.

$$Cl_2 + H_2O \rightleftharpoons HOCl + H^+ + Cl^- \quad (1)$$

$$HOCl \rightleftharpoons OCl^- + H^+ \quad (2)$$

$$HOCl + Br^- \rightleftharpoons HOBr + Cl^- \quad (3)$$

$$OCl^- + Br^- \rightleftharpoons OBr^- + Cl^- \quad (4)$$

The combined HOCl and OCl^- species are referred to as free available chlorine (FAC). The predominant chlorine species is driven by the pH of the water (Figure 1.1), with HOCl dominating around pH 6 followed by an increase in the proportion of OCl^- at higher pH values eventually dominating near pH 8.

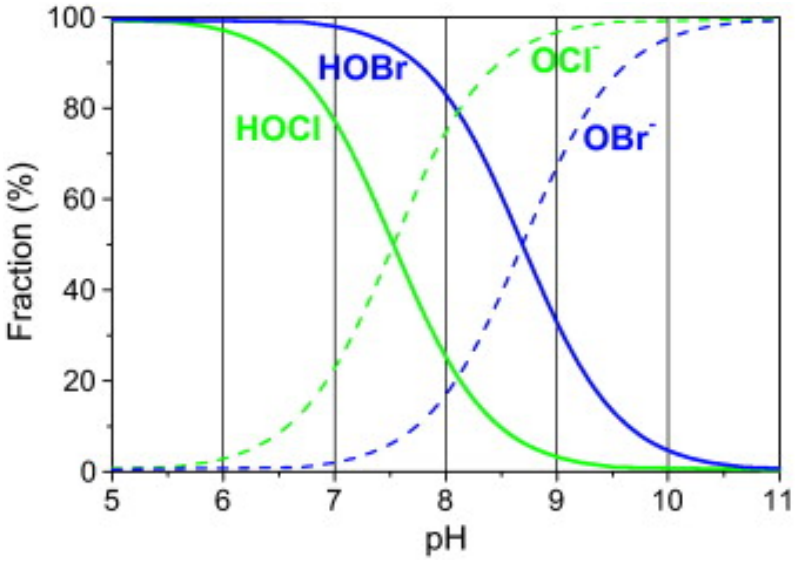

Figure 1.1 Chlorine and bromine species at differing pH values

Although bromide, an inorganic ion, does not react directly with natural organic

matter (NOM), it is oxidized by chlorine to hypobromous acid (HOBr). When oxidants are used in brackish and seawater, HOCl reacts with naturally present bromide which is oxidized to form HOBr (eq. 3), which then undergoes an acid base speciation to form hypobromite (OBr^-) (eq. 4). The combined HOBr and OBr^- species comprise the bulk of active bromine pool with other lesser bromine species (Br_2, BrCl, Br_2O) that can also react with inorganic and organic compounds (Heeb et al., 2014). A similar primary oxidant speciation curve is seen with chlorine and bromine species (Figure 1.1). However, the curve for HOBr dominance is shifted to the right so that HOBr is dominant at pH 7, where a relative increase in OBr^- starts and becomes dominant closer to pH 9. Both active chlorine and active bromine are extremely reactive with cell components and microorganisms making them effective disinfectants. The amount of active bromine will depend on the content of bromide ion, which varies proportionally to the salinity natural waters (Table 1.2). Fresh water concentrations of bromide range from trace to approximately 0.5 mg l^{-1}. The oxidant chemistry in marine waters is largely controlled by the high content of chlorine (Cl^-: 19,000 mg l^{-1}) and bromine (Br^-: 65 mg l^{-1}). At the mesohaline salinities of ballast water in this dissertation research (i.e. 5.2 – 7.1), the TRO distribution is theoretically comprised predominantly of active bromine (Table 1.2).

Table 1.2 Summary of active oxidants for a dose of 10 mg l^{-1} available chlorine

Water	Salinity (PSU)	Active biocidal agent
Seawater	32	Completely available bromine
Brackish mesohaline	5 – 18	Predominantly available bromine
Brackish	3 – 5	Mostly available bromine
Slightly brackish	< 3	Mixed available bromine/chlorine
Fresh water	< 0.3	Completely available chlorine

1.6.1 DICD Chemistry

In Chapters 2 and 3 of this dissertation, a BWMS was tested that was based on injection of a solution of DICD, to produce HOCl, followed by direct injection of DICD solution into ballast water during uptake. It is well established that dichloroisocyanurate or DICD ($C_3N_3O_3Cl_2$) equilibrates with HOCl in solution (Wojtowicz, 1993) as seen below in Figure 1.2.

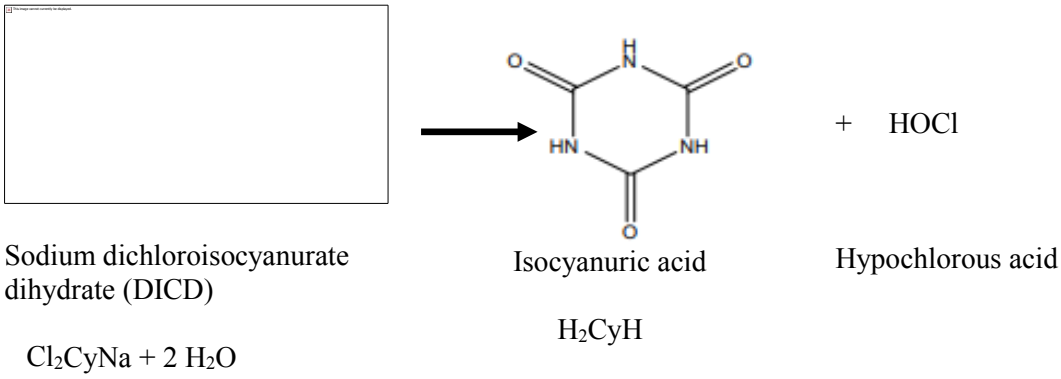

Figure 1.2 Release of free available chlorine (HOCl) from DICD in solution.

In fresh water systems with high demand from microorganism and chemical species, DICD equilibrates as HOCL is consumed by a rapid hydrolysis reaction (Jensen and Johnson, 1990). However, in low chlorine demand fresh water applications, the cyanurate ring on DICD holds chlorine until needed for disinfection. Therefore with high chlorine demand, conversion of chloroisocyanurates to HOCl is thought to be very rapid and complete (eq. 5 and 6) (Wojtowicz, 1993), with disinfection accomplished using only free available chlorine (HOCl and OCl^-). Like other chlorination techniques (e.g. electrochlorination), in the presence of bromide ion in an aqueous solution (e.g. brackish or seawater) the bromide ion is oxidized rapidly to available bromine (HOBr and OBr^-) and if enough bromide ion is present all available chlorine is converted to available

bromine. Although complete conversion to available bromine is assumed, there is no published data on the effects of cyanuric acid (H_2Cy^-) or isocyanurates on the oxidation of the bromide ion by HOCl (eq. 7) (Farkas et al., 1949).

$$Cl_2Cy^- + H_2O \rightarrow HClCy^- + HOCl \quad (5)$$

$$HClCy^- + H_2O \rightarrow H_2Cy^- + HOCl \quad (6)$$

Cl_2Cy^- = dichloroisocyanurate, $HClCy^-$ = chloroisocyanurate, and H_2Cy^- = cyanurate

$$HOCl + Br^- \rightarrow HOBr + Cl^- \quad (7)$$

1.6.2 Electrochlorination Chemistry

Electrochlorination has the advantage of using seawater to produce HOCl on demand, without the need for loading and holding chlorine in a concentrated liquid (e.g. sodium hypochlorite) or solid crystalline or powder form (e.g. DICD). In Chapter 2, three BWMS were tested that were based on electrochlorination of incoming ballast water to produce HOCl. The general method of electrochlorination is the production of TRO by running an electrical current through saltwater. Although details of the BWMS studied in this dissertation research are confidential, the electrochlorination of seawater is a well described technology and is widely used in treatment of seawater for swimming pools, desalination membrane fouling control, and biofouling control in industrial and power plant cooling waters. The chemical reactions take place in an electrolyzer unit which applies direct current to the ballast water. The electrolyzer is composed of anodes and cathodes potentially made of different electrode materials. Newer units are composed of titanium anodes with metal oxide coatings (e.g. iridium or rubidium oxides) with the Ti/RiO_2 combination producing the highest level of free chlorine (Jeong et al., 2009). The water flows between an anode (eq. 8) and a cathode (eq. 9), producing

chlorine on the anode, and hydrogen and sodium hydroxide by hydrogen-generation reaction on the cathode. The reaction in the solution is shown below (eq. 10 and 11) with the overall reaction (eq. 12) showing the final production of sodium hypochlorite (Black and Veatch Corporation, 2010).

$$\text{(Anode):} \quad 2Cl^- \rightarrow Cl_2 + 2e^- \qquad (8)$$

$$\text{(Cathode):} \quad 2H_2O + 2e^- \rightarrow 2OH^- + H_2 \qquad (9)$$

$$\text{Solution:} \quad NaOH + Cl_2 \rightarrow NaClO + NaCl + H_2O \qquad (10)$$

$$NaClO + H_2O \rightarrow HOCl + Na^+ + OH^- \qquad (11)$$

$$\text{Overall Reaction:} \quad 2Cl^- + 2H_2O \rightarrow 2HOCl + H_2 \qquad (12)$$

The formation of HOCl is directly proportional to the salinity (i.e. chloride concentration) and the applied specific charge (Jeong et al., 2009). Because the electrolysis voltage increases with low water temperature, a minimum water temperature is typically assessed to alleviate problems with electrode degradation and the need for increased power supply. As seen with DICD chlorination in brackish water, any HOCl or OCl^- produced is rapidly converted HOBr or OBr^- (eq. 3 & 4) due to the natural presence of the bromide ion, and the active substance is considered the combined effect of residual oxidants (i.e. TRO).

1.7 Formation of Disinfection By-products (DBPs)

The initial detection of DBPs was with small chlorinated DBPs in chlorinated drinking water (Rook, 1974). The discovery of chlorinated DBPs was followed by the identification of analogous brominated DBPs in marine waters (Rook et al., 1978). It is now recognized that bromide containing compounds represent the largest group of DBPs in marine waters (Helz et al., 1984; Werschkun et al., 2012). Although approximately 700 DBPs have been characterized in drinking water (Richardson, 2018), the GC-MS and

LC-MS quantified DBPs only account for a portion of TOX formed after chlorination (Krasner et al., 2006; 2009; Chen et al., 2015), and the additional TOX is presumed to be from the incorporation of halogens into higher molecular weight dissolved organic matter (DOM) (Zhang and Minear, 2002; 2006).

As stated previously, the active substance TRO is the same for DICD treatment and electrochlorination, and in mesohaline water the oxidant primarily consists of active bromine (HOBr and OBr^-). In mesohaline water, the reaction kinetics are dominated by the protonated form of bromine, HOBr, as the stronger electrophile compared to OBr^- and other lesser contributors of active bromine (e.g. Br_2, BrCl, Br_2O). The TRO species combine with natural organic matter (NOM) to form halogenated organic compounds (Figure 1.3), while also disinfecting the ballast water. In addition to being the primary oxidant in saline water (Table 1.2), HOBr is also more likely to participate in substitution reactions with DOM compared to HOCl (Uyak and Toroz, 2007; Sharma et al., 2014). Electrophilic substitution reactions on DOM moieties reacting with HOBr (Criquet et al., 2015) form primarily brominated DBPs (Br-DBPs) in saline waters (Langsa et al. 2017). Humic substances in DOM are the primary target of halogenation reactions as they contain unsaturated structures with double bonds and aromatic rings. Additional moieties such as hydroxyl and amino groups further increase reactivity (Criquet et al., 2015). Additional halogenation can result in ring opening and saturation of bonds with halogens forming HAAs and THMs. A comprehensive review of HOBr reactions with organic compounds and micro pollutants was carried out by Heeb et al. (2014). Because the majority of ballast water treatment takes place in estuarine and marine waters, the formation of Br-DBPs has been the focus of research on oxidant treated ballast water.

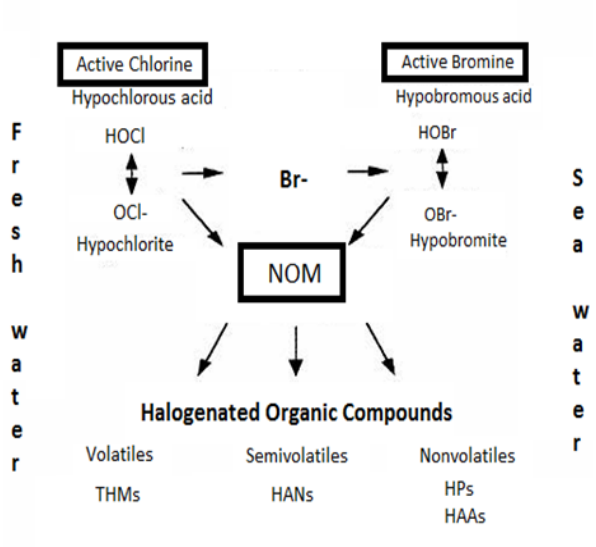

Figure 1.3 Overview of small traditional DBPs forming during chlorination of natural fresh water and sea water.

It is estimated that close to 99% of the applied oxidant dose reacts with DOM and the majority of DBPs are generally formed as a reaction with humic substances in DOM including primarily humic and fulvic acids. However, the structure of these natural biopolymers are complex, and are not definitively characterized. The chemical oxidants react with various DOM moieties via electrophilic substitution forming a diverse pool of small traditional DBPs and higher molecular weight DBPs, the sum of which is estimated by sum parameters of organohalogens (Hua and Reckhow, 2006; Langsa et al., 2017). Unlike the formation of other compounds, the lack of well-characterized DBP precursors hinders the prediction of DBPs by applying oxidant reaction pathways. For this reason, the study of DOM and high molecular weight DBPs is primarily limited to analytical chemists who have used increasingly sensitive methods to analyze DOM precursors and DBPs. Throughout this dissertation, the term high molecular weight DBPs is used for DBPs identified by high resolution MS and is used as a relative term in comparison to lower molecular weight DBPs that have been studied rather extensively and are identified

by GC-MS or LC-MS. As discussed in Chapters 3 and 4, the actual molecular weight of what is identified as high molecular weight DBPs is 160 – 600 Da.

Because the active substance, TRO, is the same for DICD treatment and electrochlorination, the production of DBPs is thought to be similar between BWMS employing chlorination. This similarity can be seen in Chapter 2 of this dissertation for small traditional DBPs that were quantified in treated ballast water. Although similar DBPs will be produced, the quantity and specific DBP production will vary with factors such as treatment type and dose, reaction time, temperature, and characteristics of the uptake water (Hua and Reckhow, 2008; Padhi et al., 2012; Shah et al., 2015). While formation of DBPs in drinking water is complicated, several additional factors increase this complexity in ballast water treatment, especially with regard to brackish water and seawater. These factors include lack of filtration, presence of bromide, higher DOM content and higher doses of oxidant. As previously discussed, the presence of bromide in brackish water leads to more abundant brominated DBPs with the possibility of some chlorinated and mixed halogenated DBPs (Shah et al., 2015). Unlike drinking water, the ballast water intake is not pretreated before adding oxidant, other than the possibility of course filtration (approximately 40-50µm), resulting in the presence of algae in the uptake water that can lead to nitrogen-containing DBPs. The chlorination of NOM during drinking water treatment uses rather low oxidant concentrations (approximately 0.5 mg l^{-1}) compared to ballast water treatment which can be as high as 20 mg l^{-1} TRO. Research has shown that the type of oxidant or the increased dose of oxidant alone can result in higher DBP concentrations and different patterns of both small traditional DBPs (Padhi et al., 2012; Shah et al., 2015) and high molecular weight DBPs (Lavonen et al.,

2013). Lastly, a myriad of DBPs can form from reaction with additional precursors such as pharmaceuticals, personal care products (PCPs), polyaromatic hydrocarbons (PAHs) and pesticides (Richardson, 2018), all of which are potentially found in uptake water from urban centered ports and harbors where ballast water treatment is typically conducted.

Over the past decade, a large data set for small traditional DBPs (e.g. HAAs, THMs, oxyhalides and HANs) formed in ballast water treatment has been gathered by the BWWG from BWMS manufacturers applying for IMO Procedure (G9) approval (i.e. *Procedure for approval of ballast water management systems that make use of Active Substances*). Although valuable, this information has been criticized because details of test conditions and DOC in test water are often lacking (Werschkun et al., 2012), and methods of DBP analysis of treated water have been called into question especially for DBPs in saline waters (Lee et al., 2018). Unfortunately, very little of the IMO approved testing has compared the differences in DBP profiles between water treated with different oxidant-based disinfection methods (e.g. DICD, electrochlorination, sodium hypochlorite) used in ballast water treatment.

In Chapter 2 of this dissertation, a DICD solution was used by the BWMS in the treatment of estuarine ballast water, and treated water was analyzed for small traditional DBPs. In fresh water use of DICD (e.g. swimming pools), the cyanurate ring on chloroisocyanurates can serve as a reservoir for available chlorine (Tachikawa et al., 2002; Yang et al., 2016), thereby moderating formation of traditional small DBPs (Scotte, 1984; Feldstein et al., 1985). However, Feldstein et al. (1985) determined that cyanurate would only be expected to inhibit DBP formation if the process was chlorine-dose

dependent because the presence of cyanurate would change the HOCl concentration. It was also determined that in river water with a chlorine dose of 13 mg l^{-1}, and corresponding cyanurate concentration of 175 mg l^{-1}, the total THM concentration was reduced by 24% after 7 days. The authors concluded that even at high concentrations of cyanurate, only minor effects on THM formation can be expected because only a small portion of THM formation reactions are affected (Feldstein et al., 1985). In recent aquaculture research, the total organic bromine (TOBr) concentrations were similar after treatment with two cyanurate-based disinfectants, trichloroisocyanuric acid (TCCA) and sodium dichloroisocyanurate (DICD), although no comparison to direct chlorination with sodium hypochlorite was conducted (Wang et al., 2017). The authors noted that total organic bromine (TOBr) increased over a five day hold time in the dark, after initial chlorination, and suggested that Br-DBPs were formed continuously over 5 days due to slow release of HOCl from cyanurates. No other research was identified to further support the concept of slow release of active chlorine from cyanurates in marine/brackish waters. In typical ballast water with high oxidant demand, chloroisocyanurates in solution are assumed to rapidly release HOCl and equilibrate with HOBr, making the use of DICD for disinfection functionally equivalent to other methods using strong oxidants. However, in water with a lower oxidant demand, or high concentration of chloroisocyanurates, residual cyanurates may hold available chlorine, thereby potentially affecting formation of both small traditional DBP and high molecular weight DBPs.

1.7.1 Nitrogenous DBPs

The formation of nitrogen-containing DBPs is of increasing concern as research has shown that they are generally more toxic than Br-DBPs without nitrogen (Plewa et

al., 2008; Wagner and Plewa, 2017). It has been recognized that nitrogenous DBPs can form in oxidation reactions with proteins and peptides (Diehl et al, 2000; Sivey et al., 2013). In both drinking water and ballast water treatment the primary source of nitrogen-containing Br-DBPs may be precursors derived from algae present in the water before treatment. Algal organic matter (AOM) has lower aromaticity than other components of NOM, reducing THM formation, but also has higher organic nitrogen, as shown by the higher DOC/DON ratio of AOM (Zhou et al., 2014), leading to nitrogen-containing DBPs (Li and Mitch, 2018). In ballast water treatment with strong oxidants, high algae concentrations in uptake water increases the source of organic nitrogen leading to increased nitrogen-containing Br-DBPs. Although most research on DBP precursors has concentrated on allochthonous sources of DOM, such as the previously mentioned humic substances, precursors from algae such as those found in algal cell exudates and intracellular organic matter released after cell lysis (Bond et al., 2012; Chen et al., 2015) will be significant in eutrophic systems with the extreme conditions found during algal blooms (Yang et al., 2011; Tomlinson et al., 2016; Hua et al., 2017). In the water, these nitrogenous biopolymers can be found in extracellular organic matter (EOM) from algal cell exudates, or intracellular organic matter that that can be released during cell lysis as a result of oxidative treatment (Bond et al., 2012; Chen et al., 2015). The characterization of AOM from different algal species has revealed that DBP formation potentially changes with species and growth phase (Pivokonsky et al., 2014), and that diatoms specifically contribute substantially to production of nitrogen-containing DBPs (Goslan et al., 2017). In other research, chlorination (NaDDC) of artificial seawater resulted in only 2 nitrogen-containing Br-DBPs, versus 32 nitrogen-containing Br-DBPs in chlorinated raw seawater

with algae showing the importance of naturally present algae as a source for DBP precursors.

1.8 Analytical DBP Methods

1.8.1 Sum Parameters of Organohalogens (OX)

Total organic halogen (TOX) and other sum parameters of halogenated DBP are measures of a combination of operationally defined halogenated organic species in a water sample which can include quantified low molecular weight halogenated compounds, and unquantified halogenated compounds of low and high molecular weight. The small traditional DBPs that are normally measured account for only 30-60% of the halogens as measured by TOX in chlorinated water (Richardson, 2002; Krasner et al., 2006; 2009; Chen et al., 2015), and with the diversity and complexity of precursors the total number of DBPs in drinking water alone will likely exceed 1,000 (Li and Mitch, 2018). For this reason, the sum parameters are used to measure the sum of organic halogen in a water sample as measured by a given analytical technique. The difference in quantified DBPs and TOX is most likely due to chlorination products that are not amenable to GC-MS or are not stable enough to survive sample preparation procedures. A large portion of unquantified DBPs are likely high molecular weight halogenated DBPs that are the focus of Chapters 3 and 4 of this dissertation. This is due to the fact that high molecular weight DBPs are not amenable to chemical analysis methods used for smaller DBPs (e.g. HANs, HAAs, THMs, oxyhalides), such as gas chromatography/mass spectrometry (GC-MS) or GC-electron capture detector (GC-ECD) techniques which cannot detect large or polar Br-DBPs (Richardson, 2002; Zhai and Zhang, 2011). Interestingly, research has shown that for the same source water, the TOX level is

positively correlated with toxicity (Hua and Reckhow, 2007; Li and Mitch, 2018) suggesting an additional contribution to toxicity by unquantified DBPs.

All sum parameters of halogens are based on an electro-chemical method called micro-coulometry, although extraction procedures differ for each method. Adsorbable organic halogen (AOX) designates organohalogens that are adsorbable on activated carbon and includes both polar and non-polar DBPs, but is not well suited for marine waters as high amounts of inorganic halogens (e.g. chloride) disturb the adsorption of organic chlorine. Purgeable Organic Halogen (POX) includes all volatile compounds a large portion of which are THMs. The sum of AOX and POX comprises TOX (Khalanski and Jenner, 2012). Sum parameters adapted to marine waters are: POX and Extractable Organic Halogen (EOX). EOX, initially designed for salt water sediments, designates organohalogens extracted in an organic solvent (Jenner et al., 1997), but is hampered by the significant loss of organic halogens during the extraction process (Han et al., 2017).

1.8.2 Quantification of Traditional DBPs

The quantification of traditional DBPs formed with the oxidation of natural waters is complicated by the diversity of DBPs including differences in volatility, number and type halogens, types of moieties, and molecular weight. As a result of this diversity, many methods are required for the extraction/concentration, separation and detection of DBPs. A summary of analytical methods for small traditional DBPs that are often regulated in drinking water is given in Figure 1.4.

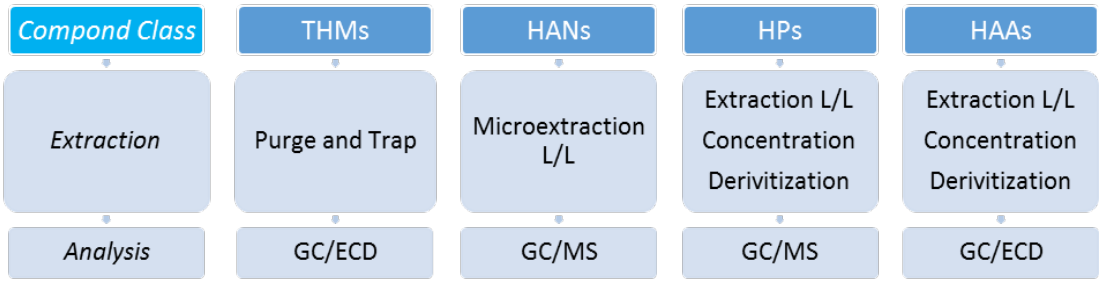

Figure 1.4 Analytical methods used for the determination of the different classes of relatively low molecular weight DBPs.

Because DBPs are found at rather low concentrations, samples are usually concentrated before DBP detection. Early DBP research focused on the identification of small halogenated DBPs identified by using GC-MS and then LC-MS. The progression of analytical techniques from GC/MS, LC/MS and GC/IR for identifying unknown polar and nonpolar DBPs in drinking water is thoroughly reviewed in Richardson (2002). Because of their volatile nature, compounds such as THMs are analyzed by purge and trap method directly without extraction. Semipolar and polar DBPs can be extracted with resin (e.g. polypropiolactone (PPL), C-18) or by liquid-liquid extraction prior to analysis. Spectroscopic methods such as gas chromatography/mass spectrometry (GC/MS) are used to obtain a mass spectra followed by unknown compound identification by comparing their spectra with those in mass spectral libraries. However, many unregulated DBPs are not present in any databases. Early analytical improvements coupled high resolution MS to GC to provide exact mass data which can be used to determine empirical formulas for unknown structures, and fragments of unknown structures, of small volatile or semi-volatile compounds (Richardson, 2002). Also, derivatization techniques are often added to include some polar classes of compounds

(carboxylic acid, polar aldehydes) before GC/MS analysis. LC-MS techniques were later developed to include the separation and detection of non-volatile DBPs, including DBPs with higher molecular weights that were previously unquantified by GC-MS (Richardson, 2002). The analysis of small traditional DBPs is still performed using GC-MS (or GC - ECD), or possibly newer LC-MS methods, for measuring most of the 18 DBPs that are typically regulated in drinking water including five haloacetic acids (HAA5), four THMs, chloral hydrate, dichloroacetonitrile, dibromoacetonitrile, trichloroacetonitrile, cyanogen chloride, formaldehyde, 2,4,6 –trichlorophenol, bromate and chlorate. However, human risk assessments based on epidemiologic studies have shown that the toxic potential of only regulated DBPs such as THMs does not add up to the magnitude of health risks observed (Costet et al., 2011; Neale et al., 2012). The additional toxicity that was unaccounted for by regulated DBPs led to interest in discovering new DBPs, and development of techniques that could identify DBPs that may contribute to the overall toxicity of oxidant treated waters. Research has also shown that a large number of emerging DBPs are cytotoxic, neurotoxic, mutagenic, genotoxic, carcinogenic and teratogenic (Richardson et al., 2007; Wagner and Plewa, 2017).

In addition to DBPs with natural organic precursors, DBPs that have precursors of anthropogenic origin have also been identified as summarized in the annual literature review of emerging contaminants (Richardson and Ternes, 2018). These DBPs precursors include many pharmaceutical and personal care products (PCPPs), herbicides, artificial sweeteners, estrogens and PAHs. Many of these anthropogenic precursors are expected to be more problematic in certain applications such as treatment of fresh and saline wastewater effluents, or possibly ballast water treatment when water is taken from

industrialized ports with urban surroundings.

1.8.3 Detection of High Molecular Weight DBPs

The vast majority of DBP analysis on oxidant treated water have been conducted with gas chromatography/mass spectrometry (GC-MS), a method which cannot detect large or polar DBPs (Richardson, 2002; Zhai and Zhang, 2011). However, the difference in TOX and quantified individual DBPs is substantial, and a large portion of the difference is likely due to high molecular weight halogenated DBPs. The primary DBP precursor in natural waters is humic substances in DOM, which are comprised of large complex biopolymers that are not definitively characterized. A possible structure for humic acid (Figure 1.5) shows the complexity of humic substances and is representative of the overall complexity of DOM (Stevenson, 1994). Chemical oxidants can combine via electrophilic substitution with DOM moieties forming high molecular weight halogenated DBPs that are largely uncharacterized, in addition to relatively small characterized DBPs.

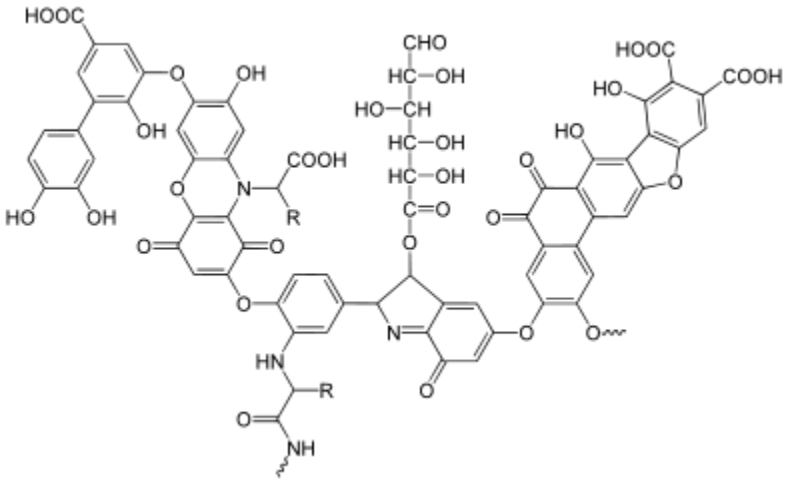

Figure 1.5 Proposed structure of humic acid including quinone and phenol moieties as well as other components.

As a result, a significant portion of TOX formed in chlorinated water was not identified until recently with new analytical techniques. These techniques include ultrahigh resolution FT-ICR MS (Zhang et al., 2014; Gonsior et al., 2015), electrospray ionization-triple quadrupole mass spectrometry (UPLC/ESI-tqMS) (Zhai and Zhang, 2011; Zhai et al., 2014) and Orbitrap MS (Postigo et al., 2016). The development of these new analytical techniques has continued to close the gap between identified DBPs and TOX. One emerging method ultrahigh resolution FT-ICR MS uses non-target analysis which is able to identify numerous DBPs in water samples despite the presence of high DOM concentrations. FT-ICR MS is the most powerful HR mass spectrometer, with sufficient resolution to identify the molecular composition of DBPs by mass measurement alone (Stenson et al., 2003). This allows the determination of molecular formulas of numerous DBPs, as well as the chemical structure of single halogenated DBPs up to eight carbons and even larger multiple halogenated DBPs. The use of FT-ICR MS in DBP research was pioneered by Zhang et al. (2012) initially to investigate high molecular weight chlorinated DBPs, and later used to characterize brominated DBPs (Zhang et al., 2014). The technique has since been reported in other studies for use in monitoring the incorporation of chlorine (Lavonen et al., 2013; Gonsior et al., 2014b), and bromine (Gonsior et al., 2015) into DOM.

Recently, LC-Orbitrap instruments have been used to detect halogenated DBPs (Postigo et al., 2016; Wang and Helbling, 2016; Shao et al., 2018). Although FT-ICR MS sensitivity (i.e. resolution and mass accuracy) is unparalleled, Orbitrap MS is similar in many respects and can more easily be coupled to chromatography systems.

Liquid direct infusion using electrospray ionization with tandem mass

spectrometry (ESI-MS/MS) and triple quadrupole MS detection (ESI-tqMS) have been used extensively for DBPs in drinking water and wastewater. The ESI-MS/MS technique was initially developed by Zhang et al. (2005) to identify high molecular weight chlorinated DBPs in drinking water and was later modified to ESI-tqMS in order to selectively detect brominated and iodinated DBPs by using paired precursor ion scans of *m/z* 79 or 81 (Zhang et al., 2008) and *m/z* 126.9 (Ding and Zhang, 2009) for bromine and iodine, respectively. The ESI-tqMS technique has also been coupled with ultra-performance liquid chromatography (UPLC) to help determine structures of newly identified DBPs (Zhai and Zhang, 2011; Pan and Zhang, 2013; Zhai et al., 2014; Pan et al., 2016a), including cyclic or aromatic brominated and iodinated DBPs (Ding and Zhang, 2009; Pan and Zhang, 2013; Pan et al., 2017). Most relevant to this dissertation, the ESI-tqMS technique has been successfully employed to identify and provide structures for brominated DBPs in chlorinated saline wastewater (Ding et al, 2013; Gong and Zhang, 2015).

The exploration of methods such as FT-ICR MS, Orbitrap MS and UPLC/ESI-tqMS for identification of higher molecular weight DBPs is an important step towards fully evaluating oxidant treated discharge waters and potentially the surrounding environment. The focus of Chapters 3 and 4 are high molecular weight DBPs formed in DICD (Chapter 3) and electrochlorination (Chapter 4) treatment of ballast water as identified with FT-ICR MS. As discussed in Chapter 4, these large and potentially aromatic DBPs are thought to be more lipophilic with higher logP values and have an increased potential to permeate cells and bioaccumulate compared to smaller aliphatic DBPs.

1.8.4 Degradation and Stability of DBPs

Knowledge of the stability of DBPs can help determine the likelihood of a DBP to persist in the environment and as a result enable accurate exposure assessments to humans and aquatic organisms. Documentation of traditional DBPs in treated waste water (Krasner et al., 2009) and drinking water systems (Krasner et al., 2006), including some brominated DBPs, is widely available as are mechanisms for DBP degradation. However, documentation of high molecular weight DBPs and research on mechanisms of degradation and environmental stability are absent. Most degradation studies of DBPs have been conducted with regulated DBPs in freshwater systems where it was shown that DBPs are subject to many abiotic and biotic transformation processes, and that hydrolysis is an important mechanism of DBP removal (Liang and Singer, 2003; Zhang et al., 2009). The degradation information for a variety of DBPs from six different compound classes representing both regulated DBPs (i.e. THMs and HAAs) and non-regulated DBPs found that the relative importance of hydrolysis, abiotic reductive dehalogenation, and biodegradation depends on the DBP structure and environmental conditions (i.e. pH, temperature, dissolved oxygen, bacteria present, etc.) (Hozalski et al., 2008). Most brominated DBPs are susceptible to abiotic reductive dehalogenation, some are susceptible to hydrolysis, and brominated HAAs are readily biodegraded under aerobic conditions (Chen et al., 2008; 2011). In fact, the primary mechanism of small HAA transformation is through aerobic biodegradation, with larger HAAs less vulnerable to these mechanisms (Landmeyer et al., 2000; Hozalski et al., 2008). THMs, although universally volatile, are relatively recalcitrant to aerobic biodegradation but degrade extensively in anaerobic conditions (Vogel, 1993). Importantly, although most bacteria

that degrade multi-halogenated DBPs can also degrade mono-halogenated DBPs, the opposite is not true so that a greater number of bacteria are available for transformation of less halogenated DBPs. Similar to the mechanisms of DBP formation, the degradation mechanisms for high molecular weight DBPs are complicated because of their highly complex structure.

1.9 Rationale for Dissertation Research

At the start of this research, limited toxicity test data suggested that ballast water treated with oxidants had only minor impacts on toxicity test organisms from three trophic levels; vertebrates, invertebrates and algae. However, algal toxicity testing was mostly limited to two diatom species, *Skeletonema costatum* and *Phaeodactylum tricornutum*, which did show some minor toxic effects from oxidant treated ballast water. With an understanding that toxicity of many compounds can vary widely between species of algae, I started testing ballast water with a new species, *Isochrysis galbana*. In past years I had had some experience cultivating this species as a food for bivalve larvae and recognized that it would be a good candidate species for toxicity testing. Initially, I also attempted to use several other species of algae that I was less familiar with, but hoped that they would be acceptable for toxicity testing. However, these other species were eliminated as candidates for toxicity testing for several reasons including primarily slow growth and an inability to thrive at the estuarine salinities required for testing waters from Baltimore, USA. The Tahitian Isochrysis (T-ISO) strain of *Isochrysis galbana* proved to be robust at a wide range of salinities, and could easily achieve the minimum growth requirements that were standard in other fresh water and marine algal toxicity test methods (USEPA, 2002; OECD, 2011; ISO, 2016). Although several test methods were

considered, the USEPA (2002) method for fresh water algae was selected and adapted for estuarine/marine algae testing. Once *Isochrysis galbana* was selected for toxicity testing, I became immediately aware that this species was substantially more sensitive to oxidant treated ballast water compared to previously tested species.

Preliminary research conducted in 2009 and 2010 at the Wye Research and Education Center (WREC) showed algal toxicity using *Isochrysis galbana* in ballast water after treatment by a BWMS employing electrochlorination (Figure 1.6; Ziegler et al., 2010). All toxicity tests were conducted on samples that were electrochlorinated and stored for five days in ballast tanks, followed by dechlorination at the time of discharge. Statistical analyses were based on algal cell counts, after conversion into cell densities, using ToxCalc™ 5.0 software (Tidepool Scientific Software, McKinleyville, CA, USA). Mean cell density values were tested for growth reductions compared to the control treatment with a one-tailed Dunnett's test and a p value of 0.05 was used for all hypotheses testing. The BWMS used relatively high levels of chlorination with TRO values measured between 8 - 12.2 mg/L. The dechlorination of treated water after the five day storage time was accomplished with either bisulfite (Trials 1and 2) or thiosulfate (Trials 3-5), resulting in final measured TRO values below the detection limit of 0.02 mg L^{-1} as measured by colorimetric assay using a handheld Hach Pocket Colorimeter™ II (Hach, Model No. 58700, U.S.A). Toxicity was found in water samples from all 5 trials (Figure 1.6). Initially, only control and 100% treated water samples were toxicity tested (Trials 1 and 2), followed by tests with dilution series v/v of treated water consisting of either a 32 – 100% series (Trials 3 and 4) or a full 0.56 dilution series (10, 18, 32, 56 and 100%).

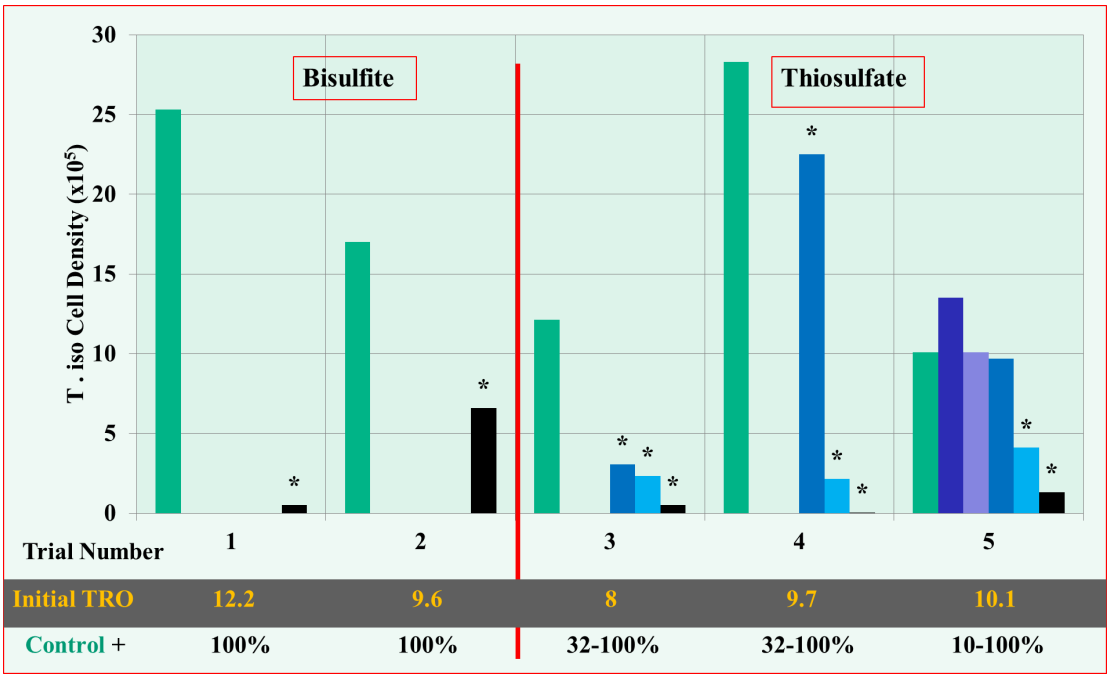

Figure 1.6. Algal toxicity (*Isochrysis galbana*) of ballast water (5 trials) after electrochlorination, and de-chlorinated (sulfur compounds) after 5 day ballast tank holding time.

* Statistically different from control.

Toxicity testing of chlorinated (sodium hypochlorite) and dechlorinated (bisulfite or thiosulfate) estuarine water also revealed that toxicity is species specific with *Isochrysis galbana* exhibiting decreased growth when TRO was below the limit of detection (Figure 1.7A). In contrast, the same treated and dechlorinated water samples were non-toxic to the diatom *Phaeodactylum tricornutum* (Figure 1.7B).

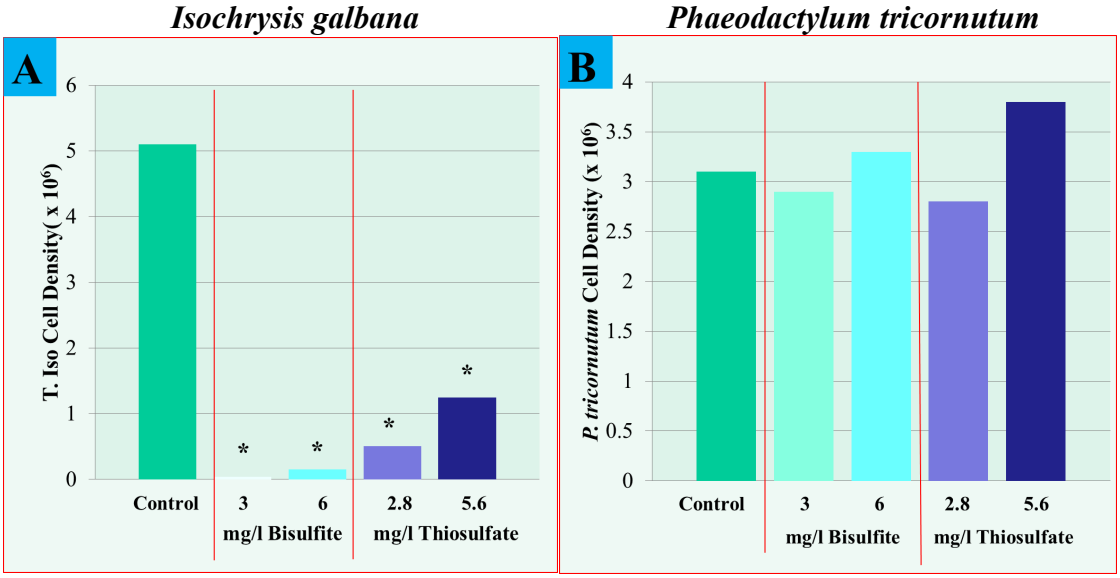

Figure 1.7 Algal toxicity of chlorinated estuarine water after five days holding time and de-chlorination. Initial TRO = 6.9 mg l^{-1}. Test species are A) *I. galbana* and B) *P. tricornutum*. Sulfur dechlorinating agents are bisulfite (3 and 6 mg l^{-1}) and thiosulfate (2.8 and 5.6 mg l^{-1}). * Statistically different from control.

A literature review of available toxicity test data revealed that most of the algal toxicity testing on oxidant treated water used either *P. tricornutum* or another diatom species *Skeletonema costatum* as the test species. In 2010, these were the preferred algal species recommended by the GESAMP-Ballast Water Working Group to satisfy the toxicity testing requirement for approval of BWMS under IMO guideline G9 "*Procedures for the Approval of Ballast Water Management Systems That Make Use of*

Active Substances". The BWWG Methodology (IMO, 2012) recommended algal toxicity testing according to ISO-10253, Water quality – Marine algal growth inhibition test with *Skeletonema* sp. and *Phaeodactylum tricornutum*. This recommendation by the BWWG resulted in the availability of algal toxicity test data for only these two algal species, which revealed that oxidant treated ballast water is occasionally toxic to *S. costatum* (Delacroix et al., 2013), while *P. tricornutum* is mostly resistant (Figure 1.6; Ziegler et al., 2010).

1.10 Dissertation Framework

BWMS have been developed to minimize the release of potential invasive species in discharged ballast water. The most common ballast water treatment methods rely on strong oxidants which form numerous small DBPs and can result in toxicity to microalgae. The research presented in this dissertation was conducted to advance the understanding of treated ballast water toxicity and to document the formation of high molecular weight DBPs using ultrahigh resolution mass spectrometry. Commercial BWMS that are based on treatment with strong oxidants, either DICD addition or electrochlorination, were used to produce the treated water that was studied in this dissertation. The BWMS were tested in estuarine waters with a salinity range of 5.2 – 7.1 PSU. Chapters 2, 3 and 4 of this dissertation are research chapters written and organized as manuscripts for publication in scientific journals. Chapter 2 of this dissertation reports on the longevity of toxic effects of treated ballast water from four oxidant-based BWMS to a sensitive marine microalgae species (*Isochrysis galbana*), and is published in Marine Pollution Bulletin (Ziegler et al., 2018). Three of the BWMS used electrochlorination of ballast water for treatment, while the other BWMS was based on direct injection of a

DICD solution. Chapter 3 focuses on the transformation of DOM and formation of high molecular weight DBPs after treatment with a DICD-based BWMS, and is published in Environmental Science and Technology (Ziegler et al., 2019). Chapter 4 reports on the relative persistence of some dibrominated high molecular weight DBPs formed after ballast water treatment by electrochlorination. Finally, Chapter 5 gives some final thoughts and direction for future research.

Chapter 2

Long-term Algal Toxicity of Oxidant Treated Ballast Water

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Abstract

National and international regulations require that ships' ballast water is treated to minimize the risk of introducing potentially invasive species. A common approach employed by commercial ballast water management systems is chlorination. This study presents the algal toxicity findings for three chlorination-based BWMS and their implications to environmental safety of port waters receiving treated ballast water from ships. Discharged treated ballast water from all three BWMS was toxic to algae with IC25s (25% growth inhibition) ranging from 9.9% to 17.9%, despite having total residual oxidant concentrations below 0.02 mg l^{-1}, based on Whole Effluent Toxicity assays. When held at 4 °C, some of the ballast water samples continued to exhibit toxic effects with no observed effect concentrations as low as 18% after a 134 day holding time. Thirteen individual disinfection by-products were measured above the detected limit at the time of discharge. No correlation between DBPs and algal toxicity was observed.

2.1 Introduction

Ballast water is used by modern ships to maintain balance, maneuverability and structural integrity. However, the discharge of ballast water can lead to the release of a

variety of non-indigenous species (NIS) at ports around the world (Ruiz et al., 1997). Introduced NIS propagules can lead to invasions and result in extensive economic, ecological, and human health impacts (Carlton, 1985; Ruiz et al., 1997, 2000; Drake et al., 2007). Although there are a number of unquantified variables (NRC, 2011), the implementation of ballast water management strategies, such as open ocean ballast water exchange (BWE) and ballast water treatment, can limit invasion success by reducing the number of propagules discharged in ballast water. Assuming a dose-response relationship for propagule pressure and establishment success, a reduction in establishment of new invasive species is expected with reduced propagule supply (Ruiz and Carlton, 2003; Lockwood et al., 2005).

To address this significant environmental and economic problem, the U.S. Coast Guard and the International Maritime Organization (IMO) have established ballast water regulations to minimize the introduction of potentially invasive species from ships. The International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM Convention) of the IMO (IMO, 2004, 2008a, 2017a), and similar regulatory instruments implemented by individual countries (e.g. USCG 33 CFR 151, 2012; USEPA, 2013a; 2013b; NZMPI, 2016), require ships to treat their ballast water with certified Ballast Water Management Systems (BWMS) and to meet numeric discharge standards for live organisms in different size classes. To date, over 70 BWMS have been Type Approved by IMO, and the USCG has Type Approved six systems. Approximately 26% of the 69 IMO Type Approved BWMS use some form of chlorination (e.g. electrochlorination, dichloroisocyanurate dihydrate, hypochlorite), and almost all of these systems have the ability to neutralize treated water before discharge.

Procedure (G9) of the BWM Convention, "Procedures for the Approval of Ballast Water Management Systems That Make Use of Active Substances" (IMO, 2004), calls for an overall review of BWMS including environmental safety of discharged ballast water. Under Procedure (G9), BWMS are evaluated following a methodology specifically designed for evaluating BWMS (IMO, 2012). The Methodology calls for toxicity testing of discharged ballast water with a vertebrate, invertebrate and algal species according to internationally accepted toxicity test methods (e.g. OECD, ISO, USEPA).

Toxicity test results from scientific presentations (Ziegler et al., 2010) and peer-reviewed journal articles (Delacroix et al., 2013; Park et al., 2017), as well as toxicity test data submitted by BWMS manufacturers for Procedure G9 review (www.imo.org), show frequent algal toxicity of discharged ballast water when strong oxidants are employed as the treatment biocide. Algal toxicity testing outside of the ballast water realm has also shown that chlorinated water can remain toxic to micro algae after the loss or neutralization of TRO (Gentile et al., 1976; Sanders, 1984; Ziegler et al., 2010; Lee et al., 2015; Rhie, 2016).

The chemistry of chlorinated fresh water is complicated, involving a cascade of reactions which can lead to small, well defined disinfection by-products (DBPs), as well as larger halogenated organic molecules that are not typically identified during DBP analysis (Richardson, 2003). The production of DBPs results from the interaction between oxidants and natural organic matter (NOM) in water (Westerhoff et al., 2004). The addition of chlorine to fresh water results in rapid hydrolysis, forming active chlorine (HOCl and OCl^-) and leading to chlorinated DBPs in fresh water, with the inclusion of brominated DBPs (after reaction with HOBr and OBr^-) in estuarine and marine waters

(Ichihashi et al., 1999; Nokes et al., 1999; Werschkun et al., 2012; Shah et al., 2015). The quantity and type of DBPs can vary and is related to multiple factors including oxidant type/dose, contact time, dissolved organic matter (DOM) concentration and composition, temperature, bromine content and pH (Chowdhury et al., 2009; Shah et al., 2015; Hao et al., 2017). Research has identified smaller traditional DBPs as well as over 600 higher molecular weight DBPs in drinking water (Richardson, 2011; Zhai and Zhang, 2011; Ding et al., 2013), and 462 brominated DBPs in ballast water following treatment by electrochlorination (Gonsior et al., 2015). There is also the possibility of oxidant reactions with other pollutants found in urban waters which can result in additional halogenated compounds (Benitez et al., 2011; Acero et al., 2013; Heeb et al., 2014).

The vast majority of available information on toxicity of chlorination based BWMS is from dossiers submitted to IMO (IMO, 2016a) during the IMO approval process under Procedure G9 (IMO, 2008b). Typically, DBP analysis and toxicity testing of treated ballast water is conducted at 0, 1 or 2 days, and 5 days (to link with IMO G8 Guidelines for efficacy testing), and is assumed to incorporate the "worst case scenario" for DBP concentrations. Ballast water risk assessments include possible toxic effects of individual DBPs measured in ballast water, while combined effects of DBPs and any residual oxidant are addressed with whole effluent toxicity (WET) testing, which is considered a more realistic measure of mixture toxicity of effluents (Johnson et al., 2006).

The persistence of algal toxicity in discharged ballast water after 5 days has not been addressed scientifically. A review of available data revealed that there can be an increase in some DBPs over a 5-day holding time (IMO, 2014), presumably as a result of

the continuing interaction between organics and TRO, or as breakdown products of larger halogenated molecules. To the authors' knowledge, no long-term (i.e. >5 days) toxicity testing or DBP analysis of treated ballast water has been conducted. Here, we present the results of algal toxicity tests conducted in 2015 and 2016 from 3 different oxidant-based BWMS, investigating the longevity of treated ballast water toxicity after storage at 4°C. BWMS tests were conducted in accordance with the collaborative USEPA/USCG, Environmental Technology Verification (ETV) Protocol (USEPA, 2010). Smaller traditional DBP compounds (haloacetic acids (HAAs), haloacetonitriles (HANs) and trihalomethanes (THMs)) were only measured at the time of discharge in an attempt to correlate observed toxicity to the initial concentration of DBPs.

2.2 Materials and Methods

2.2.1 Test Site Facility

The test site facility was located in Port Covington, Baltimore, Maryland, USA, adjacent to a large commercial port in an industrial area of Baltmore City. Estuarine salinities at this location are typically in the range of 5 – 11 PSU. The testing of each BWMS was carried out following USCG performance standards outlined in the ETV protocol (Table 2.1). Control and treated ballast tanks were thoroughly cleaned by pressure washing between all treatment events, and all piping was flushed with potable water from a municipal source.

2.2.2 Uptake

Test waters were drawn from the surface of Winans Cove (Baltimore, MD, USA) through a flexible inlet pipe allowing uptake from different depths. There was no manipulation or addition to the natural plankton community. However, dissolved organic

carbon (DOC), total suspended solids (TSS), and particulate organic carbon (POC) were enhanced to coincide with ETV challenge water conditions. Amendment of uptake water included the addition of sodium citrate dihydrate (Fisher Scientific, USA), Arizona fine test dust (Arizona Powder Technology, Inc.; Burnsville, Minnesota) and Micromate-micronized humate (Mesa Verde Resources; Placitas, New Mexico) for increasing DOC, TSS, and POC, respectively (Table 2.1). A slurry containing TSS, POC and DOC amendments was injected during ballast water uptake before separating into untreated and BWMS treated ballast water lines. The slurry was mixed with a propeller mixer (Brawn™ Mixer Inc., model MD75-870) in a cone-bottom HDPE tank (1.1 m^3). Delivery of slurry into the ballast water uptake line was by peristaltic pump (Eccentric Pumps LLC, model SLP-218). The exact slurry recipe was based on estimates of ambient water conditions, targeted flow rate through the intake pipe, and tank volume. Water was taken from the surface with no manipulation of ambient salinity. Control and treated waters were delivered to independent control and treated water ballast tanks, and held for 48 h (Systems 1B, 2 and 3), or 72 h (System 1A) in closed ballast tanks.

Parameter	ETV	IMO G8	System 1 A		System 1 B		System 2		System 3	
			Amb	**Adj**	**Amb**	**Adj**	Amb	Adj	Amb	Adj
TSS mg l^{-1}	**≥ 24**	**≥ 50**	**4.9**	**31.6**	**3.3**	**33.6**	**18.5**	**57.1**	**11.7**	**34.5**
POC mg C l^{-1}	**≥ 4**	**> 5**	**1.1**	**4.8**	**1.2**	**5.3**	**8.0**	**14.7**	**1.2**	**4.7**
DOC mg C l^{-1}	**≥ 6**	**> 5**	**3.1**	**7.7**	**3.3**	**7.9**	**4.6**	**8.2**	**3.9**	**8.2**

Table 2.1 Minimum USCG and IMO concentrations for DOC, POC and TSS compared to ambient (Amb) and adjusted (Adj) concentrations of uptake water for each ballast water treatment event. Amendments of uptake water included sodium citrate, Arizona fine test dust, and Micromate for increasing DOC, TSS and POC, respectively.

2.2.3 Discharge

At discharge, untreated water samples were collected in 20-L polycarbonate carboys directly from a hatch on the untreated ballast tank for use as control and dilution water in toxicity tests. A continuous, time-integrated sample of treated ballast water was collected by an in-line sample port and delivered to a 100-L fiberglass sample container. The treated water sampling was conducted during the entire treated water discharge process of approximately 1 h. When necessary to meet the local TRO discharge standard, treated water was neutralized by the BWMS before sample collection. Treated samples were collected from the 100-L container by gravity flow into 20-L glass carboys, which were immediately transferred to ice filled coolers for transport to the University of Maryland Wye Research and Education Center (WREC) for toxicity testing.

2.2.4 Ballast Water Management Systems

Three BWMS (Systems 1, 2 and 3) were tested that employed filtration and treatment with strong oxidants (Table 2.2). Systems 1 and 3 employed in-situ electrochlorination, and System 2 used sodium dichloroisocyunarate dihydrate (DICD) granules dissolved in water with direct injection of the disinfecting solution. Each BWMS had a target TRO dose, or an initial TRO dose range, for treatment of ballast water during uptake. System 1 was tested at two different target TRO doses, 6 mg l^{-1} (System 1A) and 8 mg l^{-1} (System1 B). System 2 had a target TRO dose between 11 and 13 mg l^{-1}, and System 3 had a target TRO dose of 15 mg l^{-1} (Table 2.1). After treatment, water was held in ballast tanks for 2 d (Systems 1B, 2 and 3) or 3 d (System 1A). Each system had the ability to add neutralizer during discharge to keep the TRO below 0.1 mg l^{-1}, the local maximum acceptable discharge limit, in accordance with the local discharge

permitting authority, the Maryland Department of the Environment (MDE). Neutralization of TRO in discharged ballast water with sodium sulfite (System 1A) or sodium bisulfite (Systems 1B, 2 and 3) injection was adjusted by the BWMS.

	BWMS Type	Target TRO Dosage (mg l^{-1})	Discharge TRO (mg l^{-1})
System 1A	Electrochlorination	6	0.01
System 1B	Electrochlorination	8	0.00
System 2	DICD	11-13	0.02
System 3	Electrochlorination	15	0.00

Table 2.2 Ballast water management systems with target TRO dose and average TRO measured in ballast water discharge samples. Discharge TRO is the average of measurements taken at beginning, mid-point and end of the discharge event.

2.2.5 *Chemical Analysis*

2.2.5.1 *POC, DOC and TSS*

Chemical analyses of POC, DOC and TSS were carried out for each BWMS on water collected at uptake, both before and after the addition of compounds, to reach ETV minimum POC, DOC and TSS concentrations (Table 2.1). Water samples were collected and shipped according to the analytical laboratory's instructions. Analysis was conducted according to USEPA Standard Methods: Method 160.2 for TSS (USEPA, 1979), and Method 415.1 for POC and DOC (USEPA, 1999).

2.2.5.2. *Total Residual Oxidant*

Measurements of the TRO concentration in discharged ballast water were made with a handheld colorimetric TRO meter based on the DPD (n,n-diethyl-p-phenylene diamine) method. The DPD method is recommended by the USEPA (method 330.5) for monitoring oxidants in wastewater discharge (USEPA, 1983). TRO measurements were

taken in triplicate on samples collected at the beginning, middle and end of the overall discharge period (approximately 1 h). The DPD reagent reacts with oxidants to form a magenta solution (i.e. Würster dye), proportional to total oxidant concentration, and the DPD Würster dye is measured at 530 nm. The TRO of discharged ballast water was measured with a handheld Hach Pocket Colorimeter™ II (Hach, Model No. 58700, U.S.A) in Low-Range mode (0.02-2.0 mg l^{-1} TRO as Cl_2), following the manufacturer's instruction manual (Hach Company, 2013; USEPS, 1983). When TRO was measured in untreated uptake water (considered interference), the treated water TRO value was adjusted by subtracting the TRO value of untreated water to obtain the final reported TRO concentration. TRO is a measure of combined and free oxidants including hypochlorite ion, hypochlorous acid, hypobromite ion and hypobromous acid. Although TRO can include chlorine and bromine, TRO values are reported in chlorine equivalents (i.e. as Cl_2).

2.2.5.3 D*isinfection By-products*

Chemical analysis for 24 DBPs (Table 2.3) was conducted on control ballast tank water and treated ballast water (after neutralization where indicated) at the time of discharge. Treated water samples for DBP analysis were collected from the time integrated sample container (100-L fiberglass) that was also used for collecting algal toxicity test samples. Samples for DBP analysis were collected and handled according to directions provided by the analytical laboratory conducting the analysis. All DBP analyses were conducted at National Environmental Laboratory Accreditation Program (NELAP) accredited laboratories according to USEPA methods (Table 2.3).

Chemical Group	USEPA Method	Compounds
Halogenated Methanes	524.2	Trichloromethane, Dichlorobromomethane, Dibromochloromethane, Tribromomethane
Halogenated Hydrocarbon	524.2	1,2,3-Trichloropropane
Halogenated Acetic Acids	552.2	Monochloroacetic acid, Dichloroacetic acid, Trichloroacetic acid, Monobromoacetic acid, Dibromoacetic acid, Bromochloroacetic acid, Dibromochloroacetic acid, Tribromoacetic acid, total HAAs
Halogenated Acetonitriles	551.1	Monobromoacetonitrile, Dibromoacetonitrile, Bromochloroacetonitrile, Chloroacetonitrile, Dichloroacetonitrile, Dichlroacetonitrile, Tricloroacetonitrile
Inorganics	300.0	Chlorate, Bromate
Halogenated Propionic Acid	515.3	Dalapon
Halogenated Nitroalkane	551.1	Chloropicrin

Table 2.3 List of analytes, with chemical group and method of analysis, for land-based BWMS testing.

2.2.6 *Toxicity Test Methods and Experimental Design*

2.2.6.1 *Algal Cultures*

The microalgal strains used were *Isochrysis* aff. *galbana* (UTEX LB 2307), a marine haptophyte, and *Phaeodactylum tricornutum* (UTEX 646), a marine diatom. Both species were obtained from University of Texas (UTEX) Culture Collection of Algae. *Isochrysis galbana* is in the subclass prymnesiophycidae and the specific strain is often referred to as Tahitian Isochrysis (T-ISO). Algae was cultured at 20 °C in f/2 medium (Guillard and Ryther, 1962), with the addition of sodium silicate for *P. tricornutum*, under continuous fluorescent lighting. The algal growth media for stock cultures was prepared by the addition of f/2 micro nutrients, macro nutrients, and vitamins to filtered (0.22 μm) estuarine water (Wye River, MD, USA). The salinity was adjusted in stock cultures with CrystalSeas Marine Mix Bioassay Formula® salts to approximately match the toxicity test salinity.

2.2.6.2 *Algal Growth Inhibition Tests*

Algal toxicity tests were conducted at the University of Maryland WREC. Algal toxicity testing protocols followed the method given in "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms" (USEPA, 2002), with several adaptations for testing estuarine water samples. Briefly, the freshwater USEPA method was adapted for estuarine water by changing the algal growth media to f/2 (Guillard and Ryther, 1962), running tests at 20°C, and conducting tests with appropriate algal species (*I. galbana* and *P. tricornutum*) that were able to meet minimum growth criteria for estuarine salinities.

A summary of the toxicity test method and performance criteria is given in Table 2.4. For ballast water testing with *I. galbana*, a dilution series (0.56) of treated and untreated ballast water samples was prepared in 250-ml beakers after warming ballast water samples to 20°C. All beakers received equal quantities of micro- and macronutrients that make up f/2 algal growth media. Six replicate flasks (150-ml) were prepared for control water, and 4 replicate flasks were prepared for each treated water dilution. Test flasks were inoculated with algae from a stock culture in log growth phase to obtain an initial cell density of 2×10^4 cells ml^{-1} in each test flask. The algal toxicity tests were carried out under controlled conditions consisting of 20 ± 1 °C and continuous illumination (cool white light of 11,000 lux). During toxicity testing, flasks were continuously agitated on a compact rotator (Thermo Scientific, Model 88880025) at 100 rpm. Water quality data was taken on Day 0 and included pH, dissolved oxygen and salinity for each test treatment. Toxicity tests were conducted for either 72 or 96 hours, depending on the time needed for control replicates to reach a minimum density

of 1.0 x 10^6 cells ml^{-1}. At test completion, 20 µl of hydrochloric acid was added to a 1 ml sample of algae to stop cell movement and assist in counting. Final cell counts were done using a hemacytometer (Bright-Line, Reichert) or a Sedgewick-Rafter counting chamber (Graticules Ltd., England) on a compound light microscope (Leitz-Laborlux D, Germany). Average cell counts from four discreet samples were used to determine the cell density in each replicate as needed for statistical analysis. A minimum growth requirement of 1 x 10^6 cells ml^{-1} for control replicates was applied to all toxicity tests.

Test type:	Static**,** non-renewal
Test duration:	72 or 96 hours
Temperature:	20 ºC ± 1ºC
Light quality	"Cool white" fluorescent lighting
Light intensity:	86 ± 8.6 µE/m^2/s
Photoperiod:	Continuous illumination
Salinity:	12 psu
Test chamber size:	250-ml Erlenmeyer flasks
Test solution volume:	100 ml
No. replicate chambers per concentration:	4, 6 control replicates
Renewal of test solutions:	None
Age of test organisms:	Log growth phase
Initial cell density in test chambers:	2 x 10^4 cells ml^{-1}
Shaking rate:	100 rpm, continuous on an orbital shaker
Aeration:	No
Nutrient solution:	f/2 culture media
Dilution water:	Untreated ballast water
Test concentrations:	At least 4 concentrations and a control
Dilution factor:	0.56 dilution series
Endpoint:	Population growth (cell counts)
Performance criteria:	≥ 1 x 10^6 cells ml^{-1} control mean. Cell density variability (CV%) ≤ 20% among control reps.

Table 2.4 Summary of test method for the algae *Isochrysis galbana* and *Phaeodactylum tricornutum* growth tests.

2.2.7 Statistical Analyses

Algal growth (cell density) estimates were based on final cell counts. USEPA recommended statistical tests (USEPA, 2002) were used as a means of quality control for toxicity test results. The homogeneity of variance was determined by Bartlett's test, and the normality by the Shapiro-Wilk's test. Inhibition concentration percentage (ICp) values (IC_{25} = 25% reduction in cell count compared to control) for algal tests were estimated using the USEPA suggested Linear Interpolation Method (USEPA, 2002, Appendix M). All statistical tests were performed using ToxCalc™ 5.0 software package (Tidepool Scientific Software, McKinleyville, CA, USA).

2.2.8 Laboratory Toxicity Tests

Different species of algae may have varying susceptibilities to toxicity from oxidant treated water. To assess the potential differences, laboratory tests were conducted with *I. galbana* and *P. tricornutum* using sodium hypochlorite as an oxidant. Culturing and testing of *P. tricornutum* followed *I. galbana* protocols, but with the addition of sodium silicate to the f/2 medium used for culturing and toxicity testing.

Reagent grade sodium hypochlorite (Fisher Sci., 5% total Cl) was added to filtered (0.45 μm) estuarine water in an acid washed 15-L glass carboy to attain an initial TRO of 6.9 mg l^{-1}. Treated water was held at room temperature in the dark for 5 days followed by neutralization with sodium thiosulfate or sodium bisulfite. After 5 days, the 15-L sample was split into four glass beakers (2-L). Each beaker received a dose of neutralizer for final concentrations of thiosulfate (2.8 and 5.6 mg l^{-1}) or bisulfite (3 and 6 mg l^{-1}). Only undiluted samples of treated and neutralized water were tested with statistical comparison to untreated control water. Algal nutrients were added into

control and each treatment (2-L beakers) before distribution into test flasks (150-ml) and inoculation with algae. Final TRO concentrations were measured before the addition of algae to test flasks.

2.3 Results and Discussion

2.3.1 *Toxicity of Discharged Ballast Water*

Basic water quality parameters of discharged ballast water are given in Table 2.5. Day 0 algal toxicity tests were started within 2 hours of a ballast water discharge event.

Parameter	System 1A	System 1B	System 2	System 3
pH	7.41	8.21	7.96	M
DO (mg l^{-1})	8.6	5.7	8.5	6.8
Salinity (PSU)	6.5	5.2	7.1	5.4
Temp. (°C)	22.4	27.2	23.8	29.1

M = missing data

Table 2.5 Basic water quality of uptake water for ballast water testing events conducted on BWMS test barge in Baltimore, Maryland, USA.

Discharged ballast waters from all four discharge events, including three different chlorination based BWMS, were toxic to *Isochrysis galbana* in algal toxicity tests based on inhibition of population growth (Table 2.6). IC_{25} values for initial tests were 17.9%, 10.7%, 9.9% and 11.9% for Systems 1A, 1B, 2 and 3, respectively (Table 2.6). Toxicity testing included discharge water from two electrochlorination based systems, one of which (System 1) was tested at two different TRO target doses, and a system based on injection of a DICD solution (System 2). Although the algal toxicity test is designed to quantify a reduction in population growth compared to control, we also observed negative population growth due to mortality in higher concentrations of treated water from all systems. This resulted in lower final algal cell densities compared to initial cell

densities. Enumeration of algal cells at lower densities was conducted on a Sedgwick-Rafter counting chamber instead of a hemocytometer. Comparison of the cell density data collected by these two counting methods is thought to be problematic as the determination of algal cell vitality leads to an uncertainty in final cell counts. Because there was no definitive way of determining cell viability, and the endpoint in the algal toxicity tests was a reduction in population growth, all intact algal cells were counted. Ultimately, however, IC_{25} values were unaffected by the negative population growth counts because the IC_{25} endpoint is based on treatments producing only 25% reductions in cell density.

BWMS Treatment Type TRO Target dose	Discharge TRO* (Neutralization)	Days after neutralization	IC_{25} (%)
System 1A Electrochlorination 6 mg l^{-1} as Cl_2	0.01 mg l^{-1} as CL_2 (Sulfite neutralization)	0	17.9
		35	36.2
		59	36.9
		97	66.7
		126	>100
		161	>100
System 1B Electrochlorination 8 mg l^{-1} as Cl_2	0.00 mg l^{-1} as CL_2 (Sulfite neutralization)	0	10.7
		32	15.9
		60	20.61
		92	29.0
		131	28.2
System 2 DICD 11-13 mg l^{-1} asCl_2	0.02 mg l^{-1} as CL_2 (Bisulfite, 5 min. start-up neutralization only)	0	9.9
		36	>100
		64	>100
System 3 Electrochlorination 15 mg l^{-1} as Cl_2	0.00 mg l^{-1} as CL_2 (Bisulfite neutralization)	0	11.9
		34	10.5
		102	20.9
		134	16.4

Table 2.6 Algal toxicity test results from 3 BWMS (Systems 1 – 3). Tests were conducted on day of discharge, followed by approximately monthly toxicity testing, until treated water sample was exhausted. * Discharge TRO corrected for interference (i.e. background TRO measurement in control tank).

The majority of toxicity research with oxidants in water, and all research with chlorination based BWMS, has been conducted with short hold times after treatment (i.e. toxicity tests are started after 5 days or less). Although an attempt is made to compare these oxidant toxicity tests, in some instances it is complicated because of differences in how the tests were conducted, the endpoints that are estimated (cell density, ATP, inhibition of photosynthesis, cell division), and how the results are presented (e.g. NOEC, LOEC, EC_X, IC_x, relative growth, percent reduction in endpoints). With these limitations

in mind, the initial algal toxicity observed in the current research seems more severe than algal toxicity reported in other oxidant-based BWMS studies, including results presented at meetings, in published papers, and in BWMS dossiers submitted to IMO for Procedure (G9) approval. Research conducted by Delacroix et al. (2013) and Park et al. (2017) showed varying results for algal toxicity tests of oxidant-based BWMS. Toxicity testing of five chlorination based BWMS with *Skeletonema costatum* was conducted by Delacroix et al. (2013). Results showed that 75% of discharged ballast water samples were toxic, with 25% of these toxic samples having what was considered an acute effect (50% reduction in growth or EC_{50}), and the remainder having only a chronic effect (EC_{10}). Park et al. (2017) used *I. galbana* to test the toxicity of electrochlorinated seawater that included organic carbon additives to increase DOC. Electrochlorinated water was held for one day and neutralized before toxicity testing. The sample containing lignin as the organic carbon additive showed a toxic effect with a complete inhibition of algal growth in the undiluted sample. The potential for increasing algal toxicity with organic carbon additives used to boost DOC is discussed further in section 3.3.

2.3.2 *Algal Toxicity and TRO Concentrations*

TRO concentrations in all discharged ballast waters were below 0.1 mg l^{-1}, the limit required by the local jurisdictions' toxic materials discharge permit. After subtracting the background control tank TRO, the final reported TRO measurements were 0.00 for two discharges (System 1B and System 3), and 0.01 mg l^{-1} and 0.02 mg l^{-1} for System 1A and System 2, respectively (Table 2.2). Systems 1A, 1B and 3 added neutralizer during the entire discharge process, while System 2 only neutralized during an

initial startup period of 3 minutes. Algal toxicity was similar for all treated discharge waters with IC_{25} values ranging from 9.9% to 17.9% (Table 2.6). Notably, the lowest IC_{25} was observed with System 2 (IC_{25} = 9.9) which had the highest calculated discharge TRO of 0.02 mg l^{-1}, and was only neutralized during the initial 3 minutes of discharge. Delacroix et al. (2013) observed no correlation between residual oxidant (i.e. FRO) and algal toxicity in oxidant-based BWMS testing of brackish waters ($R^2 < 0.01$).

Although published results of BWMS toxicity testing are limited, algal toxicity results from specific oxidants are available. As with oxidant treated ballast water, the rapid decay of oxidants complicates the toxicity testing procedures, and results are often difficult to compare. The toxicity values found in most published papers are related to the initial dose of a specific oxidizing chemical or solution. In contrast, our results measured the TRO of discharged ballast water after several days of reaction time with estuarine water in ballast tanks. Sathasivam et al. (2016) reported a 72h median EC_{50} value of 0.071 mg l^{-1} based on algal cell counts for the green algae, *Clostriu ehrenbergii*. Earlier work by the same group with marine dinoflagellates reported EC_{50} values of 0.584 mg l^{-1} and 1.177 mg l^{-1} for *Cochlodinium polykrikoides* and *Prorocentrum minimum*, respectively (Ebenezer and Ki, 2013). Another study of marine species reported an EC_{50} of 2.91 mgl^{-1} NaOCl for *Dunaliella salina*, and an EC_{50} of 1.73 mg l^{-1} NaOCl for *I. galbana* (López-Galindo et al., 2010). NaOCl toxicity results for several freshwater chlorophytes have also been reported with an EC_{50} of 1.6 mg l^{-1} for *Pseudokierchneriella subcapitata*, and 5.1 mg l^{-1} for C*hlorella salina* (Pitanga, 2011). In the current research, toxicity was found in all treated ballast waters (Table 2.6) despite adjusted TRO

measurements at or below the minimum detection limit for the Hach DPD method (0.02 mg l^{-1}).

2.3.3 *Disinfection By-products Concentrations*

Overall, for all treated ballast water discharge samples, 13 of the 24 individual DBP compounds were measured above the detection limit (Table 2.7).

System #		1		2	3
		A	B		
Treatment type		Electrochlorination		DICD	Electrochlorination
Target TRO (mg l^{-1} as Cl_2)		6	8	11 -13	15
Substance (ug/l)	**EPA Method**				
Bromate	300.0	BDL	BDL	BDL	BDL
Bromoacetonitrile	551.1	BDL	13	8	21
Bromochloroacetic acid	552.2	1.1	2.4	<1	8.7
Bromochloroacetonitrile	551.1	BDL	BDL	BDL	BDL
Chlorate	300.0	BDL	BDL	BDL	BDL
Chlorodibromoacetic acid	552.2	13	21	19	39
Dibromoacetic acid	552.2	9.4	31	11	140
Dibromoacetonitrile	551.1	BDL	BDL	BDL	BDL
Dibromochloromethane	524.2	40.1	56.2	52.1	67
Dichloroacetic acid	552.2	<1	<1	<1	<1.0
Dichloroacetonitrile	551.1	19	BDL	BDL	BDL
Dichlorobromomethane	524.2	7.6	9.9	8.6	10.9
Tribromoacetic acid	552.2	83	200	81	190
Tribromomethane (bromoform)	524.2	210	31.6	225	684
Trichloroacetic acid	552.2	1.3	1.8	<1	1.3
Trichloromethane (chloroform)	524.2	2.1	4.1	2	2
1,2,3-Trichloropropane	524.2	BDL	BDL	BDL	BDL
Dalapon	515.3	BDL	BDL	BDL	BDL
Monobromoacetic acid	552.2	<1	1.3	<1	1.8
Monochloroacetic acid	552.2	<2	<2.0	<2	<2.0
Total HAA5	552.2	10.7	34.1	11	143.1
Chloral Hydrate	551.1	110	0.82	ND	ND
Chloroacetonitrile	551.1	1.5	0.8	0.96	1.3
Chloropicrin	551.1	BDL	BDL	BDL	BDL
Trichloracetonitrile	551.1	BDL	BDL	BDL	BDL
Sum of all DBPs		509	408	419	1310

BDL = Below detection limit
Highest concentrations of each DBP highlighted.

Table 2.7 List of disinfection by-products and concentrations found in discharged ballast water including analytical methods. Samples collected from three BWMS during land-based testing on barge located in Baltimore, MD, USA.

A summary of DBPs with concentrations above 10 µg l^{-1} as well as total HAA5 is shown in Fig. 2.1 for each BWMS treatment event. The three systems with a TRO target dose under 13 mg l^{-1} (Systems 1A, 1B and 2) had similar overall DBP production, with the sum of all DBPs ranging from 408 to 508 µg l^{-1}. In contrast, System 3 with the highest TRO target dose had the highest concentrations of 8 of the 14 measured DBPs. Some of the individual DBP concentrations were much higher in System 3 treated water, leading to substantially higher values for the sum of all DBPs (1,310 µg l^{-1}) and total HAA5 (143 ug l^{-1}) compared to all other treatment systems, with HAA5 values below 35 µg l^{-1} and total DBPs below 509 µg l^{-1} (Table 2.7). Interestingly, the second highest value for the sum of all DBPs was found in System 1A treated water with the lowest TRO dose (Target TRO = 6 mg l^{-1}).

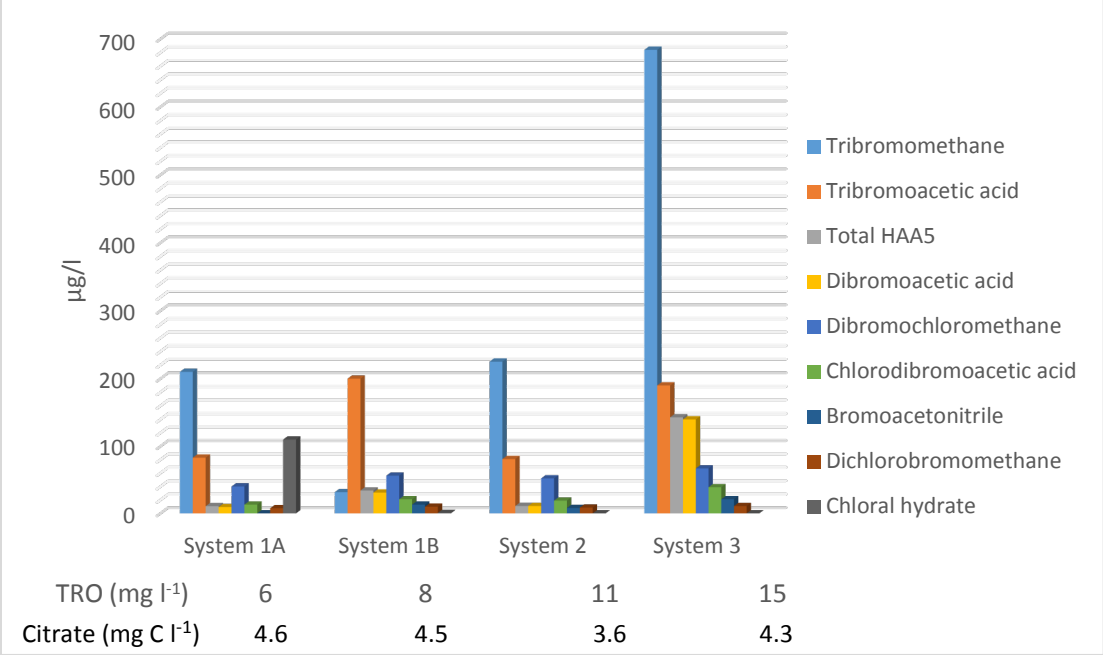

Figure 2.1 Concentration of 8 DBPs and total HAA5 in discharged ballast waters collected on day of discharge (Day 0) from 4 discharge events. TRO values are target treatment concentrations for BWMS presented in order of lowest to highest target TRO dose concentration. Citrate was added to uptake water before treatment to increase DOC content.

Discharge waters from all electrochlorination based systems (Systems 1A, 1B and 3) had DBP concentrations that generally followed a trend of increasing DBP production with increasing TRO target dose. As already stated, System 3 (TRO target = 15 mg l^{-1}) contained the highest DBP concentrations for 8 of the measured DBPs (Table 2.7). System 1B (TRO target = 8 mg l^{-1}) had the highest concentrations of tribromoacetic acid, trichloroacetic acid and trichloromethane. System 1A, with lowest TRO dose (TRO target = 6 mg l^{-1}), had the highest concentrations for chloroacetonitrile and chloral hydrate, and was the only treated water with dichloroacetonitrile. Although the highest concentration of tribromomethane was found in System 3 treated water (with the highest TRO dose), the concentration of tribromomethane in System 1B treated water was 31.6 µg l^{-1}, substantially lower than all other systems which were above 210 µg l^{-1}.

Interestingly, System 2 (the only DICD based BWMS, TRO target = 11-13 mg l^{-1}) never contained the highest DBP concentrations, and concentrations of DBPs were often lower than those found in System 1B (TRO target = 8 mg l^{-1}) treated ballast water. Also, for 5 DBP compounds (bromoacetonitrile, bromochloroacetic acid, tribromoacetic acid, trichloromethane and trichloroacetic acid), concentrations were lower in System 2 than in all other systems (Table 2.7). Although other factors could be involved in the formation of DBPs (e.g. concentration and composition of DOC in uptake water), System 2 with DICD disinfection and TRO target dose of 11-13 mg l^{-1} produced discharge water with lower concentrations of many DBPs compared to the electrolysis based system (System 1) with lower target TRO doses of 6 or 8 mg l^{-1}.

As described in Section 2.1.1, ambient water was amended with sodium citrate, Arizona Fine Test Dust, and Micromate for increasing DOC, TSS and POC, respectively.

Additives were used to increase ambient levels of these parameters to reach minimum concentrations outlined in the ETV Protocol. The ambient and final adjusted DOC, TSS and POC concentrations are given in Table 1.2 for all BWMS test events. Of the three classes of additives, the DOC concentration and content is thought to have the greatest impact on the formation of DBPs. However, in the current research, the concentrations of individual DBPs and the sum of all DBPs did not show a correlation with the amount of citrate added to the ambient water (Table 2.7, Fig. 2.1). This is most evident in System 2 which had the highest citrate concentration and none of the highest measured DBP concentrations. The ambient DOC, and hence the quantity of citrate needed to reach the ETV minimum concentration, was similar for all tests ranging from a high of 4.6 mg Cl^{-1} for System 2 to a low of 3.1 mg C/l for System 1A (Table 1.2). These similar initial DOC measurements resulted in final citrate additions that increased the DOC by 4.6, 4.6, 3.6 and 4.3 mg C l^{-1} for Systems 1A, 1B, 2 and 3, respectively (Table 1.2).

In a review of dossiers submitted to IMO for BWMS approval, we found that chlorinated brackish water typically resulted in higher THM concentrations than chlorinated sea water. Werschkun et al. (2012) reviewed DBP concentrations in brackish water (i.e. 18.8 - 22.6 psu) treated by 10 oxidant-based BWMS and, in agreement with the current research (Table 2.7) and Delacroix et al. (2013), reported that tribromomethane was the primary THM compound. HAA concentrations, however, were dominated by dibromoacetic acid in other publications (Werschkun et al., 2012; Delacroix et al., 2013) in contrast to the current research which identified tribromoacetic acid as the most abundant HAA for all BWMS (Fig. 2.1).

2.3.4 Algal Toxicity and Disinfection By-products Concentrations

Algal toxicity tests and DBP analyses were conducted on samples collected on the day of treated water discharge (Day 0). Plots of IC_{25} values versus DBP concentrations are shown in Fig. 2.2 for the eight most abundant DBPs (i.e. > 2 μg l^{-1}), total HAA5, and sum of all DBPs. System 1A, with the lowest TRO dose, had the lowest concentration of 7 DBP categories, and was the least toxic sample (IC_{25} of 17.9%) in algal tests (Table 2.6). Other than System 1A, there is no clear correlation shown between individual DBPs, or DBP groups, and initial algal toxicity across the four treated ballast water discharges (Fig. 2.2). Similar to the current research, Delacroix et al. (2013) found no correlations between algal toxicity (*S. costatum*) and measured DBPs in discharge water from five oxidant-based BWMS with TRO values below 0.08 mg Cl l^{-1}.

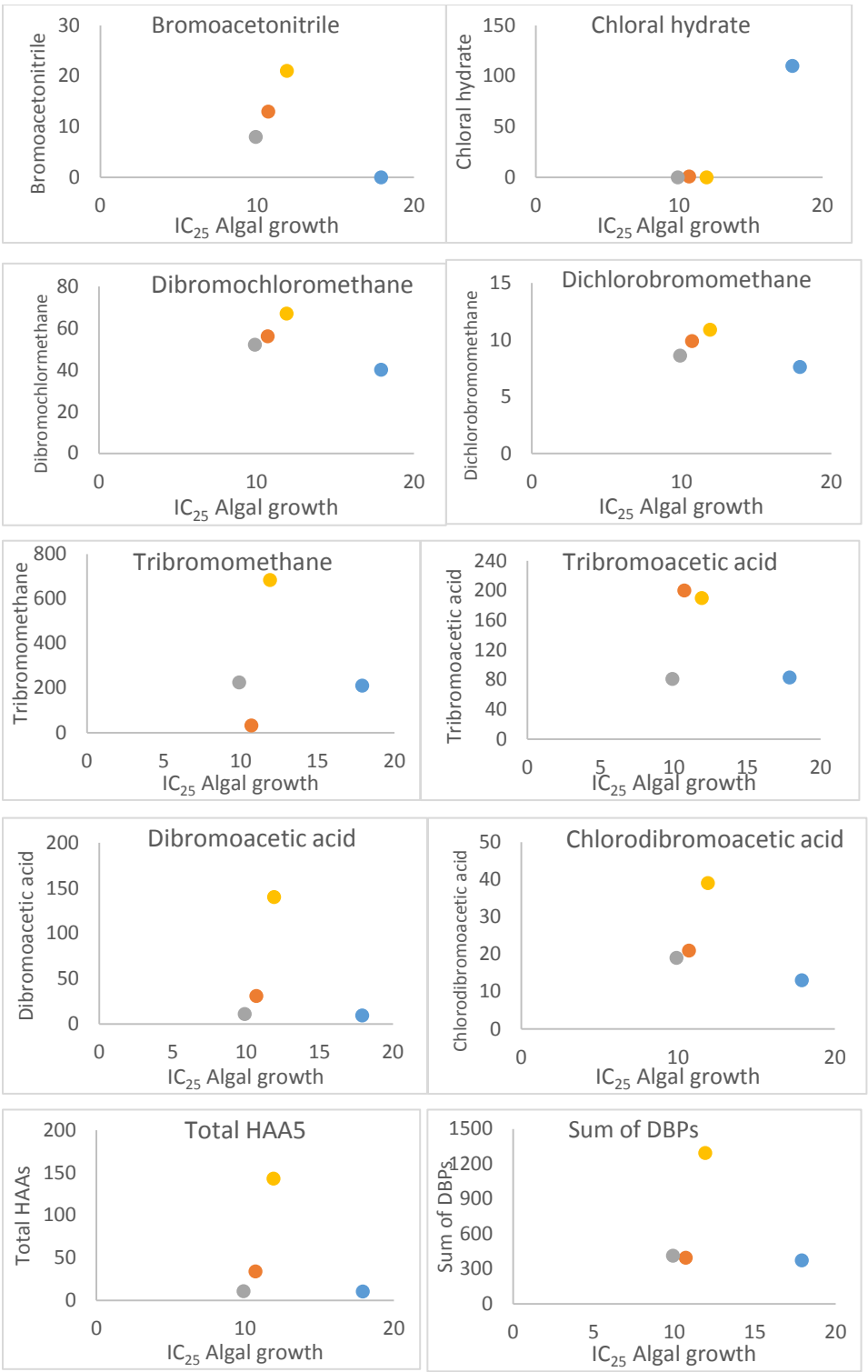

Figure 2.2 Concentration of DBPs (µg l^{-1}) and the IC_{25} value (as percentage of treated water) for algal toxicity in treated discharge samples from ● System 1A, ● System 1B, ● System 2 and ● System 3. DBP samples were collected, and toxicity tests started, on the day of discharge (Day 0).

In order to assess the potential toxicity of individual DBPs to algae, a direct comparison was made of DBP concentrations and ecotoxicity values. Table 2.8 lists the lowest algal ecotoxicity values identified in the literature alongside the highest concentrations of DBPs that were detected in the current research (Table 2.7). No algal ecotoxicity data was identified for five DBPs including: bromoacetonitrile, bromochloroacetic acid, chlorodibromoacetic acid, dichloroacetonitrile and chloroacetonitrile (Table 2.8). The ecotoxicity values for three DBPs (dichlorobromomethane, tribromomethane, chloral hydrate) were more than one order of magnitude greater than the concentrations of DBPs found in treated discharge water. The remaining six DBPs (dibromoacetic acid, dibromochloromethane, tribromoacetic acid, trichloroacetic acid, trichloromethane, and monobromoacetic acid) had algal ecotoxicity values that were at least two orders of magnitude greater than the highest DBP concentrations found in treated ballast water (Table 2.8).

	Lowest identified ecotoxicity data for algae				Highest Conc.
DBP compound	Species	End point	Conc. (mg l^{-1})	Ref.	Mg l^{-1}
Bromoacetonitrile		No data identified			0.021
Bromochloroacetic acid		No data identified			0.0087
Chlorodibromoacetic acid		No data identified			0.039
Dibromoacetic acid	*Isochrysis galbana*	NOEC, 96 h growth salt water	98	3	0.140
Dibromochloromethane	*Pseudokirchneriella subcapitata*	EC50, 72 h growth fresh water	6.1	2	0.067
Dichloroacetonitrile		No data identified			0.019
Dichlorobromomethane	*Pseudokirchneriella subcapitata*	NOEC, 72 h growth fresh water	0.8	2	0.0109
Tribromoacetic acid	*Isochrysis galbana*	NOEC, 96 h growth salt water	250	3	0.200
Tribromomethane	*Pseudokirchneriella subcapitata*	NOEC, 96 h growth fresh water	10	4	0.684
Trichloroacetic acid	*Pseudokirchneriella subcapitata*	NOEC, 48 h growth fresh water	1.0	5	0.0018
Trichloromethane	*Chlamydomonas reinhardii*	EC10, 72 h growth fresh water	3.6	6	0.0041
Monobromoacetic acid	*Scenedesmus subspicatus*	EC50, 96 h growth fresh water	0.2	7	0.0018
Chloral hydrate	*Scenedesmus quadricauda*	EC3, 7 d growth fresh water	2.8	1	0.110
Chloroacetonitrile		No data identified			0.0015

(1) Bringmann and Kuhn (1980). (2) Japanese Government (2007). (3) Fisher et al. (2014). (4) USEPA (1978). (5) Garten and Frank (1984). (6) Brack and Rottler (1994). (7) Kühn and Pattard (1990).

Table 2.8. Lowest algae ecotoxicity values and highest concentration of DBPs found in ballast water discharge (IMO, 2017b).

The DBP profile, and potentially its influence on algal toxicity, in treated water can vary with multiple factors, including oxidant dose and the concentration and composition of DOC (Werschkun et al., 2014). The algal toxicity of oxidant treated water has been shown to increase with rising DOC concentrations (Lee et al., 2015). Algal toxicity was also shown to vary with the composition of organic matter (Park et al., 2017). Lee et al. (2015) found that the addition of starch before chlorination (sodium hypochlorite) caused a reduction in population growth despite neutralization (bisulfite). Toxicity testing was conducted on several freshwater (*Selenastrum capricornutum* and *Scenedesmus obliquus*) and saltwater species (*I. galbana* and *P. tricornutum*). Although limited to five days, researchers found that an increase in reaction time between organic matter (i.e. starch) and TRO resulted in greater algal toxicity. They also observed a greater reduction in *I. galbana* growth compared to the other species tested. The authors suggested that an increase in 'reaction products', resulting from an increase in chlorine contact time, was responsible for increased algal toxicity, although no analysis for halogenated by-products (e.g. DBPs) was conducted (Lee et al., 2015). In a follow up study at the same laboratory and employing the same chlorination and treatment methods (Rhie, 2016), greater algal toxicity was observed with increased storage time (5 days) using a different organic matter additive (glucose), and two different species of algae (*Raphidoceli subcapitata* and *Chlorella vulgaris*). As with the earlier study (Lee et al., 2015), Rhie (2016) found that one species was more sensitive noting a greater reduction in algal population growth in tests using *R. subcapitata* as the test species. A comparison of results between these two studies (Lee et al., 2015; Rhie, 2016) shows that the type of

organic carbon (e.g. glucose versus starch) present in the uptake water prior to chlorination can affect algal toxicity.

Park et al. (2017) used microalgae, *I. galbana*, and bioluminescent bacteria, *Vibrio fischeri*, to compare the toxicity of electrochlorinated seawater containing starch (to increase POC), and one of several other organic carbon additives (glucose, sodium citrate, Metamucil®, lignin, and methyl cellulose) to increase DOC. When neutralized after one day, the only toxicity observed was in the lignin-amended water where the researchers found a complete inhibition of algal growth in the undiluted sample. A similar level of algal toxicity (i.e. complete inhibition of growth) was observed in the same lignin-amended water, but with a test that started after a treated water holding time of 5 days post-neutralization (Park et al., 2017), showing the persistence of toxicity. While other studies have shown limited algal toxicity of treated ballast water (Delacroix et al., 2013; Lee et al., 2015; Rhie, 2016), the initial toxicity of chlorinated and neutralized water observed by Park et al. (2017) is the only instance where toxicity (complete algal growth inhibition) seems comparable to that observed in the current research. Park et al. (2017) concluded that algal toxicity was caused by DBPs, but no comparison of DBP concentrations with algal ecotoxicity values was conducted. Although several studies (Lee et al., 2015; Rhie, 2016; Park et al., 2017) show a change in toxicity due to DOC content in oxidant treated water, no direct link has been shown between quantified DBPs, or groups of DBPs, and toxicity.

2.3.5 *Longevity of Algal Toxicity*

The initial algal toxicity of treated ballast water was similar for all BWMS (Table 2.6), and did not correspond to TRO target dose, measured DBP concentrations, or

BWMS treatment method (i.e. electrochlorination or DICD solution). The follow up toxicity tests, however, showed substantial differences in toxicity between BWMS employing different treatment methods (i.e. electrochlorination or DICD solution). System 2 treated ballast water, based on injection of a DICD solution, showed a dramatic reduction in algal toxicity in the first follow up toxicity test after a 36 day holding time (Fig. 2.3, Fig. 2.4C). In fact, no toxicity was observed in this first follow up toxicity test of DICD treated water, compared to the initial IC_{25} of 9.9% (Fig. 2.3). Interestingly, System 2 had the second highest TRO target dose (11-13 mg l^{-1}) and the highest calculated discharge TRO (0.02 mg l^{-1}). System 1A, with the lowest TRO target dose and a calculated discharge TRO of 0.0 mg l^{-1}, also lost all toxicity in follow up algal testing, but not until after a much longer holding time of 126 days (Table 2.6, Fig. 2.3). Systems 1B and 3, both electrochlorination based systems, continued to show adverse effects in algal growth tests for the duration of follow up testing (Table 2.6, Fig. 2.4), which concluded with toxicity tests that started on Day 131 (System 1B), and Day 134 (System 3). Target TRO dose and discharge TRO values were 8 mg l^{-1} and 0.01 mg l^{-1} for System 1B, and 15 mg l^{-1} and 0.0 mg l^{-1} for System 3 (Table 2.6). Results show that like initial toxicity, there is no correlation between TRO target dose or discharge TRO, and the longevity of treated discharge sample toxicity. Although many other factors may be relevant, these results indicate that the treatment method itself could be a factor in the longevity of discharge water toxicity.

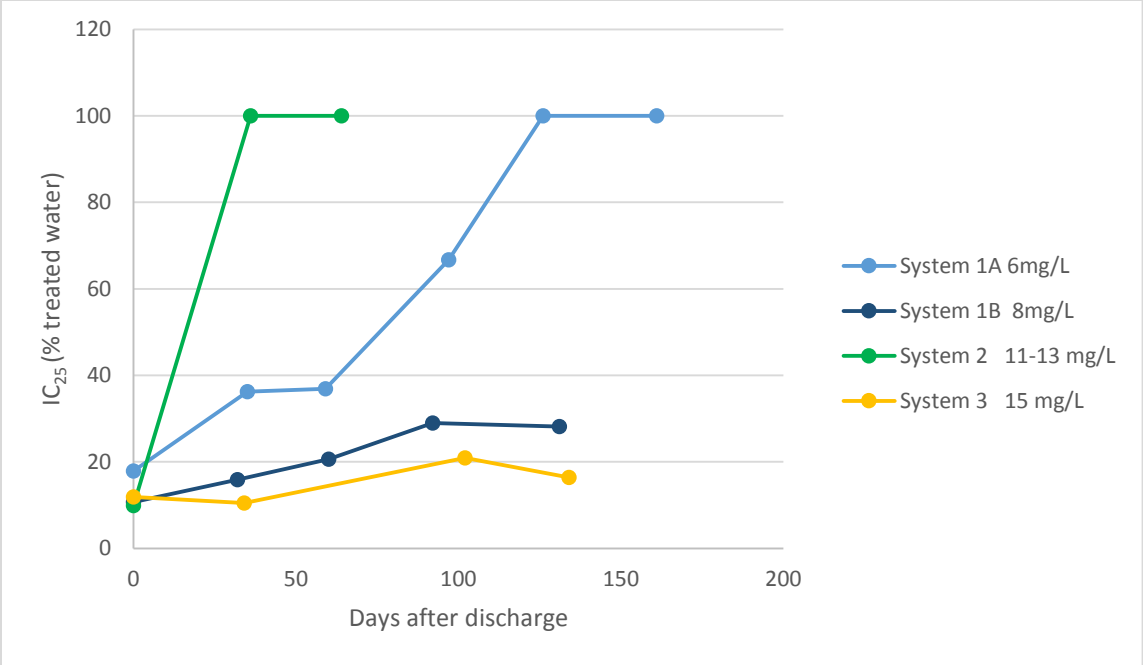

Figure 2.3 Algal toxicity (IC_{25}) of treated ballast water on the day of discharge, and after a storage period in the dark at 4 °C, for four discharge events. Legend includes BWMS and target TRO dose concentration.

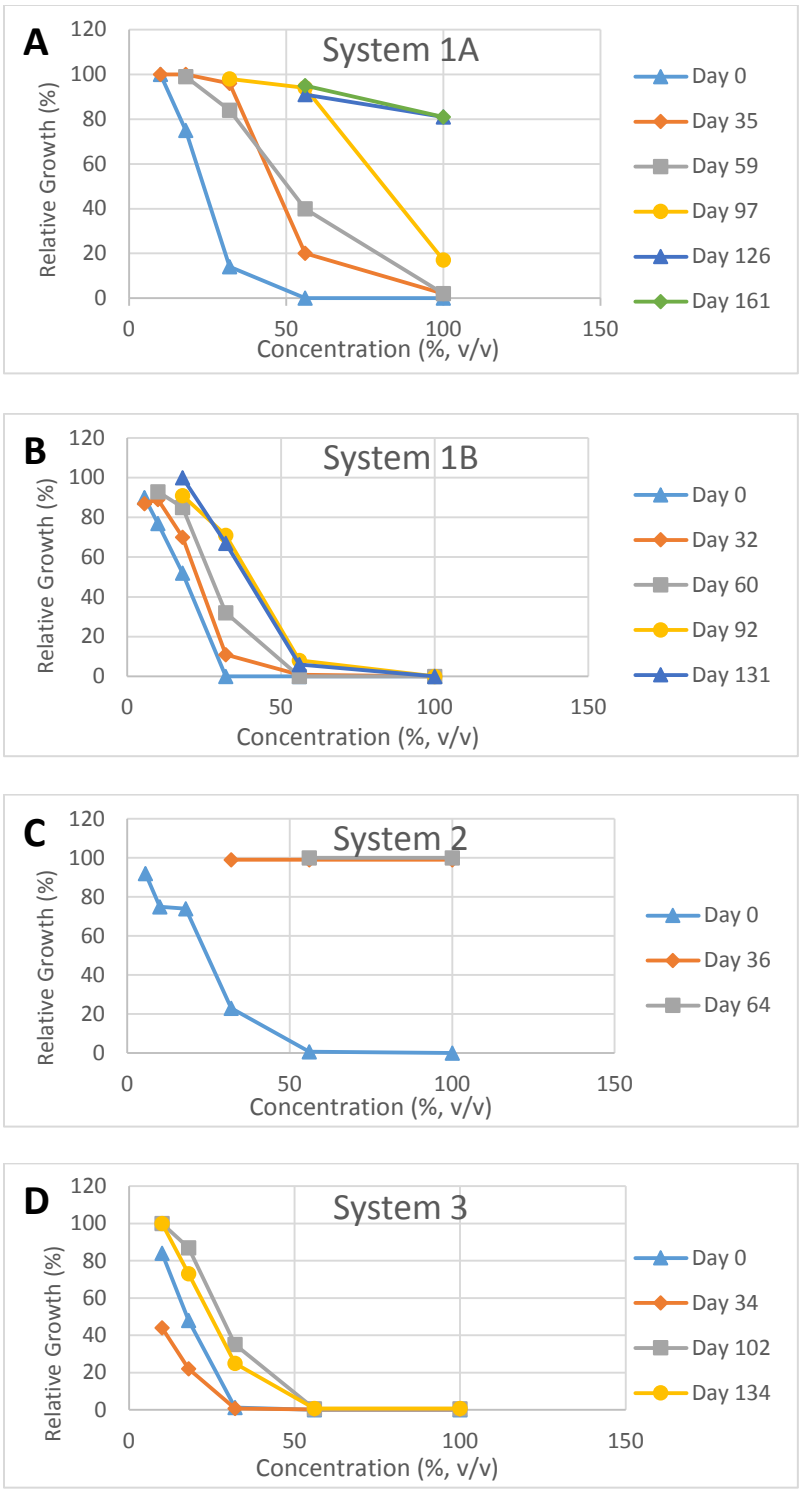

Figure 2.4 Relative growth rate (%) of *I. galbana* compared to control for dilutions of treated ballast water from four discharge events. Results are shown for algal toxicity tests started on Day 0, and after storage at 4 °C

2.3.6 *Laboratory Testing: Algal Species Comparison*

After the addition of neutralizers and f/2 media components, TRO was at the detection limit (0.02 mg l^{-1} as Cl_2) for all sodium hypochlorite treated and neutralized samples. Algal toxicity test results on neutralized samples show a substantial difference in algal sensitivities (Fig. 2.5). After neutralization with sodium thiosulfate and sodium bisulfite, all samples remained toxic to *I. galbana,* with significant reduction from control density of 4.8 x 10^6 cells ml^{-1} (Fig. 2.5A). Final cell densities for thiosulfate neutralized water were 0.51 x 10^6 cells ml^{-1} for the low concentration (2.8 mg l^{-1}), and 1.2 x 10^6 cells/ml for the high concentration (5.6 mg l^{-1}), while final cell densities were 0.04 x 10^6 and 0.15 x 10^6 cells ml^{-1} for low (3 mg l^{-1}) and high (6 mg l^{-1}) bisulfite concentrations, respectively. Toxicity tests with *P. tricornutum*, however, revealed no significant reduction in population growth compared to control in any of the treated and neutralized water samples (Fig. 2.5B). Algal toxicity tests with neutralizing agents alone (i.e. without prior sodium hypochlorite addition) showed that thiosulfate and bisulfite were not toxic to these algal strains at concentrations of 100 mg l^{-1} (data not presented). Interestingly, in hypochlorite treated water algal growth for both species was greater in the samples with higher neutralizer concentrations for both bisulfite and thiosulfate, suggesting that there may be some unquantified toxic compounds that are lost with the higher neutralizer doses.

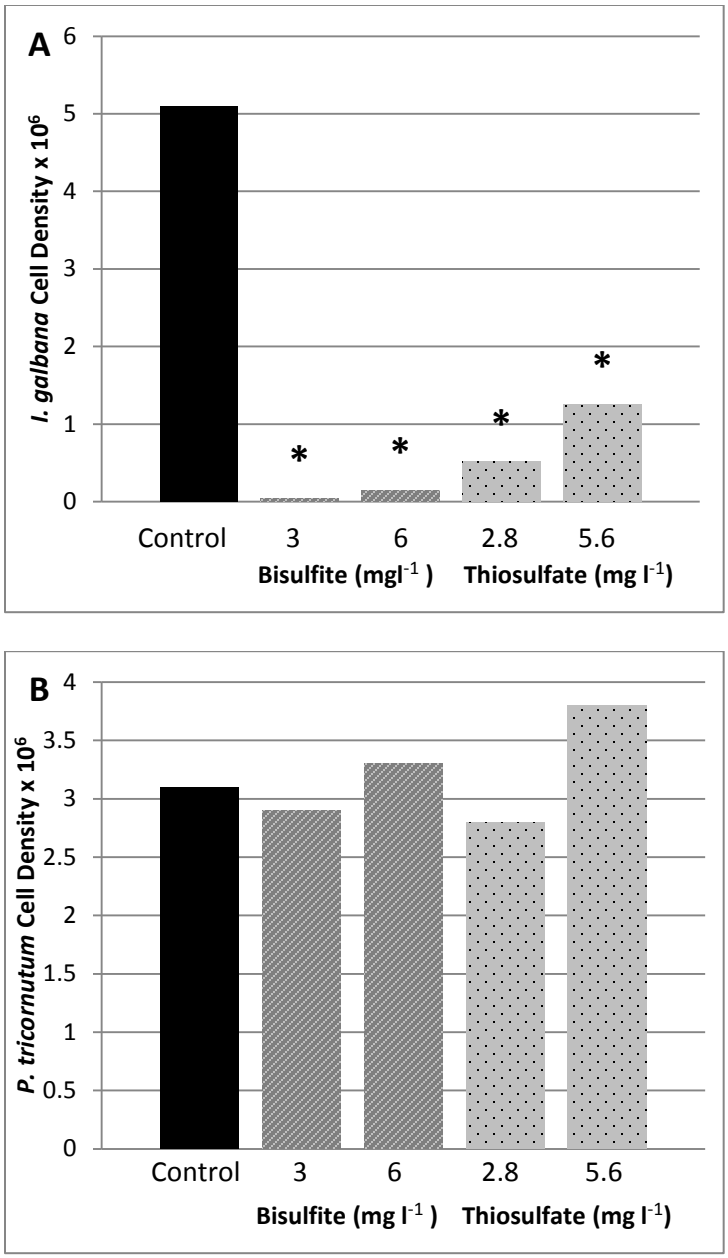

Figure 2.5 Final cell densities of *I. galbana* (A) and *P. tricornutum* (B) in sodium hypochlorite treated water (TRO = 6.8 mg l^{-1}) after neutralization with bisulfite or thiosulfate. * Treatment significantly less than control (α=0.05)

2.4 Conclusions

No definitive cause was identified for the observed algal toxicity in the current research. Toxicity may be caused by halogenated molecules, very low levels of

oxidant, or other factors specific to the water source used for BWMS testing. The focus of our research was to measure the persistence of previously observed algal toxicity of treated ballast water (Ziegler et al., 2010). All previous toxicity testing of chlorination based BWMS has been conducted immediately after ballast water discharge. Although an attempt was made to compare our Day 0 toxicity test results to results from other studies, comparisons are complicated by differences in test methodologies and how the results are presented. Despite these limitations, the initial algal toxicity from treated ballast water observed in the current research seems more severe than algal toxicity reported in other studies of oxidant treated water.

In the current research, the short half-life of oxidants and the addition of neutralizers to discharged ballast water would seemingly eliminate the possibility of oxidant toxicity over the relatively long period of time observed. However, if all of the organic carbon has reacted with oxidants while in the ballast tank, any remaining oxidant may have a much longer half-life than expected. Also, because TRO decay is dependent on temperature (Duan et al., 2016), the immediate chilling of treated ballast water samples may have helped preserve any remaining oxidant. In the current research, the collection and holding of samples (sealed container in the dark at 4 °C) before toxicity testing may have also affected TRO decay and toxicity test results. On the other hand, preparation of the treated ballast sample for toxicity testing involves filtration, the addition of algal nutrients, and the process of making treated water dilutions, all of which could consume any remaining oxidant before the start of toxicity tests. In fact, earlier research conducted by Sathasivam et al. (2016) found that the

addition of algal nutrients (i.e. f/2 algal media) before toxicity testing had a substantial oxidant demand.

Low concentrations of oxidant that may be present in discharged ballast water are typically consumed by the interaction with organic matter following discharge into a natural body of water. However, any chemical reactions, including the decay rate of any remaining oxidant, are affected by the temperature and DOC (concentration and composition) in the receiving water. In low temperature and low DOC waters, such as those found in arctic regions, any remaining oxidant in treated ballast water discharges could create a particularly hazardous situation and pose a threat to algae populations in the receiving environment. If the observed algal toxicity in the current research is due to TRO concentrations that are below detection, the longevity of toxicity may have been extended by cold storage. To the authors' knowledge, no algal toxicity tests have been conducted on cold water species, and there are currently no toxicity test guidelines for use in cold water. Arctic environments are of particular concern in the future as the warming of arctic water reduces ice volumes (NSIDC, 2017), and opens new passages allowing for arctic freighter transport, drilling and exploration, and tourism (Miller and Ruiz, 2014; IMO, 2016b). The opening of Arctic trade routes will thereby expose these areas to the risks associated with greater nautical activity, such as the introduction of non-indigenous species, release of chemicals (e.g. DBPs and biocides from discharged ballast water), and oil spills.

Another possible cause of algal toxicity in oxidant treated water could be residual organic chloramines that have shown resistance to neutralization by sulfite (Bedner et al., 2004). This residual chlorine pool can be missed during analysis

because the dechlorinating agent itself can interfere with chlorine analysis (Helz and Nweke, 1995). The sulfite resistant fraction is thought to be composed of chlorinated amines and peptides formed by the transfer of Cl^+ to organic amines, such as protein degradation products, nucleic acids, amino sugars, and aliphatic amines (Bedner et al., 2004). Yonkos et al. (2001) found that the TRO concentration measured by amperometric titration was not a good predictor of *Daphnia magna* toxicity, and suggested that halogenated residuals that were resistant to sulfite neutralization (e.g. chlorinated amines) were responsible for observed daphnid toxicity. Halogenated amines that are not detected as TRO by the DPD method may also have played a role in the algal toxicity of oxidant treated estuarine water samples in the current research.

The longevity of algal toxicity in treated ballast water may also be due to a combination of traditional DBPs or unquantified larger halogenated organic compounds. In the current research, the lack of correlation between the initial concentration of traditional DBPs and algal toxicity (Fig. 2.2) is not a definitive rejection of DBPs as a contributing factor to algal toxicity in such a complex mixture. While the initial concentrations of commonly identified DBPs were quantified, the production of additional, unidentified halogenated compounds is probable. Like traditional DBPs, the production of these large unquantified halogenated compounds is also primarily dependent on the uptake water's DOC composition and concentration (Heeb et al., 2014). However, other chemicals that may be present in uptake water, including emerging contaminants (ECs), can also react with oxidants forming additional halogenated compounds (Deborde et al., 2004; Benitez et al., 2011; Acero et al., 2013). The possibility of mixture toxicity to algae has been discussed as a way of

addressing combinations of chemicals (Petersen et al., 2014) with the potential for synergistic toxic effects. These authors found that algal toxicity tests with *S. costatum* showed synergistic toxic effects when exposed to a combination of contaminants that could potentially be present in the commercial harbor water used in the current project.

Algal toxicity of chlorinated (e.g. sodium hypochlorite) and neutralized water has been reported for multiple species in laboratory studies (Gentile et al., 1976; Sanders, 1984; Lee et al., 2015; Rhie, 2016). Currently, there is no unilaterally accepted algal species or taxonomic group that is considered more sensitive to chlorine toxicity, or to chemical toxicity in general. Therefore, the main factors in species selection for toxicity testing have been rapid growth and ease of culture, with little emphasis on species sensitivity. Results from several studies observed species dependent differences in sensitivity of algae exposed to chlorinated and neutralized water (Lee et al., 2015; Rhie, 2016). In the current research, a dramatic difference in species sensitivity was demonstrated in algal toxicity tests of neutralized water after the addition of sodium hypochlorite (Fig. 2.5). Toxicity tests with *I. galbana* showed reductions in growth after neutralization with two concentrations of two different neutralizers, while *P. tricornutum* was completely resistant to negative effects in any of the treatments. Lee et al. (2015) also noted greater sensitivity of *I. galbana* compared to *P. tricornutum* as well as two other algal species, S*elenastrum capricornutum* and *Scenedesmus obliquus*. Two diatoms (i.e. *S. costatum* and *P. tricornutum*) are most frequently used as test species because of their rapid growth rate, ecological relevance, and internationally established test guidelines (ISO, 2008; 2016). Although *P. tricornutum* seems resistant to toxic effects of water chlorination, toxicity tests with *S.*

costatum have shown susceptibility to oxidant treated discharge water. However, no toxicity tests have been conducted to compare *S. costatum* and other algal species. The paucity of algal toxicity data is a concern in light of the wide variability in species' sensitivities observed in testing of oxidant treated water.

The current research was conducted on only three BWMS, representing two types of treatment methods employing oxidants: electrochlorination and injection of a DICD solution. Future research is needed to more clearly establish the factors that influence DBP production and toxicity of treated ballast water. Despite not identifying the cause of algal toxicity, the longevity of toxic effects found in the current study is of particular concern. Although only a small percentage of ships currently treat ballast water, this number is likely to increase as maximum discharge regulations are enforced and ballast treatment becomes mandatory. If treatment systems that employ strong oxidants are adopted by a large proportion of ships, the environmental impact of potentially toxic ballast water could be amplified on a local as well as a global scale. Ongoing research is focused on identification of additional halogenated compounds that are produced by oxidant-based BWMS. The ultimate goal of our continued research is to identify factors that relate to observed toxicity and the production of DBPs. Identification of these factors can be useful in the continued development and use of BWMS to help reduce the negative ecological impact of ballast water treatment.

Chapter 3

Formation of Brominated Organic Compounds and Molecular Transformations in Dissolved Organic Matter (DOM) after Ballast Water Treatment with Sodium Dichloroisocyanurate Dihydrate (DICD)

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Abstract

Estuarine water treated with a ballast water management system (BWMS) using a solution of dissolved dichloroisocyanurate dihydrate (DICD) resulted in the formation of newly described brominated disinfection by-products (Br-DBPs). Analysis of dissolved organic matter (DOM) in untreated water with ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) identified 3,897 *m/z* ions and their exact molecular formulas. After DICD treatment, a total of 213 halogenated molecular ions with relative abundance of at least 1% were assigned and confirmed using isotope simulation. Halogenated ions were assigned in four DBP elemental groups including CHOBr (180), CHONBr (13), CHOCl (16), and CHOBrCl (4). Forty-nine (25%) of the 197 brominated formulas have not been previously reported. We also were able to tentatively assign possible structures to the formula $C_3HBr_3N_2$ due to very limited isomeric possibilities. The tentatively assigned compound found at 6.4% relative abundance was identified as either tribromoimidazole or tribromopyrazole. Our results show the formation of complex halogenated DBPs that are formed in the treatment of water with a novel BWMS that employs granular DICD as a biocide. The toxicological

and mutagenic properties as well as the fate of these newly identified brominated DBPs are unknown.

3.1 Introduction

The *Ballast Water Management Convention* of the International Maritime Organization (IMO, 2004) and similar regulatory instruments (USCG, 2012) require the treatment of ships' ballast water to minimize the risk of the transfer, release and establishment of new invasive species. The majority of existing ballast water management systems (BWMS) use an active substance as a biocide, and the majority of these systems make use of strong oxidants (e.g. chlorine, ozone or hydrogen peroxide).

The use of chlorine in fresh water applications, such as drinking water and wastewater treatment, has been extensively employed for decades and is credited for reducing the spread of disease, virtually eliminating waterborne dysentery and cholera in industrialized nations (Ali et al., 2012; 2015). However, the undesirable side effects of chlorine have been demonstrated including the formation of disinfection by-products (DBPs), which are potentially toxic, carcinogenic, and mutagenic (Richardson et al., 2007; Farré et al., 2013). The halogenation of DOM, and resulting DBPs, is influenced by oxidant dose, temperature, DOM quantity and composition, and potentially by the mechanism of disinfection (e.g. liquid hypochlorite, electrochlorination, DICD). Research has focused on freshwater DBPs using analytical techniques such as GC-MS and LC-MS to determine rather small chlorinated DBPs, such as haloacidic acids (HAAs), trihalomethanes (THMs), haloacetonitriles (HANs) and oxyhalides (Richardson, 2002). However, as much as 50% of the total organic halogen (TOX) formed after chlorination is undefined (Krasner et al., 2006; 2009), and halogens are presumed to be

incorporated into higher molecular weight DOM (Zhang and Minear, 2002; 2006). The presence of brominated DBPs (Br-DBPs) in freshwater has also been studied, where already relatively low concentrations of bromide lead to Br-DBPs (Ichihashi et al., 1999; Nokes et al., 1999; Shah et al., 2015; Zhang et al., 2014). Compared to chlorine, bromine is 20 times more likely to participate in substitution reactions leading to Br-DBPs (Westerhoff et al., 2004; Uyak and Toroz, 2007) which are more likely to be carcinogenic and mutagenic compared to their chlorinated analogues (Echigo et al., 2004; Sharma et al., 2014). In higher salinity brackish and seawater, any addition of hypochlorite will almost instantaneously yield HOBr or OBr^- (Westerhoff et al., 2004), and the majority of halogenation will be by active bromine (Shah et al., 2015), although some research has also revealed chlorinated and mixed halogenated DBPs (Wang et al., 2018).

In drinking water treatment, the removal of precursors of chlorinated DBPs, including dissolved organic matter (DOM), has been demonstrated (Bond et al., 2012; Zainudin et al., 2018). More recently, the use of strong oxidants in estuarine and marine waters for purposes such as industrial cooling (Jenner et al., 1997), desalination (Kristiansen et al., 1996; Saeed et al., 2019) and treatment of saline wastewater (Ding et al., 2013) has led to an increased release of brominated DBPs. Ballast water treatment presents a relatively new application of strong oxidants that can lead to high concentrations of DBPs (Werschkun et al., 2012; 2014) due to increased concentration and complexity of DOM in uptake water, as well as higher oxidant concentration and DOM contact times (Chowdhury et al., 2009; Shah et al., 2015; Hao et al., 2017). Unlike drinking water disinfection, no practical methods have been developed in ballast water disinfection to limit the formation of DBPs.

Ballast Water Management Systems (BWMS) that employ active substances are required to evaluate treated ballast water for environmental acceptability under *IMO Procedure for approval of ballast water management systems that make use of Active Substances (G9)* (IMO,2008b). This requirement has led to a large data set for traditional DBPs (e.g. HAAs, THMs, oxyhalides and HANs) with analyses by gas chromatography/mass spectrometry (GC-MS), a method unable to detect large or polar Br-DBPs (Richardson 2002; Zhai and Zhang, 2011). As a result, a significant portion of the halogenated organic compounds formed in chlorination of natural water have not been identified until recently, with the use of emerging techniques such as ultrahigh-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) with electrospray ionization (Zhang et al., 2014; Gonsior et al., 2015), and electrospray ionization-triple quadrupole mass spectrometry (UPLC/ESI-tqMS) (Zhai and Zhang, 2011; Zhai et al., 2014). FT-ICR MS has sufficient resolution to identify the molecular composition and formulas of DOM and DBPs by mass measurement alone (Stenson et al., 2003). However, not all constituents in DOM and DBPs are efficiently ionized and hence the current research focused on the components that are susceptible to solid phase extraction by a polymeric resin at low pH, and that can be effectively ionized in negative mode electrospray ionization. Ultrahigh-resolution FT-ICR MS has been used to monitor the incorporation of chlorine (Zhang et al., 2012; Lavonen et al., 2013; Gonsior et al., 2014b) and bromine (Zhang et al., 2014; Gonsior et al., 2015) into DOM. More generally, FT-ICR MS and other high resolution MS techniques, such as Orbitrap, have substantially advanced the field of non-targeted analysis of organic compounds in

complex mixtures (Schymanski et al., 2015; Yang and Zhang, 2016; Hollender et al., 2017; Luek et al., 2017).

The BWMS in this project used a solution of granular DICD in potable water. The use of granular DICD as a disinfectant has the advantages of safer storage and a longer shelf life (2 y) compared to liquid sodium hypochlorite (1 y, depending on storage conditions) enabling ships to make less frequent stops to re-supply with fresh disinfectant while also avoiding the potential environmental risk of chlorate production resulting from sodium hypochlorite degradation. The use of DICD also avoids the need for a significant power supply that is required for BWMS using electrochlorination to produce disinfectant. To date, only one commercial BWMS using DICD has received Type Approval by the IMO which is required before installation on ships.

Once in solution, dichloroisocyanurate ($C_3N_3O_3Cl_2$) equilibrates with hypochlorous acid (HOCl) in solution (Jensen and Johnson, 1990), as described in equations 1 and 2 below. In freshwater ballast treatment with high chlorine demand, conversion of chloroisocyanurates to HOCl is thought to be very rapid and complete, with disinfection accomplished using only free available chlorine (i.e. HOCl and its conjugate base OCl^-). The kinetics of this reaction have been previously reported (Farkas et al., 1949; Kumar and Margerum, 1987). In the presence of bromide, available chlorine will rapidly oxidize the bromide ion to available bromine (HOBr and OBr^-) (eq. 3). The oxidation of bromide by available chlorine is only dependent on the concentration of bromide ion and is independent of high chloride concentrations found in full strength seawater. Although complete conversion to available bromine is assumed, there are no published data on the effects of cyanuric acid (H_3Cy) or chloroisocyanurates

on the oxidation of the bromide ion by HOCl (eq. 3) which can potentially influence the formation halogenated organic compounds.

$$Cl_2Cy^- + H_2O \rightarrow HClCy^- + HOCl \quad (1)$$

$$HClCy^- + H_2O \rightarrow H_2Cy^- + HOCl \quad (2)$$

Cl_2Cy^- = dichloroisocyanurate, $HClCy^-$ = chloroisocyanurate, and H_2Cy^- = cyanurate

$$HOCl + Br^- \rightarrow HOBr + Cl^- \quad (3)$$

The primary goal of this research was to identify new halogenated DBPs that are formed in ballast water treatment of estuarine water with DICD using non-targeted direct infusion FT-ICR MS. Spectrometric data of un-halogenated DOM and Br-DBPs were visualized using van Krevelen diagrams(van Krevelen, 1950), plotting H/C ratio versus O/C ratio, as well as modified Kendrick plots of halogenated DBPs to visualize homologous series of molecular formulas (Yekta et al., 2012). In addition, shifts in the molecular composition of un-halogenated DOM with DICD treatment were analyzed with van Krevelen diagrams. The DBPs identified by FT-ICR MS are structurally complex and halogenated while more traditional and regulated DBPs (e.g. HAAs, THMs and oxyhalides) were not evaluated in this study, and were not observed due to either loss in the solid phase extraction (SPE) procedure, or are outside the *m/z* window of FT-ICR MS.

3.2 Materials and Methods

3.2.1 Ballast Water Sampling and Extraction

Estuarine water was treated with a BWMS that employed a biocide solution made from dissolving sodium DICD granules in potable water, during certification testing by the Maritime Environmental Resource Center (MERC). The land-based test facility used

for BWMS testing was located in Port Covington, Baltimore, Maryland, USA, a highly-productive, mesohaline urban/industrial environment. Uptake water (salinity of 7 PSU, pH 7.9, DO 8.5 and temp. 23.8 °C) was taken from the surface of Winans Cove (Baltimore, MD, USA). Adjustments were made to uptake water to coincide with the United States Coast Guard Environmental Technology Verification (ETV) Program (USEPA, 2010), which outlines BWMS testing performance standards. Adjustments to DOC and POC, as well as ETV performance standards are provided in Table 3.1. Adjustments included addition of sodium citrate dihydrate (Fisher Scientific, USA) and Micromate-micronized humate (Mesa Verde Resources; Placitas, New Mexico), to increase final DOC (8.2 mg C l^{-1}) and POC (14.7 mg C l^{-1}), respectively (Table 3.1). Citrate is an aliphatic acid and is not thought to be a precursor to halogenated DBPs identified in the current research using FT-ICR MS. Micromate, however, is comprised of concentrated humic acids and may be a source of precursors in the formation of halogenated DBPs. Although the average particle size of dry micromate is 15 µm, in solution the dissolved portion passing through a GF/F filter (0.45 µm) before extraction would be included in the uptake DOM profile identified with FT-ICR MS. Although POC of uptake water is increased with micromate, it represents a natural source of DBP precursors. All adjustments were made in uptake water before splitting into untreated and treated ballast water lines. No salinity adjustments or additions to the natural plankton community were necessary. The DICD disinfecting solution was injected into the ballast water during uptake with a target total residual oxidant (TRO) dose of 11 mg l^{-1}. Control and treated ballast waters were delivered to independent tanks and held for 48 h in closed ballast tanks at ambient temperature.

Table 3.1 Minimum USEPA ETV (2010) and IMO (2004) concentrations for DOC, POC and TSS compared to ambient and adjusted concentrations of uptake water. Amendments of uptake water included sodium citrate, Arizona fine test dust, and Micromate for increasing DOC, TSS and POC, respectively.

	ETV	IMO G8	Test Water	
Parameter			Ambient	Adjusted
DOC mg C l^{-1}	≥ 6	> 5	4.6	8.2
POC mg C l^{-1}	≥ 4	> 5	8.0	14.7
TSS mg l^{-1}	≥ 24	≥ 50	18.5	57.1

All adjustments were made in uptake water before splitting into untreated and treated ballast water lines. No salinity adjustments or additions to the natural plankton community were necessary. The DICD disinfecting solution was injected into the ballast water during uptake with a target total residual oxidant (TRO) dose of 11 mg l^{-1}. Control and treated ballast waters were delivered to independent tanks and held for 48 h in closed ballast tanks at ambient temperature.

At discharge, an untreated (i.e. control) uptake water sample was collected in a 20 L polycarbonate carboy directly from the untreated ballast water tank. A continuous, time integrated sample of discharged treated water was collected during the entire 1 h discharge process by an in-line sample port, and delivered to a 100-L polymer fiberglass tank. The BWMS added a dose of neutralizer (sodium bisulfite) for the first five minutes of treated water discharge, after which no additional neutralizer was added. A 19-L treated water sample was collected (20-L glass carboy) from the 100-L fiberglass tank by gravity flow. Control and treated water samples were immediately transferred to ice filled coolers for transport to the University of Maryland Wye Research and Education Center (UMD/WREC) for solid phase extraction (SPE).

Isolation of DOM and DBPs was accomplished with SPE followed by methanol elution and preservation of sample. This extraction method for DOM has been published previously (Dittmar et al., 2008) and has also been used to extract DBPs from electrochlorinated marine water (Gonsior et al., 2015). Extractions were started within two hours of ballast water sample collection. The SPE method allowed for the concentration of DOM and complete desalting of the water samples, required for analysis by FT-ICR MS with electrospray ionization (Stenson et al., 2003). Briefly, water samples (1-L) were vacuum filtered (pre-combusted at 500 °C Whatman GF/F, 0.7 μm) and acidified to pH 2 with formic acid. Samples were then gravity-fed through methanol activated SPE cartridges (Agilent Bond Elut PPL), containing 1 g of highly functionalized styrene-divinylbenzene (SDVB) polymer. Cartridges were rinsed with formic acid acidified water, dried with a gentle stream of nitrogen and eluted with 10 ml of LC-MS grade methanol (Chromasolv, Sigma-Aldrich). Although not quantified in this study, previous research has shown that DOM extraction with PPL cartridges is more efficient than other SPE methods (e.g. C18, XAD-8), with an extraction efficiency between 52 and 74% (Dittmar et al., 2008; Gonsior et al., 2009; 2014a; Green et al., 2014; Lavonen et al., 2013). This SPE technique has been frequently used to extract DOM from marine waters (Medeiros et al., 2015; Timko et al., 2015) and to evaluate DBPs (Gonsior et al., 2015; Lavonen et al., 2013).

3.2.2 Chemical Analysis

3.2.2.1 POC and DOC

Analyses of dissolved organic carbon (DOC) and total organic carbon (TOC) were carried out on water collected at uptake, both before and after the addition of

compounds to reach ETV minimum concentrations of DOC and POC (Table 3.1). The high temperature combustion method was used to analyze aqueous samples for TOC and DOC using the Shimadzu TOC-L carbon analyzer. Briefly, TOC concentrations were derived from unfiltered water, and water used for DOC analysis was filtered through a 0.7 μm GF/F glass fiber filter. Samples were acidified with hydrochloric acid and sparged with ultrapure air to remove inorganic carbon. High temperature combustion (680 ºC) on a catalyst bed breaks down all carbon compounds into carbon dioxide (CO_2). The CO_2 was then quantified on a non-dispersive infrared detector (NDIR). The method detection limit (MDL) for DOC was 0.24 mg C l^{-1}.

3.2.2.2 Total Residual Oxidant

Measurements of the TRO concentration in discharged treated ballast water were made using the USEPA recommended DPD (n,n-diethyl-p-phenylene diamine) method[53] with a handheld Hach Pocket Colorimeter™ II colorimetric TRO meter (Hach, Model No. 58700, U.S.A.). TRO measurements were taken in triplicate on samples collected at the beginning, middle, and end of the overall discharge period (approximately 1 h). Colorimetric TRO measurements were made in Low-Range mode (0.02-2.0 mg l^{-1} TRO as Cl_2), following the manufacturer's instruction manual (Hach Company, 2013). When uptake water indicated the presence of TRO (considered interference), the treated water's TRO value was adjusted by subtracting the TRO value of untreated water to obtain the final reported TRO concentration.

3.2.3 Ultrahigh Resolution Mass Spectrometry (FT-ICR MS)

Mass spectrometry of SPE isolated samples was performed using a Bruker Solarix 12 Tesla Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS)

after negative ion mode electrospray ionization (ESI^-). Methanolic samples were diluted 1:20 and directly infused into the electrospray at a flow rate of 2 µl min^{-1}. The dilution of the sample was necessary to limit space charge processes, which may lead to interferences in the transient spectrum. To achieve high mass accuracy and precision, 500 scans were averaged with a time domain of 4 megaword. The voltage of the ESI^- was set to -3.6 kV. A cleaning procedure using 600 µL (50% methanol and 50% water) was implemented between each sample to avoid carryover of samples. Methanolic blanks were measured occasionally to make sure that the cleaning procedure between samples was effective. The resolution of FT-ICR MS and the formation of singly charged ions by ESI^- with a mass accuracy better than 0.2 ppm, allowed unambiguous molecular formula assignments (Koch et al., 2007). The exact molecular formula assignments were based on the following number of monoisotopic atoms $^{12}C_{0-\infty}$, $^{1}H_{0-\infty}$, $^{16}O_{0-\infty}$, $^{14}N_{0-5}$, and $^{32}S_{0-2,}$ $^{35}Cl_{0-5}$ and ^{79}Br. The corresponding isotopologues of the halogens (^{37}Cl and ^{81}Br) were used to cross-validate formula assignments based on isotope simulation comparisons. Unambiguous formula assignments were possible up to *m/z* of 800, however the entire observed *m/z* range was below this *m/z* value. Molecular formula assignments were only given to *m/z* values with a signal to noise ratio greater than 10. Calculation of elemental formulas for each *m/z* ion was undertaken with the *NetCalc* network approach with details described previously in recent publications (Tziotis et al., 2011; Hertkorn et al., 2013). The *NetCalc* generated formula assignments were further validated by manual formula assignments to check isotope pattern matching for high intensity *m/z* ions. Final formulas included elemental compositions containing C, H, O, N, S, Br and Cl that were divided into elemental groups CHO, CHON, CHOS,

CHOBr, CHOClBr, CHONBr and CHOCl, for further analysis. The *Netcalc* approach does not require limiting atomic numbers prior to formulas assignments because it is based on transformations that have to be defined. However, we restricted the atomic numbers to $C_{1\text{-}60}$, $O_{1\text{-}25}$, $N_{1\text{-}3}$, $S_{1\text{-}2}$, $Br_{1\text{-}5}$ and $Cl_{1\text{-}5}$ for traditional formula calculations to cross-validate that our allowed transformations are indeed capturing all *m/z* ions that can be assigned to molecular formulas.

Brominated molecular formulas were validated by isotope simulations to cross-validate assigned formulas, which is very robust due to the almost equal contributions of the two stable isotopes ^{79}Br and ^{81}Br. The MS spectra were pre-calibrated using arginine clusters and then again post-calibrated using known DOM molecular formulas (Gonsior et al., 2016). All assigned halogenated DBPs were checked against methanolic blanks and with samples extracted prior to the treatment.

The relative abundance of individual molecular formulas were calculated by comparison to the highest DOM *m/z* ions in each individual mass spectrum. However, the intensities after averaging 500 scans were used to compare samples, because it was not clear if highest intensities *m/z* ions were affected by the DICD treatment. We are well aware that ionization suppression, ionization efficiencies and matrix effects all influence the intensity of individual *m/z* ions, but a relative comparison of intensity of samples before and after treatment has been shown valuable in previous studies and can be used in a semi-quantitative way (Koch et al., 2005; Sleighter et al., 2012; Lavonen et al., 2013; Gonsior et al., 2014b). Although, it should be noted here that *m/z* ion intensity cannot be related to actual concentrations due to the vastly different ionization

efficiencies of different compound classes. Hence, FT-ICR MS data are not quantitative because of the absence of standards and the lack of structural information of *m/z* ions.

Multiple parameters were used to interpret FT-ICR MS spectrometric data, allowing a comparison of untreated intake water and DICD treated water. Parameters included *m/z* range, number of hydrogen and oxygen to carbon ratios (O/C and H/C), Kendrick mass defect (KMD) and the z-score (*z**). Van Krevelen diagrams (van Krevelen, 1950; Kim et al., 2003) were used to characterize molecular formulas by plotting H/C ratio against the O/C ratio of assigned formulas (Hertkorn et al., 2006; Hao et al., 2017), although this type of diagram is a projection of a number of formulas with the same atomic ratios on the same spot, and hence does not reflect the true diversity. However, this plot allows visualization of relative oxygen or hydrogen deficiency of molecular formulas, resulting in similar types of organic compounds to cluster into specific regions on the plot. Kendrick plots (Kendrick, 1963) were modified and used to visualize compositional patterns of molecular formulas along the *m/z* range (Yekta et al., 2012). This was achieved by using the KMD and the *z**, and plotting their ratio (-KMD/z*) against mass (Stenson et al., 2003).

3.3 Results and Discussion

3.3.1 Molecular Characterization of Source Water DOM

The ultrahigh resolution mass spectra of PPL extracts of DOM from untreated Baltimore Harbor water is shown in Figure 3.1A. Numerous *m/z* ions representing unique molecular formulas were identified belonging to three elemental formula groups: CHO, CHON and CHOS. The CHON elemental group was the most abundant with 2,681 peaks followed by CHO group with 2,187 peaks and CHOS with 511 peaks. The

observed mass range of DOM was approximately 150 – 600 Da, which is consistent with previous FT-ICR MS studies of estuarine or coastal DOM (Sleighter and Hatcher, 2007; Gonsior et al., 2015). DOM can be highly variable across salinity gradients and with season as previously shown for the Delaware Bay (Powers et al., 2018). .No halogenated formulas were identified in untreated control samples.

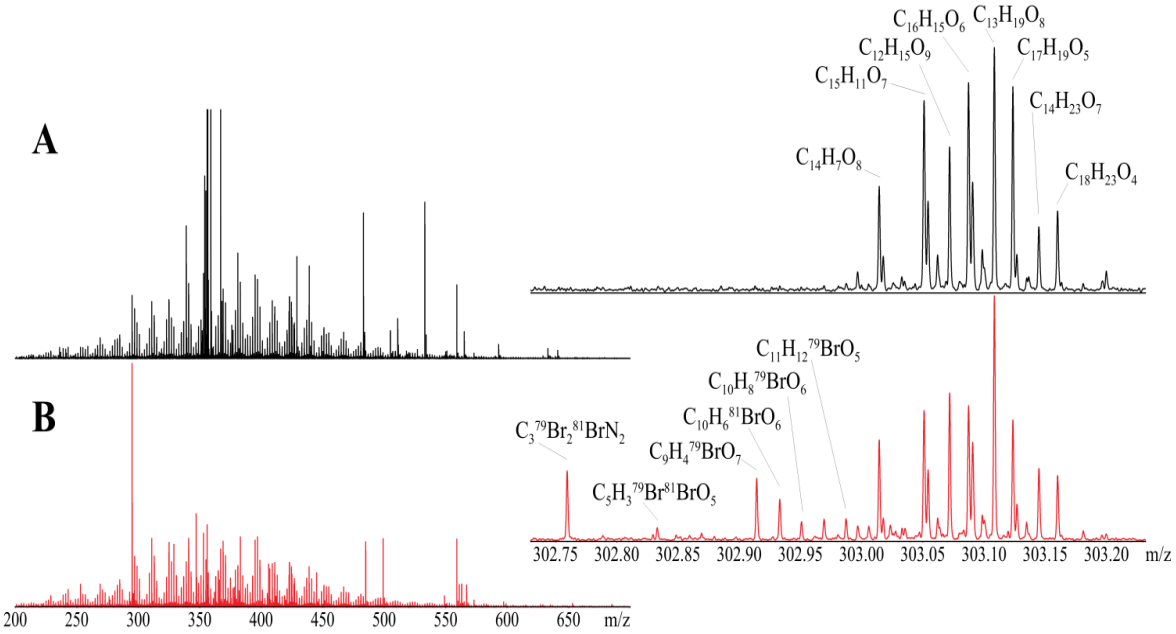

Figure 3.1 Ultrahigh resolution FT-ICR mass spectra of PPL extracts of DOM from Baltimore Harbor, USA in untreated (A) and DICD treated (B) ballast water. The enlarged area shows negative m/z ions at nominal mass 303 and their corresponding neutral formula assignments.

The van Krevelen plots of molecular formulas in DOM containing only C, H and O before and after DICD treatment (Figure 3.2) reveal a decrease in the relative abundance of ions after treatment, but very little difference in O/C or H/C ratios is visible, showing a strong similarity in DOC before and after treatment.

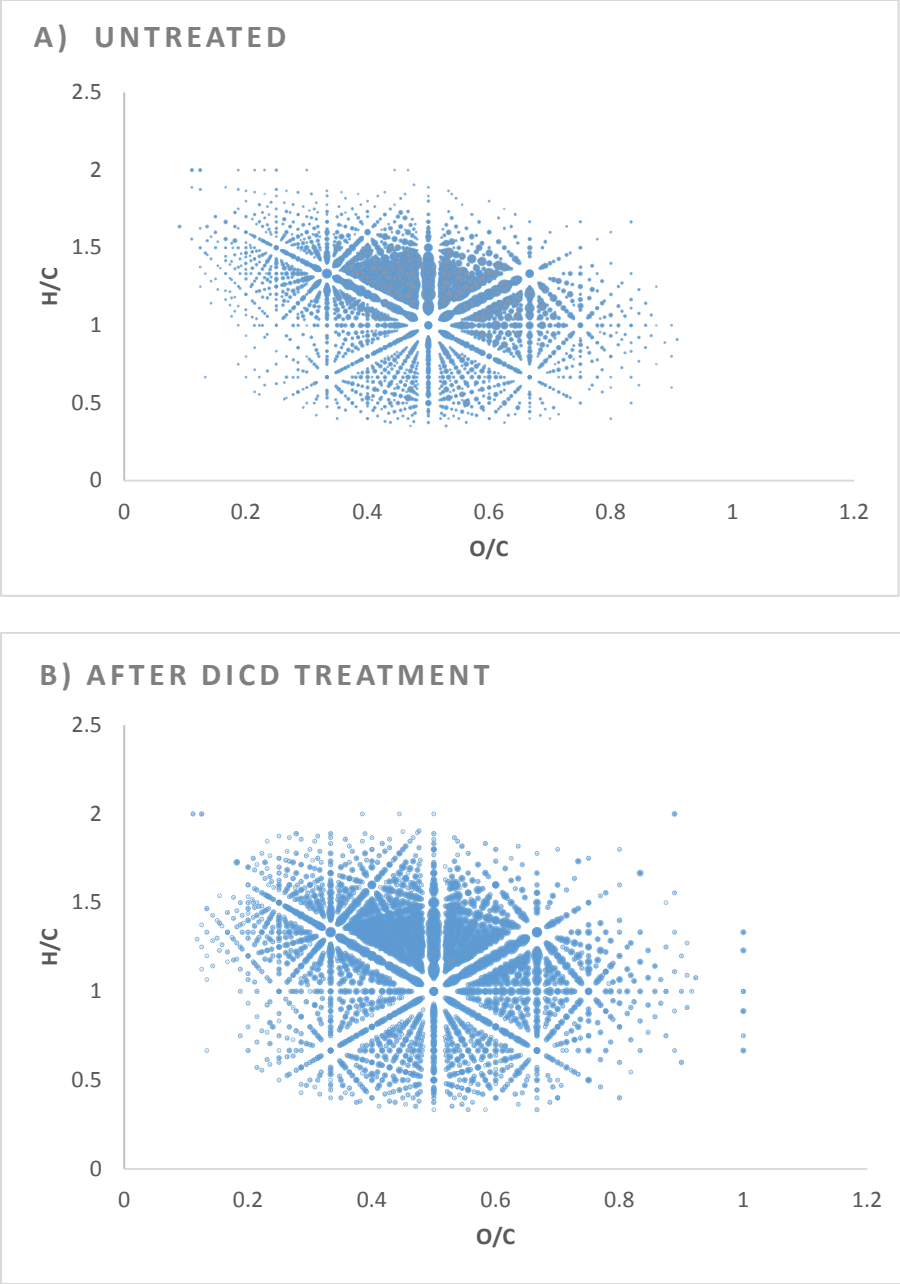

Figure 3.2 Van Krevelen diagram of CHO molecular formulas of DOM in untreated (A) and DICD treated (B) ballast water. Bubble size represents abundance.

For CHO-only formulas before treatment H/C = 1.107 and O/C = 0.463, while after DICD treatment H/C= 1.112 and O/C = 0.472 (Table 3.2). The van Krevelen analysis identifies classes of organic compounds in DOM by elemental ratios, where similar types of compounds concentrate in particular areas of the plot. The majority of

DOM molecules in uptake water had patterns of elemental ratios that are characteristic of phenol-like compounds (Figure 3.1A). These patterns are often defined in the literature as 'lignin-like' compounds with H/C = 0.7 to 1.5, and O/C = 0.1 to 0.7 (Hockaday et al., 2009; Lu et al., 2015; Ohno et al., 2010; Sleighter and Hatcher, 2007), which implies a terrestrial origin of DOC. Because the location in the current research receives a large volume of freshwater input, the majority of DOM is most likely of terrestrial origin. The distribution of O/C ratios (between 0.1 and 0.7) is also rather typical for terrestrially-derived DOM (Table 3.2).

Table 3.2 Mean values for ratios of oxygen to carbon (O/C) and hydrogen to carbon (H/C) formula groups before (CHO group only) and after DICD treatment.

Sample	Formula Group	Avg. O/C	Avg. H/C
Control	CHO	0.46303	1.10670
DICD Treated	CHO	0.471989	1.11201
DICD Treated	CHOBr	0.507733	1.01888

There are also DOM molecules on the van Krevelen diagram with signatures that may indicate highly polymerized or even condensed aromatic structures (Figure 3.2) as defined by having H/C = 0.2 to 0.7 and O/C = 0 to 0.6 (Ohno et al., 2010). Notably, all of the compounds in this area have relatively low abundance, which is expected due to their presumed rather low solubility.

As stated previously, the majority of DOM consists of organic compounds in the CHON and CHO elemental formula classes. The portion of DOM containing nitrogen atoms precludes formation by the simple breakdown of phenol-like compounds and may have formed during early stages of the microbial degradation process (Stenson et al.,

2003), during polyphenol-peptide binding reactions in the soil (Olk et al., 2006) or may be derived from algal organic matter present in uptake water.

3.3.2 Changes in DOM with DICD Treatment

A substantial change in the DOM pool was seen after DICD treatment with differences in molecular composition at each nominal mass (Figure 1B). Following treatment, unique molecular formulas were assigned to the previous elemental formula groups CHON, CHO and CHOS, as well as an additional four halogenated DBP groups: CHOBr, CHONBr, CHOCl and CHOBrCl. Figure A2.1 (Appendix 2) shows histograms of the heteroatom class distribution for Port Covington NOM before and after treatment with DICD. The same class species of CHO-only compounds are found before and after treatment, with the O8 class of compounds most abundant in both samples. However, there is an increase in the abundance of the O9 class of CHO-only compounds after treatment (Figure A2.1 B).

When looking at the entire set of peaks found in the FT-MS spectrum with relative abundance as low as 0.23%, there were 2,187 CHO elemental formulas in uptake water and 2,207 formulas identified after DICD treatment, an increase of 20 unique molecular formulas. However, when limited to more abundant ions (i.e. relative abundance of at least 1%) there were 1,495 CHO formulas before and 1,588 formulas after treatment, an increase of 93 formulas, showing the marked increase in the more abundant CHO element group ions after DICD treatment. An increase in intensity of lower mass *m/z* ions in the CHO formula group was also observed after DICD treatment, with a corresponding overall decrease in the intensity of higher mass ions (Figure 3.1). This is consistent with the ability of strong oxidizing agents like DICD to oxidize

complex organic molecules and break them down into lower molecular weight compounds. The overall shift in molecular weight of DOC is best visualized as the absolute intensity change as a function of mass (Figure 3.3).

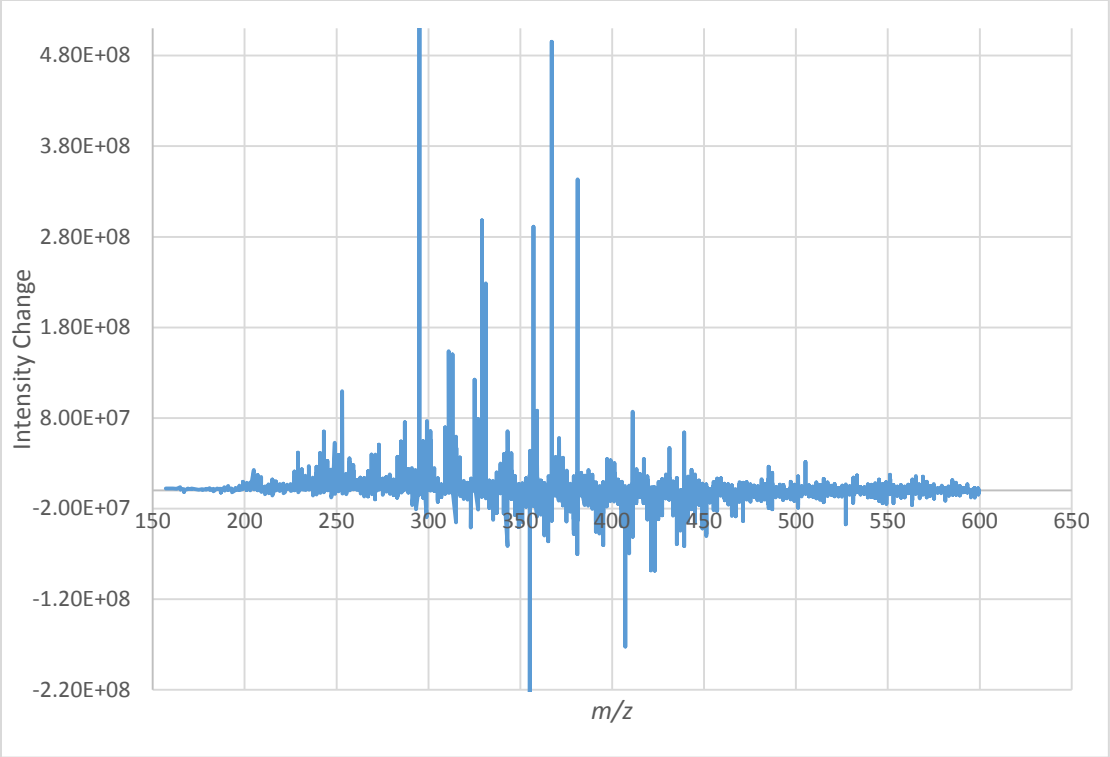

Figure 3.3. Absolute intensity change (CHO group only) after DICD treatment.

After DICD treatment, the majority of absolute *m/z* ion intensity changes in the region below 340 Da are in the positive direction, while to a smaller degree *m/z* ion intensity changes above 380 Da are primarily in the negative direction. This trend can also be observed on a finer scale by looking at individual DOM components before and after DICD treatment. The zoomed in view of the mass spectrum at nominal mass (NM) 329 before and after DICD treatment shows the increase in intensity of these relatively

low molecular weight *m/z* ions after DICD treatment (Figure 3.4), a typical trend seen in the full mass spectrum.

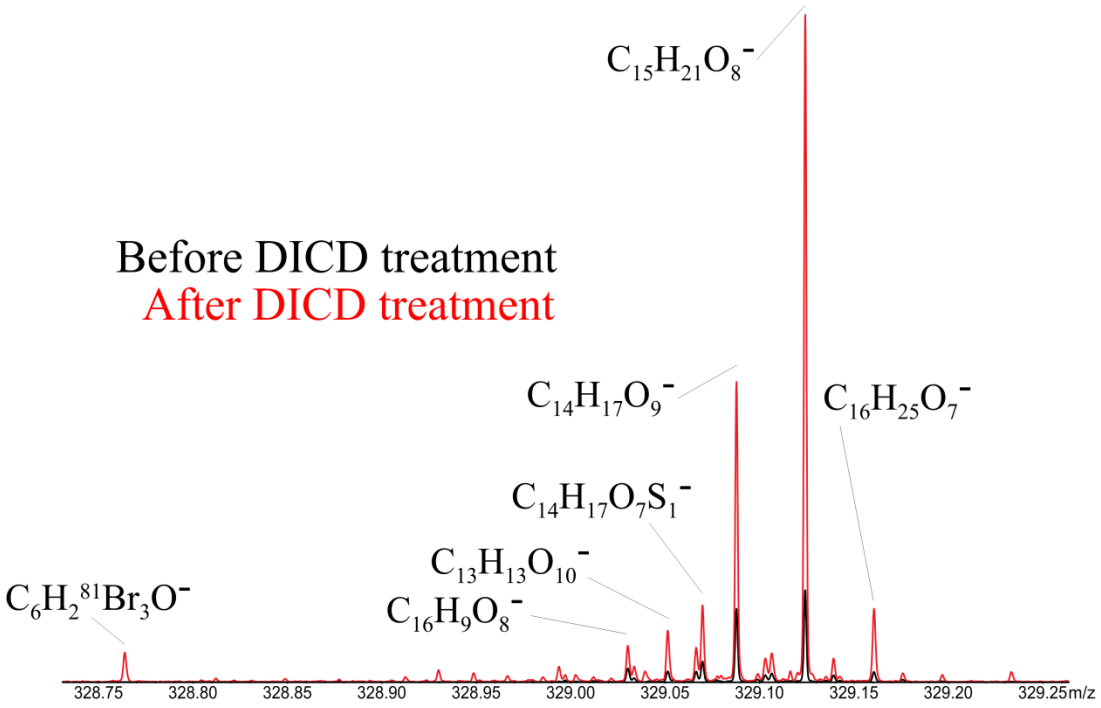

Figure 3.4 Negative-ion ESI-FT-ICR mass spectra between *m/z* 328 and 332 before and after DICD treatment.

3.3.3 DBP Formation with DICD Treatment

A comparison of the ultrahigh resolution mass spectra of extracted DOM in untreated and DICD treated ballast water revealed the formation of numerous Br-DBPs (Figure 3.1) with a mass range similar to untreated DOM (approx. 200 – 600 Da). Because the salinity of the treated estuarine sample was 7 PSU, the bromide concentration was high enough that hypochlorous acid (HOCl) initially present after the addition of DICD solution was rapidly converted into hypobromous acid (HOBr). Disinfection was therefore primarily accomplished with free bromine (HOBr and OBr^-),

resulting in the formation of mostly DBPs containing only bromine comprising 90% of determined halogenated DBPs, in addition to fewer chlorinated and mixed halogenated DBPs (Appendix 2). A total of 213 halogenated molecular ions with relative abundance of at least 1% were assigned to four DBP elemental groups: CHOBr (180), CHONBr (13), CHOCl (16), and CHOBrCl (4). It is important to point out that despite numerous DOM ions containing nitrogen (2,681) in the uptake water, only 13 of the brominated ions that were identified at ≥1% relative abundance after DICD treatment contained a nitrogen atom out of a total of 213 brominated ions. Hence, the CHO only component of DOM was responsible for the majority of observed brominated DBPs. The majority of DBPs of at least 1% relative abundance contained one bromine atom with assignments of 180 single brominated and 13 double brominated DBPs. Brominated DBPs were found in relative abundances as high as 18%, suggesting that concentrations of some DBPs are in the range of naturally occurring compounds within DOM. The formation of brominated versus chlorinated DBPs has been studied extensively in drinking water where typically low concentrations of bromide lead to few (Gonsior et al., 2014b) or no Br-DBPs (Zhang et al., 2012). In one instance, the bromide concentration of fresh water was increased to a very high 2 mg/L in an attempt to amplify Br-DBPs (Zhang et al., 2014). In control water without bromide, at a free chlorine dose of 5.0 mg/L (sodium hypochlorite) and contact time of 5 days, no Br-DBPs were identified. However under the same conditions with added bromide, molecular formulas were assigned to 441 single brominated, 37 double brominated and 139 chlorinated *m/z* ions (Zhang et al., 2014). Gonsior et al. (2015), using FT-ICR MS analysis of water samples after electrochlorination of salinity 30 ballast water, identified 462 brominated DBPs at a

relative abundance of at least 1%, and as high as 22%, and no chlorinated or mixed halogenated DBPs above 1% relative abundance. Although the bromide (Br^-) concentration of the estuarine uptake water was not measured in the current study, bromide is conservative and steadily increases with increasing salinity. The results from these studies reveal a consistent increase in the ratio of Br-DBPs to Cl-DBPs with an increase in salinity and/or bromide concentration, independent of other differences in water chemistry of treated samples. Also, in all studies, consistently fewer DBPs with multiple halogen atoms were formed as predicted by the decreasing electron density of DOM structures with halogenation (Heeb et al., 2014).

Although the majority of DBPs contained only bromine, 4 mixed halogenated and 16 chlorinated DBPs were also identified in DICD treated ballast water at relative abundance of at least 1%, and as high as 2.1% and 2.8% for mixed halogenated and CL-DBPs, respectively (Appendix 2). None of the mixed halogenated DBPs have been previously described. In fact, in other research no mixed halogenated DBPs were reported after chlorination of bromide boosted water with analysis by FT-ICR MS (Zhang et al., 2014). In marine water research also using FT-ICR MS, electrochlorinated ballast water (Gonsior et al., 2015) and chlorinated aquaculture water (Wang et al., 2018) revealed several mixed halogenated DBPs at low (<1%) relative abundance. Our research is the first reported data from FT-ICR MS analysis of oxidant treated mesohaline (i.e. 5 – 18 PSU) water. This analysis helps differentiate the proportions of brominated, chlorinated and mixed halogenated high molecular weight DBPs that are formed in different salinity waters.

Formula assignments of *m/z* ions in the mass spectra also identified four triple-brominated DBPs, however all were found at a relative abundance between 0.28 – 0.58% (Appendix 2). The triple-brominated DBPs had molecular formula assignments of $C_8H_3O_4Br_3$, $C_{14}H_{27}O_5Br_3$, $C_{18}H_{27}O_6Br_3$ and $C_{18}H_{31}O_6Br_3$. In two studies, triple halogenated DBPs appeared rarely or not at all in the mass spectra of oxidant treated water (Zhang et al., 2012; Lavonen et al., 2013). However, in another study the mass spectra of PPL extracted DBPs identified four triple brominated DBPs, including one highly abundant structure (tribromo HCD) with high relative abundance of 21% (Gonsior et al., 2014b).

The majority of formula assignments for the more abundant DBPs (i.e. with relative abundance of at least 1%) in DICD treated water were in the CHOBr elemental group with 180 confirmed formulas. Several of these Br-DBPs were highly abundant with relative abundance of 18% and 16% for $C_{15}H_{25}O_8Br_1$ and $C_{15}H_{23}O_8Br_1$ formulas, respectively. A review of available DBP formulas from previous research found that 27 of the 180 Br-DBPs in the CHOBr elemental group have not been previously described (Appendix 2). However, four of the CHOBr formulas with relative abundance of at least 0.99% match previously proposed formulas.

Gonsior et al. (2015) proposed bromo-trihydroxybenzoic acid for the formula $C_7H_5O_5Br_1$; while Wang et al (2018), and Pan and Zhang (2013) proposed 3,5-dibromo-4-hydroxybenzoic acid for the formula $C_7H_4O_3Br_2$. Two structures, dibromosalicylaldehyde (Wang et al., 2018) and dibromo-4-hydroxybenzaldehyde (Pan and Zhang, 2013) have been proposed for the formula $C_7H_4O_2Br_2$. Wang et al. (2018) also proposed 4,5-dibromophthalic acid for the formula $C_8H_4O_4Br_2$. Importantly, all of

these structural assignments contain aromatic structures, in contrast to regulated DBPs which contain no aromatic moieties. Increasingly, larger more complex DBPs with cyclic structures, including halogenated MCDs (Gong et al., 2005), HCDs (Pan et al., 2016a), pyrroles (Yang and Zhang, 2014), benzoquinones (Yang and Zhang, 2013; Wang et al., 2014), hydroxybenzaldehydes and hydroquinones (Yang and Zhang, 2014), and phenols (Liu and Zhang, 2014), have been identified as DBPs having cytotoxic, genotoxic or developmental toxic properties. In fact, research by Yang and Zhang (2013) found that the developmental toxicities of several aromatic halogenated DBPs were hundreds or thousands of times more toxic than aliphatic DBPs. Specifically, the structural assignment of bromohydroxybenzoic acid may be of significance as halogenated hydroxybenzoic acids were recently identified as having developmental toxicity in polychaete (*Platynereis dumerilii*) embryo bioassays (Pan et al., 2016b). Aromatic compounds are generally also more lipophilic (i.e. higher log P values) compared to aliphatic DBPs, increasing the chance of cellular uptake and accumulation in aquatic organisms (Wang et al., 2018; Yang and Zhang, 2013). However, structures still need to be confirmed for all the suggested compounds in this study, even though isomeric possibilities can be constrained by specific reaction pathways of free bromine.

The Van Krevelen plot of all newly formed Br-DBPs after DICD treatment shows a wide range of H/C (0.25 – 1.9) and O/C (0.17 – 0.86) values with a cluster in the area of higher H/C values and to a lesser extent lower O/C values (Figure 3.5A). This is similar to the van Krevelen diagram for the CHO elemental group before DICD treatment (Figure 3.2A). The slightly lower average H/C ratio (1.0189) in the CHOBr elemental group compared to CHO elemental group before treatment (1.1067) is presumably a

result of electrophilic substitution of a hydrogen atom by a halogen atom, in this case bromine (Table 3.2). The bromination of CHO-only structures in this area of the van Krevelen diagram, thought to be aromatic phenol-like structures, is in agreement with the general correlation between high specific UV_{254} absorbance (SUVA) values and formation of DBPs (Matilainen et al., 2011; Hua et al., 2015; Yan et al., 2018). A modified Kendrick plot of Br-DBPs formed after DICD treatment (Figure 3.5B) shows several homologous series of Br-DBPs, suggesting their similarity to CHO-only precursors present in uptake water (Zhang et al., 2012). Other research has also observed that changes in DOM after halogenation were diverse, but that many DBPs were similar to polyphenolic-like (Harris et al., 2015) or humic-like (Gonsior et al., 2015) compounds. However, it has been suggested that part of this phenomenon is a side effect of using FT-ICR MS, because the halogenated DBPs that retain their structural humic-like character are also amenable to FT-ICR MS analysis (Harris et al., 2015). Furthermore, the significant conversion of DOM to smaller halogenated compounds (e.g. HAAs and THMs) would result in their loss either during extraction (SPE) or being outside of the observable mass range of FT-ICR MS.

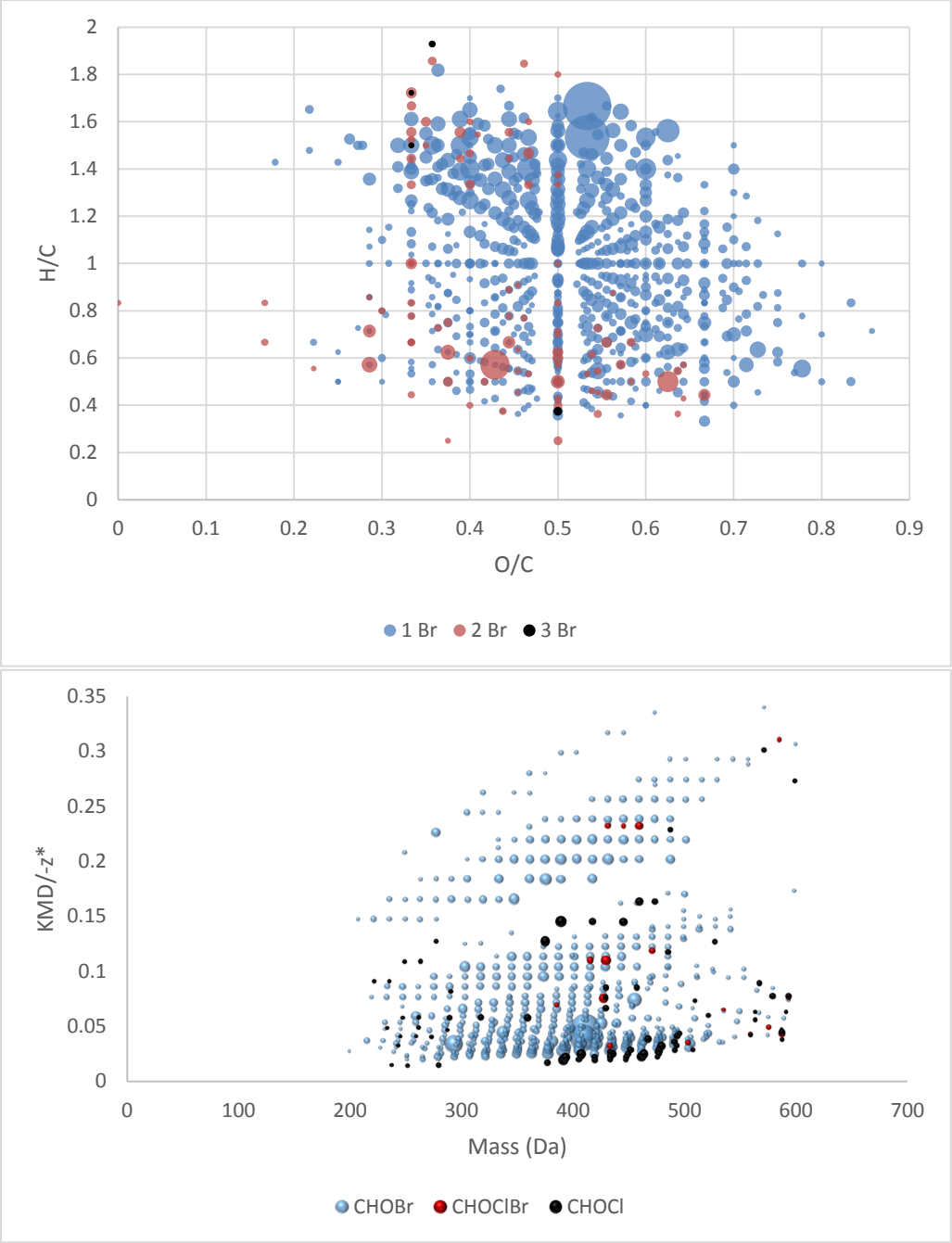

Figure 3.5 Van Krevelen diagram (A) and Modified Kendrick plot (-KMD/*z** vs mass) (B) of newly formed Br-DBPs identified after DICD treatment. The bubble sizes represent relative abundance.

A total of 13 nitrogen-containing Br-DBPs with a relative abundance of at least 1%, and as high as 3.5%, were identified and confirmed in DICD treated ballast water (Appendix 2). None of these 13 formulas have been previously described as DBPs.

However, several other nitrogen-containing DBPs found at slightly lower 0.9 % relative abundance coincide with two previously identified formulas with suggested structures of 2-bromo-4-nitrophenol and 2-bromo-1-(5-bromo-2-hydroxy-3-nitrophenyl) ethanone for $C_6H_4O_3N_1Br_1$ and $C_8H_5O_4N_1Br_2$, respectively (Wang et al., 2018). Nitrogen-containing DBPs are of increasing concern as research has shown that they are generally more toxic than Br-DBPs without nitrogen (Plewa et al., 2008; Wagner and Plewa, 2017).

In ballast water treatment with strong oxidants, algae in uptake water may be a significant source of organic nitrogen, and serve as one type of precursor leading to nitrogen-containing Br-DBPs due to relatively labile biopolymers (e.g. proteins, peptides, amino acids) in algal organic matter (AOM). Precursors from algae such as found in algal cell exudates and intracellular organic matter released after cell lysis (Bond et al., 2011; Chen et al., 2017) can also be significant in eutrophic systems (Hua et al., 2017; Yang et al., 2011). In aquaculture research, chlorination (NaDDC) of raw seawater that contained algae resulted in the production of 32 nitrogen-containing Br-DBPs, while chlorination of artificial seawater (i.e. contained no algae) resulted in only 2 nitrogen-containing Br-DBPs (Wang et al., 2018). Algae in natural fresh waters are also known to contribute to the formation of DBPs after chlorination (Hoehn et al., 1980; Liao et al., 2015; Ge et al., 2018) and chloramination (Yang et al., 2011; Chen et al., 2017). Diatoms can contribute to significant production of nitrogen-containing DBPs compared to other classes of microalgae (Goslan et al., 2017). The dominant species in the current project were diatoms of the genera *Gymnodinium* and *Prorocentrum* (data not presented), which may have contributed to the formation of the large number (244) of nitrogen-containing DBPs mostly found at low relative abundance (<1%) in DICD treated ballast water.

We also identified a newly discovered nitrogen-containing Br-DBP with formula $C_3HBr_3N_2$, found at a relative abundance of 6.4%, tentatively identified as either tribromoimidazole or tribromopyrazole (Figure 3.6). No ecotoxicity data is available for either potential structure. This compound is most assuredly a DBP generated with DICD water treatment, although there is one report of a naturally occurring tribromoimidazole (2,4,5-tribromo-1H-imidazole) found in the literature (Benkendorff et al., 2004). Although oxidation products for some amino acids have been characterized (Choe et al., 2015), no oxidation products for histidine are available in the literature. The non-halogenated imidazole is a water soluble aromatic heterocycle with a 5-membered ring (Mihajlović et al., 2017). However, the imidazole in the current research is assumed to be incorporated into protein, possibly as a histidine side chain found in many proteins. Halogenation reactions between biomolecules and HOBr may form the suggested tribromoimidazole structure by an initial electrophilic aromatic substitution reaction perhaps on a hydroxyimidazole which further activates the ring to be even more susceptible to additional electrophilic aromatic substitutions to reach full substitution with bromine. Another less likely source of imidazole is from the ship itself where imidazole derivatives are used as copper corrosion inhibitors (Mihajlović et al., 2017) and in epoxy coating formulations (Kirchgeorg et al., 2018). The other possible structural assignment for this new DBP is tribromopyrazole. Similar to imidazole, the pyrazole is an aromatic 5-membered ring comprised of the same atoms, but with adjacent nitrogen atoms (Figure 3.6). Natural pyrazoles in aquatic settings are rare presumably because the formation of the N-N bond is not easily accomplished by aquatic organisms.

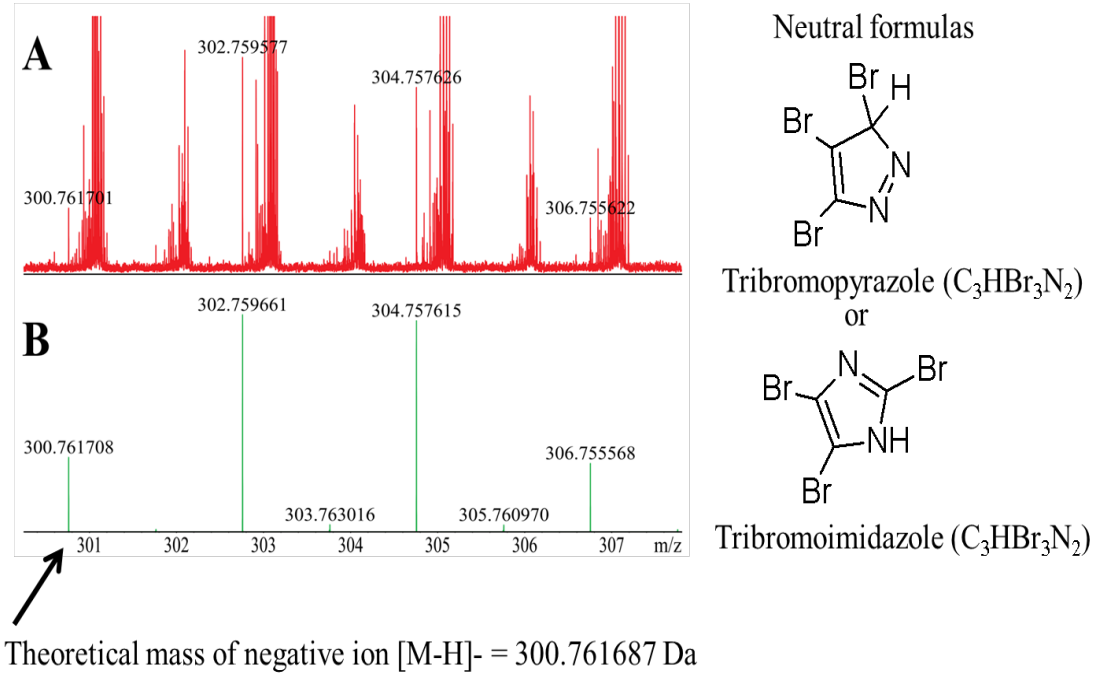

Figure 3.6 Isotope simulation of the newly discovered DBP $C_3HBr_3N_2$ tentatively identified as tribromoimidazole or tribromopyrazole.

A large number of *m/z* ions with a sulfur atom were found in uptake water. However, no sulfur-containing halogenated DBPs were identified and confirmed after DICD treatment of estuarine water (7 PSU) contrary to other research which has identified sulfur-containing DBPs in electrochlorinated saline ballast water (Gonsior et al., 2015) chlorinated saline sewage water (Gong and Zhang, 2015) and aquaculture water (Wang et al., 2018).

The method of oxidant treatment and sampling of ballast water as well as the extraction, processing and analysis of DOM can affect the number and type of DBPs that are formed and identified. Previous studies have often used methods that are expected to result in the maximum number of a certain group of DBPs, or have created environments that are expected to lead to new DBPs. These methods include adjusting water chemistry by artificially increasing specific DBP precursors or bromide. In the current work with a

DICD based BWMS, ballast water was amended with DOC and POC additives before treatment, and treated water was held for 48 h before collection of sample, and extraction for DBP analysis. The 48 h hold time simulated the normal operation of a ship's BWMS including ballast water treatment during uptake, followed by transport and then discharge. The TRO was not monitored during the 48 h hold time, although it is assumed that the initial oxidant demand of uptake water will have rapidly decreased the active halogen concentration resulting in an initial DBP profile. However, rapidly formed DBPs may have had time to further break down into smaller structures during the 48 h hold time. In chlorination of bromine-rich drinking water, aromatic Br-DBPs formed quickly and increased for several days, then decreased with continued contact time leading to increased concentrations of lower molecular weight (<300 Da) aliphatic Br-DBPs (Zhai and Zhang, 2011; Zhai et al., 2014). In the current project, no attempt was made to identify lower molecular weight DBPs because of the methods of extraction (SPE) and analysis (FT-ICR MS). However, in oxidant-based BWMS testing many lower molecular weight aliphatic DBPs have been shown to increase with holding time (IMO, 2014), possibly due to the breakdown of larger molecular weight DBPs.

Recent research has shown that ballast water treated with strong oxidants can remain toxic to algae, even after neutralization of TRO (Delacroix et al., 2013; Ziegler et al., 2018). In one study, only the addition of lignin before electrochlorination resulted in algal toxicity with a complete inhibition of growth (Park et al., 2017). Research on the longevity of algal toxicity of treated ballast water from a chlorination-based BWMS showed that algal toxicities of DICD and electrochlorinated waters could last up to 134 days and that toxicity did not correlate to quantified low molecular weight DBPs (Ziegler

et al., 2018), suggesting the long-term presence of some unquantified toxic component in oxidant treated water.

The disinfection of seawater with strong oxidants is used in a variety of applications including industrial cooling water, desalination, seawater toilets and aquaculture, in addition to ballast water treatment. Although the characterization of brominated DBPs formed in saline waters is ongoing, evidence so far suggests these saltwater applications increase both the number of more toxic nitrogen-containing DBPs, and aromatic DBPs that are more likely to persist in the environment. The increased use of new analytical methods to identify and characterize higher molecular weight DBPs is an important step towards evaluating their potential environmental risk.

Chapter 4

Persistence of Brominated Organic Compounds Formed after Ballast Water Treatment by Electrochlorination

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Abstract

Estuarine water treated with a ballast water management system (BWMS) using in-situ electrochlorination resulted in the formation of numerous brominated disinfection by-products (Br-DBPs) that were evaluated on the day of discharge and after 92 days. Analysis of electrochlorinated water with ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) identified 92 brominated *m/z* ions with relative abundance of at least 1% that were assigned and confirmed using isotope simulation. One hundred and thirty one days after electrochlorination, a total of 150 brominated molecular ions with relative abundance of at least 1% were identified. A comparison of electrochlorinated water on the day of treatment and after 92 days showed a similar pool of Br-DBPs, but with a substantial difference in the most abundant assigned formulas. After 92 day hold time, the two most abundant brominated ions contained two bromines, a distinct difference from primarily singly brominated ions that were abundant at the time of ballast water discharge. Previous research has demonstrated the ability of FT-ICR MS to provide the molecular composition of complex halogenated DBPs in ballast water after treatment with strong oxidants. This study is the first attempt to monitor the persistence of high molecular weight halogenated DBPs. The persistence

of high molecular weight Br-DBPs identified by FT-ICR MS is a critical factor in assessing their long-term risk in the aquatic environment.

4.1 Introduction

The treatment of ballast water to prevent bioinvasions resulting from transfer of non-indigenous species (NIS) is required in accordance with internationally recognized *Ballast Water Management Convention* of the International Maritime Organization (IMO, 2004). Under the Convention, and other governmental based regulatory programs, discharged ballast water must meet specific discharge standards of organisms in specific size classes. Typical treatment of ballast water on vessels takes place during uptake of ballast water through physical or chemical treatment by a ballast water management system (BWMS). The majority of BWMS use an active substance as a biocide, and the majority of these systems make use of strong oxidants either stored on board (e.g. sodium hypochlorite, hydrogen peroxide) or produced in-situ by electrochlorination of seawater.

Strong oxidants in the form of chlorine and bromine have been used extensively in fresh water applications such as drinking water, swimming pools, desalination membranes and wastewater treatment. However, the use of strong oxidants has the undesirable side effect of forming disinfection by-products (DBPs) after reacting with primarily dissolved organic carbon (DOC), but also with other components of dissolved organic matter (DOM) such as biomolecules (e.g. proteins and nucleic acids). The formation DBPs is influenced by oxidant dose, temperature, pH, DOM quantity and composition, and potentially by the mechanism of disinfection (e.g. liquid hypochlorite, dichloroisocyanurate dihydrate (DICD), electrochlorination). Although small chlorinated DBPs such as haloacetic acids (HAAs), trihalomethanes (THMs), haloacetonitriles

(HANs) and oxyhalides have been identified by GC-MS and LC-MS for decades (Richardson, 2002), as much as 50% of the total organic halogen (TOX) formed after chlorination is undefined (Krasner et al., 2006; 2009; Chen et al., 2015). The majority of the unidentified halogenated DBPs are presumed to be complex higher molecular weight halogenated DBPs not amenable to analysis by GC- and LC-MS (Zhang and Minear, 2002; 2006).

In the current research, estuarine water (salinity 5.2) was treated by a BWMS employing filtration (50 μm) and *in-situ* electrochlorination. Because of naturally present bromine in brackish water, any hypochlorous acid (HOCl) formed during electrochlorination treatment will almost instantaneously yield active bromine (HOBr or OBr^-) (Westerhoff et al., 2004). The combination of active chlorine and active bromine is quantified as the total residual oxidant (TRO) measured in Cl_2 equivalents (mg l^{-1} TRO as Cl_2). The electrochlorination process produces HOCl by running an electrical current through water containing chloride (e.g. brackish water or seawater). The electrochlorination technology is well developed and is widely used in treatment of seawater for swimming pools, desalination membrane fouling control and biofouling control in industrial and power plant cooling waters. Electrochlorination of ballast water uses an electrolyzer unit which is composed of an anode and cathode that can be made of a variety of materials. The different electrode materials can vary, with anodes in newer units composed of titanium with metal oxide coatings (e.g. iridium or rubidium oxides) (Jeong et al., 2009). The chemical reactions take place in the electrolyzer unit which applies a direct current to the incoming ballast water. The water flows between the anode and a cathode, producing chlorine on the anode (eq. 1), and hydrogen and sodium

hydroxide on the cathode (eq. 2). The overall reaction in the solution (eq. 3) shows the final production of sodium hypochlorite that can exist as hypochlorous acid (HOCl) or hypochlorite ion (CLO^-) depending on the pH of the solution (Black and Veatch., 2010).

$$\text{(Anode):} \quad 2Cl^- \rightarrow Cl_2 + 2e^- \qquad (1)$$

$$\text{(Cathode):} \quad 2H_2O + 2e^- \rightarrow 2OH^- + H_2 \qquad (2)$$

$$\text{Overall Reaction:} \quad 2Cl^- + 2H_2O \rightarrow 2HOCl + H_2 \qquad (3)$$

The formation of HOCl is directly proportional to the applied specific charge and salinity (i.e. chlorine concentration) of the water (Ghernaout et al., 2011; Rahmani et al., 2019). HOCl is rapidly converted into HOBr in brackish water, so that the majority of halogenation will be by active bromine. Active bromine participates in an increased number of substitution reactions compared to chlorine (Uyak and Toroz, 2007), leading to numerous brominated DBPs (Br-DBPs). The use of direct chlorination or in-situ electrochlorination of brackish and marine waters is also used for treatment of industrial cooling water (Jenner et al., 1997; Allonier et al., 1999), desalination (Kristiansen et al., 1996) and treatment of saline wastewater (Ding et al., 2013) leading to multiple sources of Br-DBPs in the aquatic environment. Ballast water treatment is a comparatively new application of chlorination that can lead to high concentrations of Br-DBPs (Werschkun et al., 2012; 2014) due to higher doses of total residual oxidant (TRO) and complexity of DOM in uptake water (Shah et al. 2015; Hao et al., 2017). Research on Br-DBPs has revealed that they are more likely to be carcinogenic and mutagenic, compared to their chlorinated analogues (Echigo et al., 2004; Richardson et al., 2007) making the analysis of brominated compounds formed in ballast water treatment of particular concern.

A large data set of relatively small traditional DBPs (e.g. HAAs, THMs, oxyhalides and HANs) formed in ballast water treatment with oxidants is available in an online database (*GESAMP-BWWG-Database of chemicals most commonly associated with treated ballast water*) located on the Global Integrated Ship Information System (GISIS) website (IMO, 2017b). However, as stated previously, a significant portion of the total organic halogen (TOX) formed after water chlorination has not been accounted for with GC-MS and LC-MS techniques that were used for quantifying the DBPs available in the BWWG Database. In the current research, we use a combination of solid phase extraction (SPE) and ultrahigh-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to identify higher molecular weight Br-DBPs. Ultrahigh-resolution FT-ICR MS has been successfully used to assess the halogenation of DOM in drinking water (Lavonen et al., 2013; Zhang et al., 2014) and oxidant treated ballast water (Gonsior et al., 2015; Ziegler et al., 2019). FT-ICR MS has sufficient resolution to distinguish individual elemental compositions in DOM and halogenated DBPs, and can define the fundamental molecular differences between components by mass measurement alone (Stenson et al., 2003). However, the DOM extraction efficiency has been estimated at around 55% due to the loss of highly volatile DBPs and DOM during solid phase extraction (Gonsior et al., 2014b). Also, all DOM and DBPs are not equally ionized during negative mode electrospray ionization. Therefore, this paper is limited to DOM and complex high molecular weight Br-DPBs that are amenable to the extraction method and are efficiently ionized.

The primary goal of this research was to record the relative persistence of high molecular weight halogenated DBPs. Persistence in the current paper was estimated by

holding electrochlorinated ballast water for a period of 92 days, followed by solid phase extraction and analysis for DBPs by FT-ICR MS. Non-targeted direct infusion FT-ICR MS was used to determine the molecular ions that were present at the time of ballast water discharge (Day 0), as well as the ions that were present in the same treated water after 92 days. Spectrometric data of Br-DBPs was visualized using van Krevelen diagrams (van Krevelen, 1950) plotting H/C ratio versus O/C ratio. The complex high molecular weight Br-DBPs identified by FT-ICR MS are within the *m/z* window of FT-ICR MS. Characteristics of DBPs after the 92 day holding time were compared to the DBP pool found at the time of treated ballast water discharge.

4.2 Materials and Methods

4.2.1 Ballast Water Sampling and Extraction

Estuarine water (salinity of 5.2) was treated with a BWMS that employed course filtration (50 μm) followed by electrochlorination. The land-based test facility used for BWMS testing was located in Port Covington, Baltimore, Maryland, USA. Uptake water (pH 8.2, temp. 27.2 °C) was taken from the surface of Winans Cove (Baltimore, MD, USA). Adjustments were made to DOC of uptake water to coincide with United States Coast Guard Environmental Technology Verification (ETV) Program (USEPA, 2010), which requires modification of test water to meet minimum values for specific water parameters. Potentially relevant to the formation of DBPs, sodium citrate dihydrate (Fisher Scientific, USA) was added to increase the ambient DOC of 3.3 mg C l^{-1} to a final DOC of 7.9 mg C l^{-1}. Addition of citrate was made in uptake water before splitting into treated and untreated ballast water lines. The TRO target dosage of the BWMS was 8 mg l^{-1} TRO as Cl_2 controlled by a continuous feedback loop from in-line TRO meters.

Control and treated ballast waters were delivered to independent tanks and held for 48 h in closed ballast tanks at ambient temperature.

An untreated (i.e. control) water sample was collected in a 20-L polycarbonate carboy directly from a hatch on the untreated ballast water tank. A continuous, time integrated sample of treated water was collected throughout the entire discharge process (approximately 1 h) and delivered to a 100-L polymer fiberglass tank. Ballast water was not treated again upon discharge, but sodium sulfite was added during the entire discharge process to neutralize any remaining TRO, after which treated water was collected for analysis. The neutralization of all remaining TRO was independently confirmed with a hand-held colorimetric TRO meter (Hach, Model No. 58700, USA) based on the DPD method of TRO quantification (USEPA, 1983). A 19 l treated water sample was collected by gravity flow in a glass carboy (20-L) directly from the 100-L fiberglass tank. Control and treated water samples were immediately transferred to ice filled coolers for transport to the University of Maryland Wye Research and Education Center (UMD/WREC) for solid phase extraction (SPE) of the untreated control and treated Day 0 samples. The remainder of the treated water sample was held in the glass carboy sealed with Parafilm™ and stored at 4 °C. After 92 days, another aliquot of treated water was taken from the 20-L carboy for extraction and analysis.

Isolation of DOM and DBPs was accomplished with solid phase extraction (SPE) on small (1 g) cartridges of highly functionalized styrene-divinylbenzene (SDVB) polymer beads (Agilent Bond Elut PPL), followed by methanol elution and preservation of sample. This extraction method for DOM has been published previously (Dittmar et al., 2008) and has also been used to extract DBPs from dichloroisocyanurate (DICD)

treated estuarine water (Ziegler et al., 2019) and electrochlorinated marine water (Gonsior et al., 2015). Extractions of the Day 0 samples were started within three hours of ballast water discharge sample collection. The SPE method allowed for the concentration of DOM/DBPs and desalting of the ballast water, required for analysis by FT-ICR MS with electrospray ionization (Stenson et al., 2003). Briefly, water samples (1 l) were vacuum filtered (pre-combusted at 500 °C Whatman GF/F, 0.7 µm) and acidified to pH 2 with formic acid. Samples were then gravity-fed through methanol activated SPE cartridges, rinsed with formic acid acidified water, dried with a gentle stream of nitrogen and eluted with 10-ml of LC-MS grade methanol (Chromasolv, Sigma-Aldrich). Previous research has shown that DOM extraction with PPL filled SPE cartridges has an extraction efficiency between 52 and 74% (Lavonen et al., 2013; Gonsior et al. 2014b; Green et al., 2014).

4.2.2 Chemical Analysis

4.2.2.1 DOC

Analyses of dissolved organic carbon (DOC) was carried out on uptake water both before and after the water amendments to reach ETV minimum concentration of DOC. The high temperature combustion method was used to analyze samples for DOC using the Shimadzu TOC-L carbon analyzer. Briefly, the water sample was filtered through a 0.7 µm GF/F glass fiber filter, acidified with hydrochloric acid and sparged with ultrapure air to remove inorganic carbon. Samples were high temperature combusted (680 ºC) on a catalyst bed to break down carbon compounds into carbon dioxide (CO_2) which was then quantified on a non-dispersive infrared detector (NDIR). The method detection limit (MDL) for DOC was 0.24 mg C l^{-1}.

4.2.2.2 Total Residual Oxidant

Measurements of the TRO concentration in discharged ballast water were confirmed using the USEPA recommended DPD (n,n-diethyl-p-phenylene diamine) colorimetric method (USEPA, 1983) with a handheld TRO meter (Hach Pocket Colorimeter™ II, Model No. 58700, U.S.A.). TRO measurements were taken in triplicate on samples collected at the beginning, middle, and end of the overall discharge period (approximately 1 h). TRO measurements on the Hach Pocket Colorimeter were made in Low-Range mode (0.02 - 2.0 mg l^{-1} TRO as Cl_2), following the manufacturer's instruction manual (Hach Company, 2013).

4.2.3 Ultrahigh Resolution Mass Spectrometry (FT-ICR MS)

Mass spectrometry of extracted samples was performed using a Bruker Solarix 12 T Fourier transform (FT) ion cyclotron resonance (ICR) mass spectrometer (FT-ICR MS) following electrospray ionization in the negative ion mode (ESI^-). Methanolic samples were diluted with methanol at a ratio of 1:20 to limit space charge processes, which may lead to interferences in the transient spectrum. Diluted samples were directly infused into the electrospray at a constant flow rate of 2 µl min^{-1} and 500 scans were averaged with a time domain of 4 megaword. The voltage of the ESI^- was set to -3.6 kV. A cleaning procedure using 600 µl blank methanol samples (50% methanol and 50% water) was implemented between each sample to avoid carryover. The resolution of FT-ICR MS of singly charged ions formed by ESI^- resulted in a mass accuracy <0.2 ppm, allowing unambiguous molecular formula assignments. The exact molecular formula assignments were based on the following atomic numbers: $^{12}C_{0-\infty}$, $^{1}H_{0-\infty}$, $^{16}O_{0-\infty}$, $^{14}N_{0-5}$, and $^{32}S_{0-2}$, $^{35}Cl_{0-5}$ and ^{79}Br. The isotopologues of the halogens (^{37}Cl and ^{81}Br) were used to cross-

validate formula assignments using simulation comparisons. Unambiguous formula assignments, only given to *m/z* values with a signal to noise ratio >10, were possible for the entire observed *m/z* range. Calculation of elemental formulas for each *m/z* ion was undertaken with the previously described *NetCalc* network approach (Tziotis et al., 2011; Hertkorn et al., 2013). The *NetCalc* generated formula assignments were also manually validated to check isotope pattern matching for high intensity *m/z* ions. Manually validated formulas included elemental compositions containing C, H, O, N, and Br. The focus in this research is the evaluation of the number and character of Br-DBPs remaining in electrochlorinated water after a holding time of 131 days.

Brominated molecular formulas were validated by isotope simulations using two stable isotopes of bromine (^{79}Br and ^{81}Br) to cross-validate assigned formulas. The MS spectra were pre-calibrated using arginine clusters and also checked post-calibrated using known DOM molecular formulas (Gonsior et al., 2016). The relative abundance of individual molecular ions were calculated by comparison to the highest DOM ion intensity in the mass spectrum for each sample. The intensities of ions in the mass spectra were also used to compare untreated samples to a treated sample collected at the day of discharge (Day 0) and after a 92 day hold time. An additional comparison was made between the electrochlorinated water samples collected on Day 0 and Day 92 from the same glass carboy of treated water. Although there is the possibility of ionization suppression and matrix effects influencing the intensity of individual *m/z* ions, a relative comparison of ion intensities before and after oxidative treatment (Koch et al., 2005; Sleighter et al., 2012; Lavonen et al., 2013), and over time (Gonsior et al., 2013) have been used in previous studies in a semi-quantitative way. Also, it should be noted that

m/z ion intensities are not uniformly related to the actual concentration due to the ionization efficiencies of different compound classes.

Several parameters were used to interpret FT-ICR MS spectrometric data, allowing for the comparison of initial uptake water, treated water at discharge, and treated water after 92 days. Parameters included *m/z* range and number of hydrogen and oxygen to carbon ratios (O/C and H/C). Van Krevelen diagrams (van Krevelen, 1950; Kim et al., 2003) were used to characterize molecular formulas by plotting H/C ratio against the O/C ratio of assigned formulas (Hertkorn et al., 2006; Gonsior et al., 2014b). This plot allows visualization of relative oxygen and/or hydrogen deficiency, resulting in the clustering of organic compounds into specific regions which can also reflect biological signatures of DOM ions (Sleighter and Hatcher, 2007; Hockaday et al., 2009; Ohno et al., 2010).

4.3 Results and Discussion

4.3.1 Molecular Characterization of Uptake Water DOM

Figure 4.1A shows the ultrahigh resolution mass spectra of the solid phased extracted DOM from Baltimore Harbor water before treatment. Numerous *m/z* ions representing unique molecular formulas were identified. These ions fell into three major organic groups: CHO, CHON and CHOS. The CHO elemental group was the most abundant with 2,222 *m/z* ions followed by CHON group with 1,954 *m/z* ions and CHOS with 436.

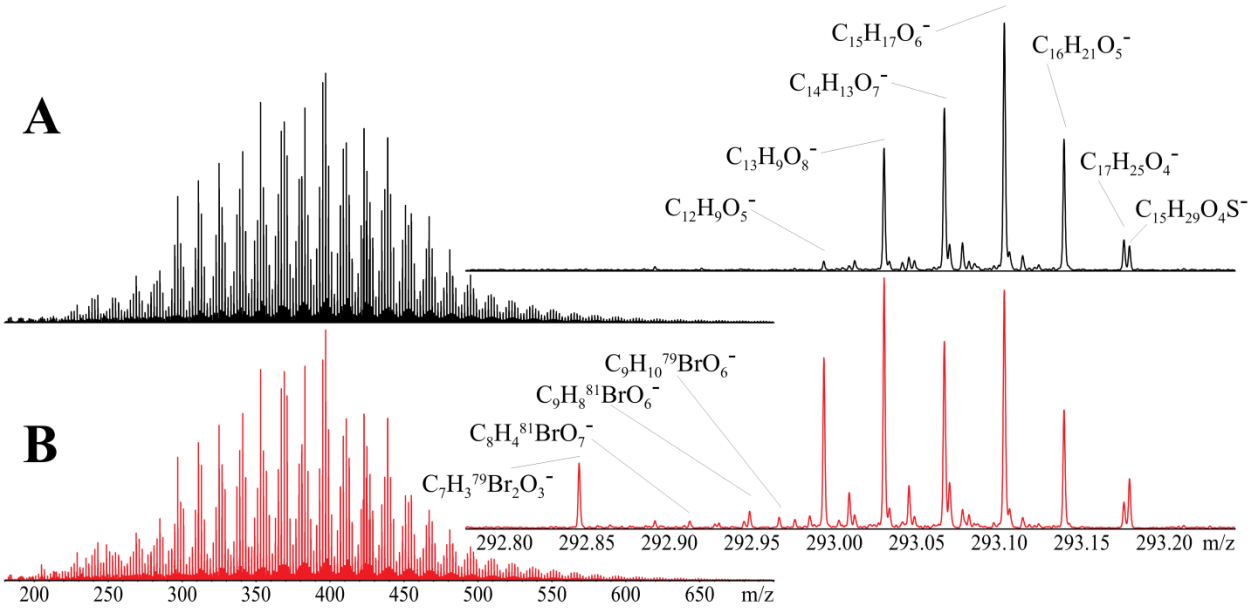

Figure 4.1 Ultrahigh resolution FT-ICR mass spectra of PPL extracts of DOM from Baltimore Harbor, USA in A) untreated water B) electrochlorinated water after 92 days. The enlarged area shows negative m/z ions at nominal mass 292.

The examined mass range of DOM was 160 – 600 Da, which is consistent with previous FT-ICR MS studies of Baltimore Harbor DOM (Ziegler et al., 2019) and other coastal sources of DOM (Sleighter et al., 2012; Gonsior et al., 2015). The van Krevelen diagram of molecular formulas in DOM containing only C, H and O (Figure 4.2) show a similar pattern to recent analysis by Ziegler et al. (2019) on samples taken from the same body of water, and using the same solid phase extraction method (PPL) and analysis (FT-ICR MS). The van Krevelen diagram clusters organic compounds in DOM by elemental ratios (i.e. O/C and H/C), where similar types of compounds are found in close proximity on the diagram. Like previous analysis of Baltimore Harbor water, the majority of DOM molecules had elemental ratios that are characteristic of terrestrially-derived DOM (Hockaday et al., 2009; Gonsior et al., 2015; Sleighter and Hatcher, 2007; Lu et al., 2015).

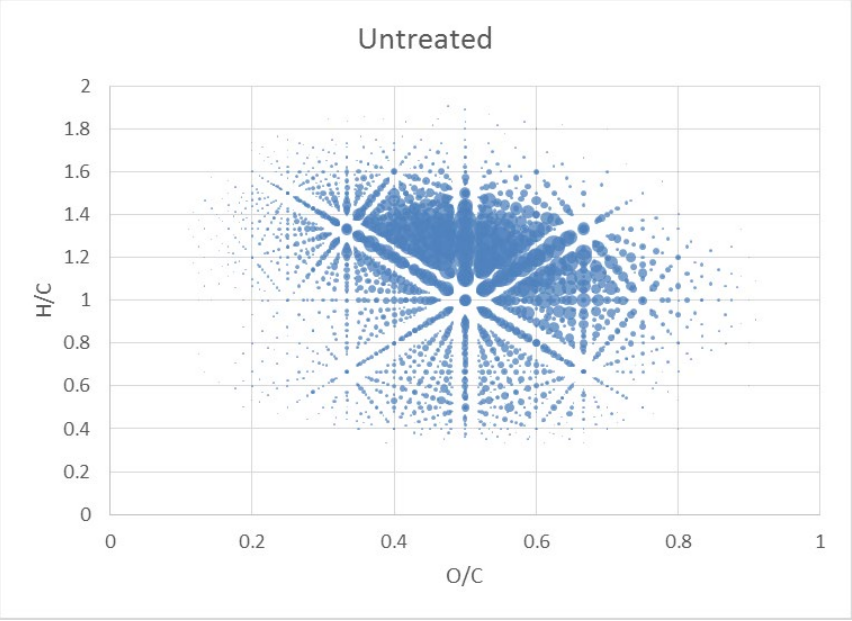

Figure 4.2 Van Krevelen diagram of CHO molecular formulas of DOM in untreated ballast water. Bubble size represents abundance.

4.3.2 Brominated DBPs after Electrochlorination

4.3.2.1 DBPs on the Day of Discharge (Day 0)

The salinity of the estuarine water in the current research was 5.2 with a high enough bromide concentration that any hypochlorous acid (HOCl) formed during electrochlorination was rapidly converted into active bromine (i.e. HOBr and OBr^-), resulting in primarily brominated DBPs. The van Krevelen diagram of all newly formed Br-DBPs after electrochlorination (Figure 4.3A) is similar to the van Krevelen diagram for the CHO elemental group before electrochlorination (Figure 4.2) with a cluster of ions in the area of H/C values between 1 and 1.4 and O/C values between 0.4 and 0.6 (Figure 4.3A). The average H/C ratio of the CHO elemental group before treatment was 1.07, while the average H/C ratio in the CHOBr elemental group after treatment was 0.95. The lower H/C ratio is presumably due to the electrophilic substitution of a hydrogen atom by a bromine atom (Ziegler et al., 2019). Following electrochlorination, 654 unique molecular formulas were assigned in the CHOBr (454) and CHONBr (200) elemental groups of Br-DBPs. Of the 654 halogenated features identified, 193 ions were identified at a relative abundance of at least 1% and as high as 2.5% in the CHOBr (190) and CHONBr (3) elemental groups.

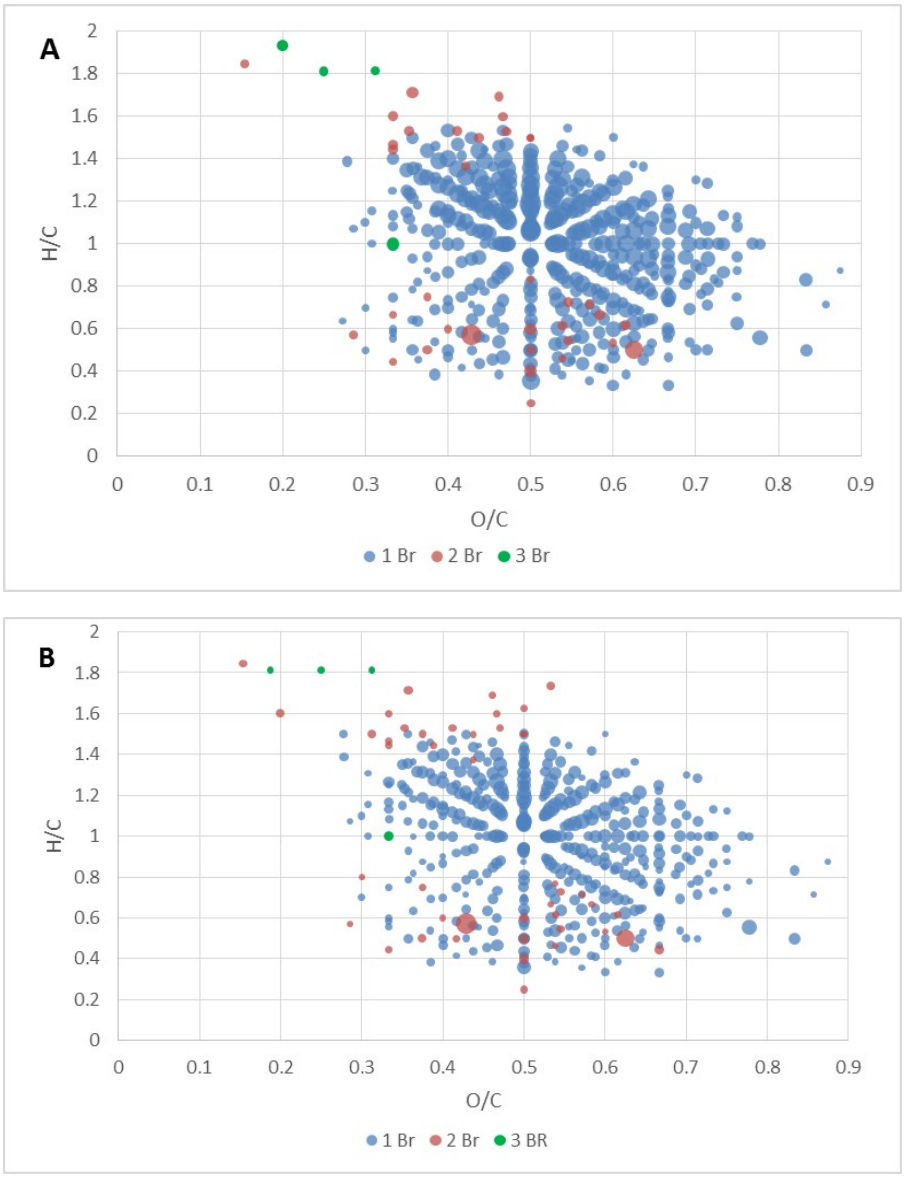

Figure 4.3 Van Krevelen diagram of newly formed Br-DBPs identified after electrochlorination of ballast water A) at the time of discharge B) after a 92 days. The bubble sizes represent relative abundance.

4.3.2.2 Br-DBPs after 92 Days

A comparison of the ultrahigh resolution mass spectra of extracted DOM in untreated water (Figure 4.1A) and electrochlorinated ballast water after 92 days (Figure 4.1B) revealed a substantial number of Br-DBPs with a mass range similar to untreated

DOM (approximately 160 – 600 Da). A similar number of Br-DBPs were identified in treated ballast water at the time of discharge and after a holding period of 92 days. Unique molecular formulas were again assigned in the CHOBr and CHONBr elemental groups in relative abundances as high as 3.3%. After the 92 day holding time, a total of 676 brominated formulas were assigned to ions in the CHOBr (462) and CHONBr (214) elemental groups of Br-DBPs. Of the 676 formulas, 150 brominated ions were identified with a relative abundance of at least 1% and as high as 3.3% in the CHOBr (145) and CHONBr (5) elemental group categories (Appendix 3).

The majority of Br-DBPs were singly brominated in treated samples from both sample times (Day 0 and Day 92). Of the 676 brominated ions in the 92 day sample, there were 616 ions with one bromine with a smaller number of dibrominated (56) and tribrominated (4) ions (Appendix 3). As observed in other studies using analysis by FT-ICR MS (Wang et al., 2018; Ziegler et al., 2019), fewer multiple halogenated DBPs were formed as predicted by the decreasing electron density of DOM structures with increased halogenation (Heeb et al., 2014).

The total number of DBPs identified by FT-ICR MS analysis was similar in the treated sample on Day 0 (654) and on Day 92 (676). However, the average relative abundance of all brominated ions decreased from 1.0% in the Day 0 sample to 0.8% in the Day 92 sample. The overall difference in relative abundance can be seen in the van Krevelen diagrams of Br-DBPs in Day 0 (Figure 4.3A) and Day 92 (Figure 4.3B) where most of the bubble sizes, representing relative abundance, are significantly smaller in the Day 92 van Krevelen diagram. It should be mentioned that an individual bubble, and

bubble size, on the van Krevelen are not necessarily representative of a single molecular formula, but rather represent all formulas with the same H/C and O/C ratios.

The number and composition of the most abundant brominated ions (i.e. at least 2% relative abundance) changed substantially after 92 days. Initially in the Day 0 sample, 17 of the 18 most abundant ions had a single bromine atom with only one dibrominated DBP (Figure 4.4A). Also in the Day 0 sample, all of the 18 DBPs in the most abundant group had similar relative abundances of between 2.0% and 2.5%. In contrast, in the 92 day sample the ions with two bromines were far more abundant than monobrominated DBPs (Figure 4.4B; Appendix 3). Analysis of the 92 day sample revealed only four Br-DBPs with relative abundance of at least 2%, and two out of the four DBPs contained two bromines. When taking the abundance of individual ions into account, dibrominated ions comprised more than half of the total of these four most abundant DBPs (Figure 4.4B). The two dibrominated DBPs in the 92 day sample, with formulas of $C_7H_4O_3Br_2$ and $C_8H_4O_5Br_2$, were found at 3.3% and 2.5%, respectively, substantially higher than the next ion which was monobrominated ($C_{15}H_{17}O_8Br_1$) with relative abundance of 2.14% (Figure 4.4B; Appendix 3).

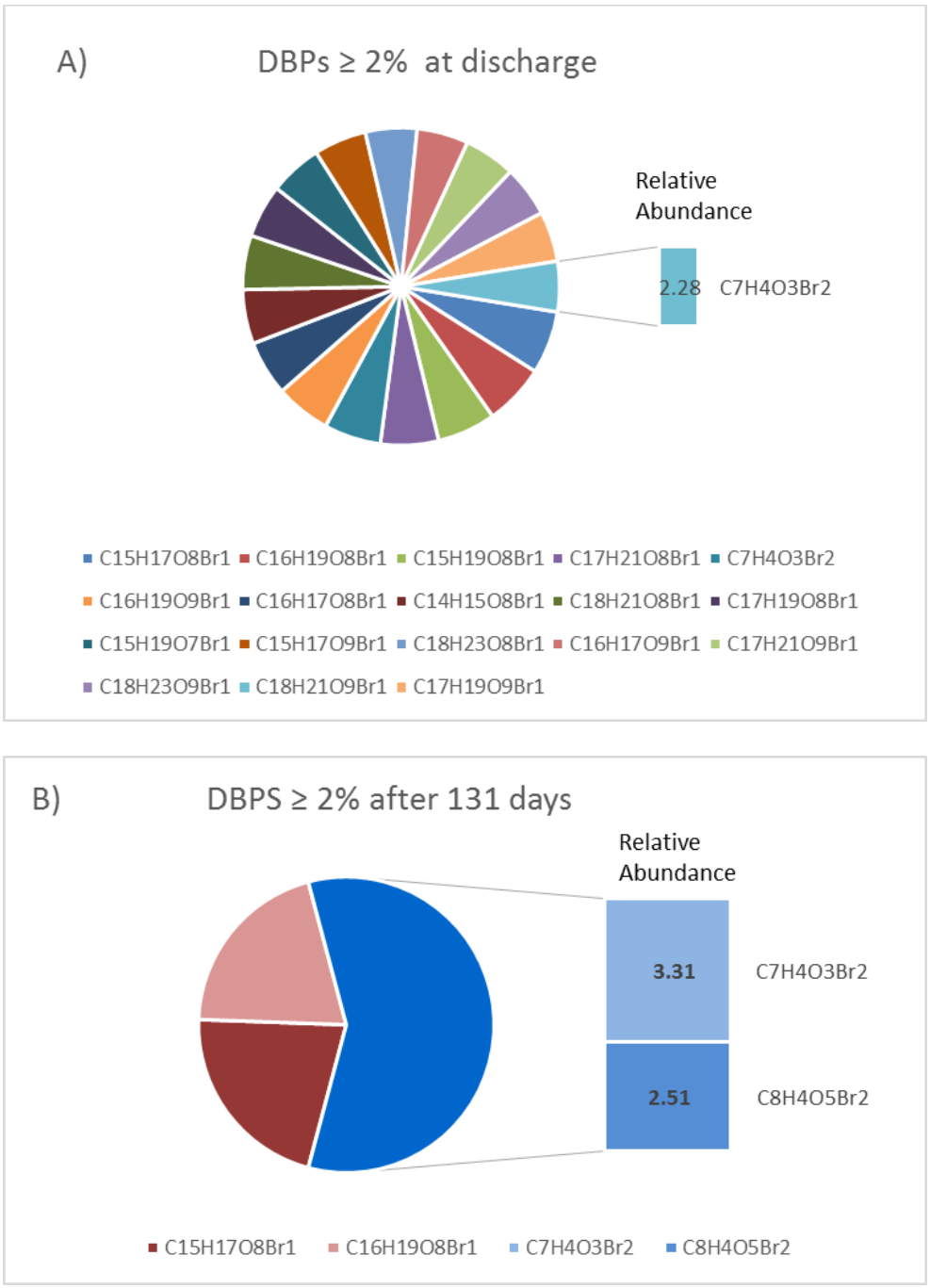

Figure 4.4 Distribution of mono- and dibrominated DBPs based on abundance A) at time of ballast water discharge B) after 92 days. Pie chart shows all DBPs with relative abundance >2%. Breakout bar graph chart shows portion comprised of dibrominated DBPs relative to all DBPs in pie chart.

The higher relative abundance of these two dibrominated ions can also be seen in the treated water van Krevelen diagrams as the only bubbles that increase in size in the 92 day sample (Figure 4.3B) compared to bubbles in the same location on the Day 0 van Krevelen diagram (Figure 4.3A). In this case, bubble size does reflect abundance of individual ions because only the mentioned dibrominated DBPs had this specific combination of H/C and O/C ratios. These dibrominated ions are also located in a similar position on the van Krevelen diagram characterized by low H/C ratio which is a characteristic of aromatic structures. In fact, the only possible structures identified for the most abundant formula $C_7H_4O_3Br_2$ have aromatic moieties. However, multiple potential structures exist for this formula including two rather common structures in the literature, 3,5-Dibromo-4-hydroxybenzoic acid (Wang et al., 2018; Chemspider, 2019a) and 3,5-Dibromosalicylic acid (Chemspider, 2019b). The most commonly referenced structure for this formula is 3,5-Dibromo-4-hydroxybenzoic acid (BrAC) which is known as a persistent breakdown product of bromoxynil (3,5-dibromo-5-hydroxybenzonitrile), a nitrile herbicide. However, theoretical isomeric possibilities are very high for these formulas. The second most abundant brominated ion identified ($C_8H_4O_5Br_2$) had a similar ratio of H/C (0.5), but with a higher O/C ratio of 0.62. No potential structure is suggested for this second dibrominated ion.

The high relative abundance of dibrominated DBPs after 92 days (Figure 4.4B) supports the general rule that persistence of a compound is increased with halogenation (Howard and Muir, 2010). Also the aromatic nature of all structures proposed for $C_7H_4O_3Br_2$ predicts that this DBP would be relatively persistent in contrast to regulated DBPs which contain no aromatic moieties and can be quite volatile as in the case of

THMs. Large complex DBPs with cyclic structures, have been identified in recent publication using new analytical techniques. These cyclic compounds include HCDs (Pan et al., 2016a), halogenated MCDs (Gong et al., 2005), pyrroles (Yang and Zhang, 2014), benzoquinones (Wang et al., 2014), phenols (Liu and Zhang, 2014), hydroxybenzaldehydes and hydroquinones (Yang and Zhang, 2014), all of which were identified as DBPs having genotoxic, cytotoxic or developmental toxic properties. Interestingly, developmental toxicities of aromatic halogenated DBPs were found to vary, and could be hundreds or thousands of times more toxic than aliphatic DBPs (Yang and Zhang, 2013). This increased toxicity has been explained in part by the higher log P values, a measure of lipophilicity which correlates with cell permeability. Aromatic DBPs tend to have higher log P values (range of 2.4 – 5.0) compared to aliphatic DBPs, increasing the chance of cellular uptake and bioaccumulation (Wang et al., 2018).

In addition to these two abundant Br-DBPs, many other higher molecular weight brominated DBPs with low H/C ratios were identified with FT-ICR MS. As stated above, the low H/C ratio is characteristic of DBPs with aromatic moieties. Generally larger halogenated DBPs with cyclic structures are also thought to be more persistent than smaller aliphatic DBPs (Howard and Muir, 2010; Yang and Zhang, 2013). The current research has identified two dibrominated ions with relative abundance increases after 92 days compared to other halogenated ions that were less abundant after the 92 day holding time. This seems to support the premise that increased halogenation results in an increase in persistence. On the other hand, the relatively low mass of these two dibrominated DBPs, relative to other high molecular weight DBPs identified by FT-ICR MS, does not support the premise of increased persistence with an increase in size.

A review of available brominated formulas presented in previous published research revealed that all of the Br-DBPs in the CHOBr elemental group with abundance of at least 1% have been previously described as DBPs. In contrast, 4 out of the 5 DBPs in the CHONBr elemental group have not been described in the literature. The only nitrogenated Br-DBP that has been reported has the formula $C_6H_3O_3N_1Br_2$ with proposed structure of 2,6-Dibromo-4-nitrophenol (Wang et al., 2018). The number of research projects and publications using ultrahigh resolution MS techniques has grown to the point that fewer high molecular weight DBPs are newly identified. However, most published research limits the list of identified formulas to DBPs with relative abundance of at least 1%. Because the structure of most of the high molecular weight DBPs identified by FT-ICR MS are unknown, even DBPs at low abundance may be relevant as DBPs have shown a wide range of developmental toxicities (Yang and Zhang, 2013) and in some cases toxicity seems to be dependent on the entire pool of high molecular weight DBPs in oxidant treated water (Lv et al., 2017). Furthermore, m/z ion intensity is not necessarily related to concentration due to drastic differences in ionization efficiencies of compounds with varying functional groups.

Formula assignments in the mass spectra of treated water at Day 0 and after 92 days holding time identified four tribrominated DBPs with molecular formula assignments. However, relative abundances of all identified formulas were below 0.8%, with only one formula with greater than 0.4% relative abundance. In some research trihalogenated DBPs appear rarely in the mass spectra of oxidant treated fresh water (Zhang et al., 2012; Lavonen et al., 2013). However, data reported by Ziegler et al. (2019) and Gonsior et al. (2014) reported four and three tribrominated DBPs,

respectively, using the same methods of extraction (PPL) and analysis (FT-ICR MS) as employed in the current research. Like the current paper, Ziegler et al. (2019) found that relative abundances of tribrominated DBPs were low, falling below 0.58% for all identified formula assignments. In contrast, Gonsior et al. (2014b) identified tribromo HCD in electrochlorinated seawater as a highly abundant structure with relative abundance of >22%. The number and abundance of trihalogenated DBPs may be dependent on some unidentified precursors in the water before treatment. In the current research, the abundance of the tribrominated Br-DBPs was lower after 92 days, similar to singly brominated DBPs and contrary to the idea that persistence is increased with halogenation.

At time of discharge, 200 nitrogen containing brominated molecular ions (CHONBr) were identified and confirmed in electrochlorinated ballast water. The number of ions in this category increased slightly to 214 molecular ions after 92 days presumably as breakdown products of larger peptides that were then halogenated and presumably formed bromoamines. However, very few nitrogen-containing Br-DBPs were identified with a relative abundance of at least 1%, with a total of three ions at the time of discharge and two ions after 92 days (Appendix 3), which might reflect that bromoamines are not stable over time. Interestingly, and similar to Br-DBPs without nitrogen, the most abundant nitrogenated Br-DBP ion (relative abundance 3.2%) identified after 92 days contained two bromines ($C_{10}H_5O_4N_1Br_2$). The number and abundance of nitrogen-containing Br-DBPs were higher in previous FT-ICR MS research on oxidant (DICD) treated ballast water (Ziegler et al., 2019) and aquaculture seawater (Wang et al., 2018). In both studies the abundant nitrogen-containing Br-DBPs were

attributed to high algae concentrations present in the water before treatment. This correlation between increase in nitrogenous Br-DBPs and algal density seems to indicate that algae and algal organic matter (AOM) serve as a significant source of precursors for nitrogenous DBPs, which is consistent with bromoamine formation from peptides. This is important because nitrogenous DBPs are generally more toxic compared to DBPs without nitrogen (Plewa et al., 2008; Wagner and Plewa, 2017). There is also the possibility of reducing the formation of toxic nitrogen-containing DBPs by limiting the oxidative treatment of water with high algae concentrations.

The ultimate fate of any persistent DBPs found in discharged ballast water may be anoxic sediments that are found in urban/industrial environments that are typical of large ports worldwide, including the port of Baltimore location studied in the current research. No research using FT-ICR MS or similar techniques has been conducted to identify high molecular weight brominated DBPs in the environment. However, research has been conducted on sediment DOM using the same techniques as those employed in the current research (i.e. SPE and FT-ICR MS). Research has shown that the composition of DOM and DBPs formed after halogenation are similar, and resemble polyphenolic-like or humic-like compounds (Gonsior et al., 2015; Harris et al., 2015) found in coastal DOM, and breakdown may be achieved by similar mechanisms. FT-ICR MS has been used to investigate sediment pore water from freshwater, estuarine and marine sediments (Schmidt et al., 2009, 2017; Tremblay et al., 2007; Valle et al., 2018). Monitoring of the anaerobic transformation of DOM in sediment pore waters with FT-ICR MS has shown DOM transformation characterized by a loss of nearly saturated CHO substances with a corresponding increase in oxygenated aromatics (Valle et al., 2018). In the current

research, there was an increase in two brominated ions with low H/C ratios, a characteristic of ions with aromatic moieties, suggesting a similarity in the transformations of DOM (Valle et al., 2018) and Br-DBPs.

Another mechanism for breakdown of DOM that could be relevant to high molecular weight DBPs is exposure to sunlight which can degrade complex aromatic DOM compounds (Gonsior et al., 2009; 2014a), substantially decreasing concentrations of lignin within just a few days (Opsahl and Benner, 1998). The photodegredation of high molecular weight Br-DBPs using FT-ICR MS analysis has been demonstrated in chlorinated aquaculture seawater (Wang et al., 2018). Similarly, the reduction of cytotoxicity from all DBPs in a high molecular weight DBP fraction (> 1 kDa), and assumed degradation of high molecular weight DBPs, has been demonstrated with UV exposure (Lv et al., 2017). However, if DBPs are in the sediment, as suggested in the previous paragraph, any exposure to sunlight will be extremely limited and is unlikely to be a factor in DBP degradation.

Because the structures of most of the brominated formulas found in the current research are unknown, the ability to predict environmental concentrations is difficult. However, there are mechanisms to predict the concentration of smaller quantified DBPs including DBPs as large as halogenated phenols. Models have been used for the predicted environmental concentration (PEC) of common ballast water DBPs using a simulated port scenario (IMO, 2012) which takes into consideration the physicochemical properties and environmental fate parameters of individual DBPs, when data is available.

Other than a few phenols, the absence of structural and physicochemical data for high molecular weight DBPs also results in a lack of persistence data from standard

biodegradation screening tests, which could be used in environmental fate modeling. Although models have been used for smaller DBPs, actual monitoring of DBPs in the environment (e.g. water or sediment) to ground truth PECs has not been conducted, and the reliability of some PECs have been called into question (David et al., 2018). To date, the actual measurements of DBP concentrations for oxidant treated ballast water (Werschkun et al., 2012; Delacroix et al., 2013; Ziegler et al., 2018), and most of the data for chlorinated cooling water effluents (Jenner et al., 1997; Allonier et al., 1999; Khalanski and Jenner, 2012) has been taken from effluent streams, rather than from the surrounding environments. The measurement of a variety of traditional DBPs in receiving waters and sediments is limited to a few published studies (Boudjellaba et al., 2016; Manasfi et al., 2018) with an additional study where only halogenated phenols were measured (Sim et al., 2009). A comprehensive study of halophenols (HPs) in the area near a chlorinated cooling water discharge found only two bromophenols, 2,4-dibromophenol (2,4-DBP) and 2,4,6- tribromophenol (2,4,6-TBP), in both water and sediment samples, while sediment samples contained six different bromophenols (2/3/4-BP; 2,6-DBP; 2,4-DBP and 2,4,6-TBP), suggesting the natural production of mono- and dibrominated phenols in sediments (Sim et al., 2009). In an investigation of a wide-range of DBPs (i.e. THMs, HAAs, HANs, trihaloacetaldehydes, HPs) in the area close to the discharge of chlorinated cooling water, only the largest DBPs (i.e. HPs) were found in sediments (Manasfi et al., 2018). This is consistent with the calculation that 2,4,6-TBP is most likely to adsorb to sediment (PubChem, 2019; WHO, 2005) compared to the other quantified DBPs. An investigation of 15 DBPs in conger eel muscle (Boudjellaba et al., 2016) identified only 2,4,6-TBP (10 out of 15 samples) in muscle samples, which was

consistent with its' relatively high bioconcentration factor (BCF) for fish of 513 (Sacan et al., 2004), suggesting the potential for bioconcentration in aquatic organisms. These studies analyzed for a wide range of DBPs, and ultimately found that all of the persistent DBPs identified in sediment and fish tissue (Sim et al., 2009; Boudjellaba et al., 2016; Manasfi et al., 2018) were the larger halogenated aromatic compounds (i.e. halophenols) that are similar to the DBPs found using FT-ICR MS in the current study.

One halophenol, (2,4,6-TBP), found in the environment in several of the studies described above (Sim et al., 2009; Manasfi et al., 2018), is also included in the BWWG-Database of the most common ballast water chemicals (IMO, 2017b). However, as stated previously, the Database only lists DBPs that have been targeted in chemical analysis for quantification by common analytical methods such as GC-MS (HANs and HPs) and GC-ECD (THMs and HAAs). Unfortunately the limitations of techniques to analyze for higher molecular weight Br-DBPs, and the lack of structural information, has resulted in a total lack of data for the presence of these complex Br-DBPs in the environment.

The lack of structural information for the numerous halogenated DBPs found by FT-ICR MS in oxidant treated water also makes it impossible to screen compounds in databases for persistence and bioaccumulation, or to monitor the environment with targeted analytical techniques (Brown and Wania, 2008). Analytical screening techniques for extraction by conventional methodologies and analysis by GC-MS have been developed to monitor for specific halogenated man-made chemicals where structures are known (e.g. polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and pesticides). Although sophisticated techniques would be needed, targeted analysis could be developed for large halogenated DBPs after the structures of

the more persistent DBPs are identified. In fact, advanced targeted analytical techniques such as ultra-performance liquid chromatography (UPLC) coupled to electrospray ionization-triple quadrupole mass spectrometry (ESI-tqMS) have already been developed for a few higher molecular weight DBPs (Ding and Zhang, 2009; Ding et al., 2013; Pan et al., 2017) in drinking water.

It is recognized that the storage conditions of electrochlorinated water in the current research (i.e. 4° C in the dark) is different from conditions after release into the environment. Specifically the longevity of DBPs will have been influenced by cold storage, lack of dilution, and potentially by additional biotic and abiotic processes. However, similar low temperature conditions that would affect any biotic or abiotic degradation processes would also be found in cold-water regions where many commercial shipping ports are located. Research has also shown the potential for toxic effects of oxidant treated water (e.g. electrochlorination) that could not be accounted for by the concentration of traditional small DBPs, suggesting the presence of some unidentified toxic component such as the high molecular weight DBPs identified in the current research. In these studies oxidant treated water; remained toxic to algae after neutralization of TRO (Delacroix et al., 2013) and storage for several months at 4° C (Ziegler et al., 2018), and that toxicity increased with lignin addition before oxidative treatment (Park et al., 2017).

The use of strong oxidants for the disinfection of seawater is used in a variety of applications with the highest oxidant doses used in ballast water treatment, and the greatest quantity of treated water contributed by industrial cooling water applications. The formation and release of potentially persistent and toxic Br-DBPs warrants the

continued research into the characteristics of higher molecular weight Br-DBPs. Identifying the structure of the more common high molecular weight Br-DBPs can be followed with the development of analytical methods to screen the environment for their presence. The non-targeted FT-ICR MS analysis of pore waters from sediments collected near chlorinated seawater discharge points such as chlorinated saline waste water (Ding et al., 2013), industrial cooling water (Sim et al., 2009; Manasfi et al., 2018) and oxidant treated ballast water (Ziegler et al., 2019) could confirm the persistence of the DBP types identified in the current research. These are important steps towards evaluating the potential environmental risk of higher molecular weight brominated DBPs.

Chapter 5

Conclusions and Future Research

The potential for undesired environmental effects of biocide treated ballast water has long been recognized. However, prior to this dissertation, limited toxicity testing had been conducted on discharged ballast water. Initial toxicity testing found only occasional toxicity of ballast water treated with oxidants. Nevertheless, some minor toxic effects of oxidant treated ballast water were being observed by ballast water test facilities around the world. This research, conducted at the Wye Research and Education Center in collaboration with the Maritime Environmental Resource Center, showed significant algal toxicity of electrochlorinated ballast water to a new test species, *Isochrysis galbana*. Thus, the initial questions addressed were the cause(s) of toxicity and longevity of the toxic effect. These questions led to the series of algal toxicity tests (Ch. 2) and DBP analysis (Ch. 3 & 4) presented in this dissertation.

Research presented in Chapter 2 demonstrates consistent algal toxicity of discharged ballast water after treatment by commercial BWMS employing either electrochlorination or direct chlorination with a solution of DICD. Initial (Day 0) IC_{25} values were similar for all treatments ranging from 9.9% to 17.9% of treated ballast water. Treated water was analyzed for 24 individual DBPs of which 17 were found above the detection limit. However, initial toxicity was not correlated to DBP concentrations, target TRO dose, or treatment method suggesting another unquantified factor was causing toxicity. I was also able to show the longevity of this toxic effect in Chapter 2 with treated waters from two electrochlorination-based BWMS lasting greater

than 130 days.

The variable susceptibility of different species of algae to the toxic effects of oxidant treated ballast water was shown in preliminary research, conducted in 2010 (Ch. 1). Research presented in Chapter 2 of this dissertation indicates that *Isochrysis galbana* is the most sensitive species of algae among the limited algal species that have been tested. However, the majority of marine/brackish algal toxicity data is based on only two species of diatoms, *S. costatum* and *P. tricornutum*. Also, toxicity testing with different species of algae has shown the phylogenetic basis for inhibitory effects of chemicals (Larras et al., 2014) and that these effects can vary widely with up to 50 times differences observed between species with some contaminants (Bi et al., 2018). In light of the large differences in species sensitivities, future research should focus on using new and multiple test species to gain a broader perspective on how diverse phytoplankton communities in coastal waters around the world could be impacted by the frequent discharge of large volumes of oxidant treated ballast water. Although no single species can represent all species from the same biological classification, testing of several species from a diverse group of genera may show a phylogenetic response to oxidant treated water. Identifying species sensitivities at the genera level could help with the development of ecological risk assessments of oxidant treated waters. Ultimately, a wide range of species sensitivities could result in driving natural algae assemblages toward species which are resistant to toxicity from oxidant treated water. This diverse multi-species approach can have broad application for toxicity testing of treated effluent water in coastal systems, far beyond ballast water.

In Chapters 3 and 4, I was able to identify numerous high molecular weight DBPs

using ultrahigh resolution FT-ICR MS. Data presented in Chapter 3 is the first demonstration of molecular transformations of DOM and formation of high molecular weight DBPs in a BWMS using direct chlorination (i.e. DICD). Analysis of DOM in untreated water identified 3,987 *m/z* ions and their molecular formulas. The breakdown of complex organic molecules in DOM to lower molecular weight formulas after DICD treatment was shown. After DICD treatment, 213 halogenated ions in four elemental groups, CHOBr (180), CHONBr (13), CHOCl (16), and CHOBrCl (4) were identified with a relative abundance of greater than 1%. Twenty-seven of the 180 Br-DBPs have not been previously described.

In DICD treated water (Ch. 3) with high concentrations of diatoms in the genera *Gymnodinium* and *Prorocentrum*, I was also able to identify 244 nitrogen-containing Br-DBPs (CHONBr), 13 of which had a relative abundance of at least 1%. None of these nitrogen-containing Br-DBPs have been previously described. In contrast, in Chapter 4 electrochlorinated water without high algae concentrations resulted in the formation and identification of only 200 nitrogen containing Br-DBPs, with only 3 at higher than 1% abundance. The increased production of nitrogen-containing Br-DBPs in the presence of high concentrations of algae is of particular concern as research has shown that nitrogen-containing DBPs are generally more toxic than DBPs without nitrogen (Wagner and Plewa, 2017). An increase in the number and size of algal blooms has been observed in numerous locations around the world in the last decade and is thought to be a significant global problem (Hallegraeff, 2014). Algae blooms have already resulted in the shutdown of desalinization plants that use chlorination to treat filtration membranes, and precautions have been suggested for other drinking water treatment processes in the

presence of algal blooms (Li and Mitch, 2018). Basic research is needed to determine how algal density and species composition effect the production of nitrogen-containing Br-DBPs in marine/estuarine waters. With this basic understanding, it may be possible to limit oxidative treatment of waters containing certain species of algae or concentrations over a certain threshold.

In Chapters 3 and 4, I used the relatively new non-targeted technique of ultrahigh resolution FT-ICR MS to identify the formulas of numerous high molecular weight halogenated DBPs in oxidant treated ballast waters. This data can be used to identify the most common molecular formulas of Br-DBPs that form during chlorination treatment of ballast water. However, in this dissertation only a limited number of structures were proposed based on steric interference and limited isomeric possibilities. The further development and application of analytical methods such as UPLC/ESI-tqMS (Ding et al., 2013; Gong and Zhang, 2015; Zhai et al., 2014) that can assign structural information to high molecular weight DBPs is now needed. Time-of-flight mass spectrometer is already capable of providing fragment information as well as exact MW so that structures of compounds can be obtained making UPLC/ESI-tqMS-ToF a particularly attractive alternative. Once structures of the more prominent newly identified DBPs are found, it should be possible to group DBPs by their structural similarities (Wang and Helbling, 2016). This structural information can be used to compare high molecular weight DBPs to available quantitative structural activity relationships (QSARs) to identify DBPs which are most likely to be of concern for coastal environmental impact (Chen, 2011; 2015).

In Chapter 4, I was able to show that high molecular weight Br-DBPs formed after electrochlorination can persist for at least 92 days under certain conditions (i.e. 4°C

in a glass carboy). Many of the Br-DBPs identified had low H/C ratios, an indication of aromatic moieties that tend to be more persistent than smaller aliphatic DBPs. Currently, little fate or persistence data is available for high molecular weight halogenated DBPs identified by FT-ICR MS. The research I conducted in this dissertation is the first attempt to show the persistence of higher molecular weight DBPs in a controlled environment. The use of a non-target analytical technique such as ultrahigh resolution FT-ICR MS to analyze environmental samples for higher molecular weight DBPs is an important next step in evaluating the fate of these compounds in the environment. In Chapter 4, I was able to show that the relative abundance of several dibrominated ions increased over time. I proposed several possible structures for the most abundant formula ($C_7H_4O_3Br_2$), all of which have aromatic moieties. Based on the observed relative persistence, I predict that these dibrominated DBPs are the most likely DBPs to be found in ports with large numbers of ships discharging oxidant treated ballast water. Although information on the aerobic or anaerobic degradation of these specific dibrominated DBPs is unavailable, the potential for burial in anoxic sediments away from UV exposure will likely increase the persistence of these DBPs based on the degradation of similar compounds (e.g. DOM; Gonsior et al., 2009: Lignin; Opsahl and Benner, 1998: High molecular weight DBPs; Wang et al., 2018). If structures for these two Br-DBP formulas can be confirmed, these would also be good candidates for targeted analysis of water and sediments. Generally, the identification of structures of high molecular weight DBPs will allow the development of targeted methods that can be used to monitor for their presence in the environment.

This dissertation targeted experiments to test whether treated ballast water may

have longer term impacts based on toxicity tests and DBP analysis which were conducted for several months after ballast water treatment. In Chapter 4, I was able to show that high molecular weight Br-DBPs can persist in electrochlorinated ballast water for at least 92 days. I also used toxicity tests in Chapter 2 to show that toxicity to algae could last for at least 134 days after electrochlorination of ballast water. The next logical step is to combine the knowledge that has been gained thus far and attempt to test whether high molecular weight DBPs have a role in causing toxicity. One way to establish this link is through the use of a toxicity identification evaluation (TIE) methodology. TIE methods have been developed to identify the toxic component of complex effluents and sediments. Initially the TIE method can be used to identify the group of toxicants responsible for toxicity, followed by more precise methods to isolate individual toxic compounds. The traditional TIE approach uses a set of guidelines that are broken into phases, and are specific to fresh or marine waters. Phase I isolates different constituents in an effluent that may be the cause of toxicity. Phase II methods attempt to narrow the focus of compounds that are causing toxicity to one or a few specific compounds. TIE Phase III methods attempt to confirm the suspected compounds as the cause of toxicity by adding the contaminant to a water sample and toxicity testing with the test organism of choice. The aquatic TIE methodology has been applied to ambient waters, discharge waters and leachates, but has not been applied to treated ballast water samples. Perhaps the best way to adapt the TIE methodology is to use the solid phase extraction (SPE) method that I employed in Chapters 3 and 4 of this dissertation. In research for this dissertation, I used Bond Elut PPL cartridges to extract organic compounds from treated ballast water. Because high molecular weight DBPs are suspected of causing toxicity, a Phase I method

could be accomplished by conducting algae toxicity tests on treated ballast water after extraction of halogenated organics on a PPL filled cartridge. If this removes toxicity, a Phase III type approach could be developed with SPE extracts (Ch. 3 and 4) added back to water samples before toxicity testing. This latter option would be substantially more complicated, as organic halogens would need to be removed from the methonolic solution, but would confirm whether the fraction removed by SPE (e.g. high molecular weight DBPs) is contributing to algal toxicity.

Combined, the proceeding chapters of this dissertation provided critical new insights into how a specific approach to addressing a significant ecological and economic problem may also have unintended impacts. Global shipping now transports greater than 90% of the world's overseas trade resulting in the transfer of 3 – 10 billion tonnes of ballast water (IMO, 2010; Tsolaki and Diamadopoulos, 2010; David, 2015). It has been estimated that globally, anywhere from 50,000 to 60,000 commercial ships will be required to install and operate BWMS systems to minimize the risk of invasive species (King, 2017; Welschmeyer et al., 2018). If even just 1/4 of those vessels are continuously treating ballast water with a chlorine-based BWMS, this could result in the glolobal release of over a billion tonnes of potentially toxic water annually. Therefore, careful consideration, and additional research, is needed on (a) production and impacts of DBPs, (b) acute and/or chronic toxicity on diverse and representative algal species, and (c) cumulative effects of continuous, large-scale discharges of treated ballast water in a single port, to ensure that benefits of biocidal treatment of ballast water (i.e., reduced risk of biological invasions) outweigh the costs (i.e., release of DBPs and aquatic toxicity).

Appendix 1

Table A1.1 List of 43 Most Common Ballast Water Chemicals

Chemical Name	CAS Number	Chemical Group
Acetaldehyde	75-07-0	Aldehyde
Bromate ion	15541-45-4	Inorganic
Bromochloroacetic acid	5589-96-8	Haloacetic acid
Bromochloroacetonitrile	83463-62-1	Haloacetonitrile
Chloral hydrate	302-17-0	Aldehyde hydrate
Chlorate ion	14866-68-3	Inorganic
Chloropicrin	76-06-2	Halonitroalkane
Dalapon	75-99-0	Halopropionic acid
Dibromoacetic acid	631-64-1	Haloacetic acid
Dibromoacetonitrile	3252-43-5	Haloacetonitrile
Dibromochloroacetic acid	5278-95-5	Haloacetic acid
Dibromochloromethane	124-48-1	Halomethane
1,2-Dibromo-3-chloropropane	96-12-8	Halopropane
1,1-Dibromoethane	557-91-5	Haloethane
Dibromomethane	74-95-3	Halomethane
Dichloroacetic acid	79-43-6	Haloacetic acid
Dichloroacetonitrile	3018-12-0	Haloacetonitrile
Dichlorobromoacetic acid	71133-14-7	Haloacetic acid
Dichlorobromomethane	75-27-4	Halomethane
1,1-Dichloroethane	75-34-3	Haloethane
1,2-Dichloroethane	107-06-2	Haloethane
Dichloromethane	75-09-2	Halomethane
1,2-Dichloropropane	78-87-5	Halopropane
Formaldehyde	50-00-0	Aldehyde
Isocyanuric acid	108-80-5	Inorganic
Monobromoacetic acid	79-08-3	Haloacetic acid
Monobromoacetonitrile	590-17-0	Haloacetonitrile
Monochloramine	10599-90-3	Haloamine
Monochloroacetic acid	79-11-8	Haloacetic acid
Monochloroacetonitrile	107-14-2	Haloacetonitrile
Sodium hypochlorite	7681-52-9	Inorganic Sodium
thiosulphate	7772-98-7	Inorganic
Tetrachloromethane	56-23-5	Halomethane
Tribromoacetic acid	75-96-7	Haloacetic acid
Tribromomethane	75-25-2	Halomethane
2,4,6-Tribromophenol	118-79-6	Halophenol
Trichloroacetic acid	76-03-9	Haloacetic acid
Trichloroacetonitrile	545-06-2	Haloacetonitrile
1,1,1-Trichloroethane	71-55-6	Haloethane
1,1,2-Trichloroethane	79-00-5	Haloethane
Trichloroethene	79-01-6	Haloethene

Trichloromethane	67-66-3	Halomethane
1,2,3-Trichloropropane	96-18-4	Halopropane

Appendix 2

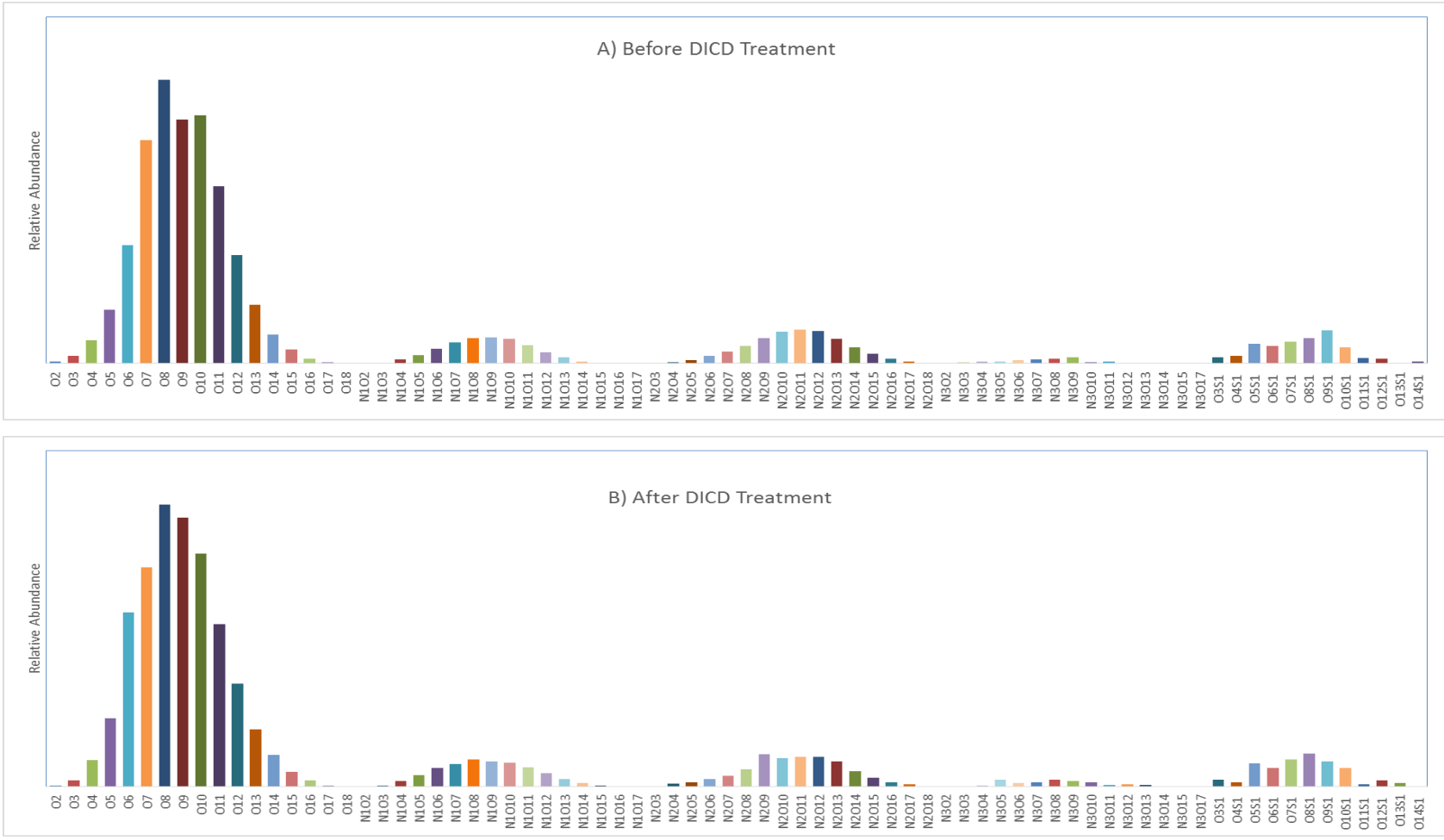

Figure A2.1. Heteroatom class distributions for Port Covington water DOM (A) before treatment and (B) after DICD treatment.

Table A2.1 All newly formed DBPs after treatment of ballast water with DICD measured by ultrahigh resolution mass spectrometry with a relative abundance of at least 1%. Possible structures are suggested for molecular formula up to 8 carbon atoms and/or multiple halogen atoms, or based on suggestions found in a literature review. Highlighted text identifies previously unreported DBPs.

Possible Structure	Mass (neutral)	Formula (neutral)	Rel. abundance	O/C	H/C
	259.9320	C8H5O5Br1	1.9	0.63	0.63
Bromo-hydroxy-nitrobenzoic acid	**260.9273**	**C7H4O5N1Br1**	**1.8**	**0.71**	**0.57**
	273.9477	C9H7O5Br1	1.1	0.56	0.78
	274.9429	**C8H6O5N1Br1**	**1.1**	**0.63**	**0.75**
Dibromosalicylaldehyde	277.8578	C7H4O2Br2	2.0	0.29	0.57
	279.9946	C9H13O5Br1	1.4	0.56	1.44
	282.9480	**C10H6O4N1Br1**	**1.2**	**0.40**	**0.60**
	285.9113	C9H3O6Br1	1.0	0.67	0.33
	289.9790	C10H11O5Br1	1.3	0.50	1.10
	291.9946	C10H13O5Br1	1.1	0.50	1.30
	292.8687	**C7H5O2N1Br2**	**1.3**	**0.29**	**0.71**
	292.9899	**C9H12O5N1Br1**	**1.5**	**0.56**	**1.33**
3,5-Dibromo-4-hydroxybenzoic acid	293.8527	C7H4O3Br2	6.9	0.43	0.57
	294.0103	C10H15O5Br1	1.1	0.50	1.50
	301.9426	C10H7O6Br1	1.9	0.60	0.70
	303.9219	C9H5O7Br1	2.5	0.78	0.56
	305.9739	C10H11O6Br1	1.1	0.60	1.10
	307.9896	C10H13O6Br1	1.3	0.60	1.30
	311.9270	C11H5O6Br1	1.2	0.55	0.45
	313.9426	C11H7O6Br1	1.0	0.55	0.64
	315.9219	C10H5O7Br1	1.2	0.70	0.50
	315.9583	C11H9O6Br1	1.1	0.55	0.82
	317.9375	C10H7O7Br1	1.7	0.70	0.70
	317.9739	C11H11O6Br1	1.3	0.55	1.00
	318.0103	C12H15O5Br1	1.2	0.42	1.25
	320.0259	C12H17O5Br1	1.3	0.42	1.42

	320.8636	**C8H5O3N1Br2**	**1.8**	**0.38**	**0.63**
	320.9848	**C10H12O6N1Br1**	**1.3**	**0.60**	**1.20**
4,5-dibromophthalic acid	321.8476	C8H4O4Br2	1.5	0.50	0.50
	322.0052	C11H15O6Br1	1.5	0.55	1.36
	322.0416	C12H19O5Br1	1.2	0.42	1.58
	324.0209	C11H17O6Br1	1.0	0.55	1.55
	326.9379	**C11H6O6N1Br1**	**2.3**	**0.55**	**0.55**
	329.9375	C11H7O7Br1	1.6	0.64	0.64
	330.0467	**C14H19O4Br1**	**1.4**	**0.29**	**1.36**
	331.9896	C12H13O6Br1	1.4	0.50	1.08
	332.0259	C13H17O5Br1	1.0	0.38	1.31
	333.9688	C11H11O7Br1	1.2	0.64	1.00
	334.0052	C12H15O6Br1	1.9	0.50	1.25
	335.8633	C9H6O4Br2	1.2	0.44	0.67
	336.0209	C12H17O6Br1	1.9	0.50	1.42
	336.8949	**C9H9O3N1Br2**	**1.0**	**0.33**	**1.00**
	337.8425	C8H4O5Br2	3.5	0.63	0.50
	338.0365	C12H19O6Br1	1.3	0.50	1.58
	338.9954	**C10H14O7N1Br1**	**1.0**	**0.70**	**1.40**
	343.9532	C12H9O7Br1	1.5	0.58	0.75
	343.9896	C13H13O6Br1	1.0	0.46	1.00
	345.9324	C11H7O8Br1	2.1	0.73	0.64
	346.0052	C13H15O6Br1	1.3	0.46	1.15
	346.0416	C14H19O5Br1	1.3	0.36	1.36
	347.9845	C12H13O7Br1	1.5	0.58	1.08
	348.0572	C14H21O5Br1	2.7	0.36	1.50
	349.8425	C9H4O5Br2	1.0	0.56	0.44
	350.0001	C12H15O7Br1	1.3	0.58	1.25
	350.0365	C13H19O6Br1	1.7	0.46	1.46
	352.0158	C12H17O7Br1	1.1	0.58	1.42
	354.0314	C12H19O7Br1	1.0	0.58	1.58
	357.9688	C13H11O7Br1	1.3	0.54	0.85
	358.0416	C15H19O5Br1	1.3	0.33	1.27
	359.9481	C12H9O8Br1	1.5	0.67	0.75
	359.9845	C13H13O7Br1	1.2	0.54	1.00

	360.0209	C14H17O6Br1	1.5	0.43	1.21
	360.0572	C15H21O5Br1	1.2	0.33	1.40
	360.1704	**C18H29O5Cl1**	**1.2**	**0.28**	**1.61**
	361.9637	C12H11O8Br1	1.2	0.67	0.92
	362.0001	C13H15O7Br1	1.7	0.54	1.15
	362.0365	C14H19O6Br1	1.8	0.43	1.36
	363.9794	C12H13O8Br1	1.2	0.67	1.08
	364.0158	C13H17O7Br1	1.8	0.54	1.31
	364.0522	C14H21O6Br1	2.2	0.43	1.50
	365.8375	**C9H4O6Br2**	**1.2**	**0.67**	**0.44**
	366.0314	C13H19O7Br1	1.4	0.54	1.46
	371.9481	C13H9O8Br1	1.1	0.62	0.69
	371.9845	C14H13O7Br1	1.2	0.50	0.93
	372.0209	C15H17O6Br1	1.3	0.40	1.13
	373.9637	C13H11O8Br1	1.1	0.62	0.85
	374.0001	C14H15O7Br1	1.6	0.50	1.07
	374.0365	C15H19O6Br1	2.4	0.40	1.27
	375.9794	C13H13O8Br1	1.3	0.62	1.00
	376.0158	C14H17O7Br1	1.7	0.50	1.21
	376.0522	C15H21O6Br1	3.1	0.40	1.40
	376.1653	**C18H29O6Cl1**	**1.9**	**0.33**	**1.61**
	377.9950	C13H15O8Br1	1.3	0.62	1.15
	378.0314	C14H19O7Br1	1.8	0.50	1.36
	378.0678	C15H23O6Br1	2.5	0.40	1.53
	380.0471	C14H21O7Br1	1.8	0.50	1.50
	382.0627	C14H23O7Br1	3.1	0.50	1.64
	385.9637	C14H11O8Br1	1.1	0.57	0.79
	386.0001	C15H15O7Br1	1.2	0.47	1.00
	386.0365	C16H19O6Br1	1.3	0.38	1.19
	387.9794	C14H13O8Br1	1.3	0.57	0.93
	388.0158	C15H17O7Br1	1.6	0.47	1.13
	388.0522	C16H21O6Br1	1.7	0.38	1.31
	389.9950	C14H15O8Br1	1.5	0.57	1.07
	390.0314	C15H19O7Br1	2.5	0.47	1.27
	390.0678	**C16H23O6Br1**	**1.7**	**0.38**	**1.44**

	390.1445	C18H27O7Cl1	2.8	0.39	1.50
	392.0107	C14H17O8Br1	1.4	0.57	1.21
	392.0471	C15H21O7Br1	4.4	0.47	1.40
	392.1602	**C18H29O7Cl1**	**2.2**	**0.39**	**1.61**
	394.0263	C14H19O8Br1	1.3	0.57	1.36
	394.0627	C15H23O7Br1	2.3	0.47	1.53
	394.1758	**C18H31O7Cl1**	**1.5**	**0.39**	**1.72**
	396.0420	C14H21O8Br1	1.4	0.57	1.50
	397.9637	C15H11O8Br1	1.1	0.53	0.73
	398.0576	**C14H23O8Br1**	**2.1**	**0.57**	**1.64**
	399.9794	C15H13O8Br1	1.2	0.53	0.87
	400.0158	C16H17O7Br1	1.1	0.44	1.06
	401.9950	C15H15O8Br1	1.3	0.53	1.00
	402.0314	C16H19O7Br1	1.7	0.44	1.19
	403.9743	C14H13O9Br1	1.1	0.64	0.93
	404.0107	C15H17O8Br1	1.8	0.53	1.13
	404.0471	C16H21O7Br1	2.3	0.44	1.31
	406.0263	C15H19O8Br1	2.1	0.53	1.27
	406.0627	**C16H23O7Br1**	**2.1**	**0.44**	**1.44**
	408.0420	C15H21O8Br1	2.6	0.53	1.40
	408.1551	**C18H29O8Cl1**	**2.1**	**0.44**	**1.61**
	410.0576	C15H23O8Br1	16.0	0.53	1.53
	412.0733	C15H25O8Br1	18.5	0.53	1.67
	414.0314	C17H19O7Br1	1.1	0.41	1.12
	415.9743	C15H13O9Br1	1.1	0.60	0.87
	416.0107	C16H17O8Br1	1.5	0.50	1.06
	416.0237	**C14H22O7Cl1Br1**	**1.0**	**0.50**	**1.57**
	416.0471	C17H21O7Br1	1.4	0.41	1.24
	416.0835	C18H25O6Br1	1.9	0.33	1.39
	417.9899	C15H15O9Br1	1.2	0.60	1.00
	418.0263	C16H19O8Br1	1.9	0.50	1.19
	418.0627	C17H23O7Br1	1.6	0.41	1.35
	418.0991	C18H27O6Br1	2.0	0.33	1.50
	418.1758	**C20H31O7Cl1**	**1.0**	**0.35**	**1.55**
	420.0056	C15H17O9Br1	1.4	0.60	1.13

	420.0420	C16H21O8Br1	1.8	0.50	1.31
	420.1148	**C18H29O6Br1**	**1.6**	**0.33**	**1.61**
	422.0212	C15H19O9Br1	1.2	0.60	1.27
	422.0576	C16H23O8Br1	2.7	0.50	1.44
	424.0369	C15H21O9Br1	1.1	0.60	1.40
	424.0733	C16H25O8Br1	1.8	0.50	1.56
	426.0525	**C15H23O9Br1**	**3.1**	**0.60**	**1.53**
	427.9743	C16H13O9Br1	1.1	0.56	0.81
	428.0107	C17H17O8Br1	1.2	0.47	1.00
	428.0237	**C15H22O7Cl1Br1**	**1.8**	**0.47**	**1.47**
	429.9899	C16H15O9Br1	1.1	0.56	0.94
	430.0263	C17H19O8Br1	1.6	0.47	1.12
	430.0394	**C15H24O7Cl1Br1**	**2.1**	**0.47**	**1.60**
	430.0627	C18H23O7Br1	1.7	0.39	1.28
	432.0056	C16H17O9Br1	1.2	0.56	1.06
	432.0420	C17H21O8Br1	1.7	0.47	1.24
	432.0784	C18H25O7Br1	2.6	0.39	1.39
	434.0212	C16H19O9Br1	1.5	0.56	1.19
	434.0576	C17H23O8Br1	1.9	0.47	1.35
	434.0940	C18H27O7Br1	2.9	0.39	1.50
	436.0369	C16H21O9Br1	1.8	0.56	1.31
	436.0733	C17H25O8Br1	1.3	0.47	1.47
	436.1097	**C18H29O7Br1**	**2.2**	**0.39**	**1.61**
	436.1864	**C20H33O8Cl1**	**1.4**	**0.40**	**1.65**
	440.0682	C16H25O9Br1	1.9	0.56	1.56
	442.0263	C18H19O8Br1	1.1	0.44	1.06
	444.0056	C17H17O9Br1	1.1	0.53	1.00
	444.0420	C18H21O8Br1	1.5	0.44	1.17
	444.0784	C19H25O7Br1	1.3	0.37	1.32
	445.9849	C16H15O10Br1	1.1	0.63	0.94
	446.0212	C17H19O9Br1	1.3	0.53	1.12
	446.0576	C18H23O8Br1	1.8	0.44	1.28
	446.0940	C19H27O7Br1	1.2	0.37	1.42
	446.2071	**C22H35O7Cl1**	**1.4**	**0.32**	**1.59**
	448.0369	C17H21O9Br1	1.4	0.53	1.24

	448.0733	**C18H25O8Br1**	**1.9**	**0.44**	**1.39**
	450.0525	C17H23O9Br1	1.3	0.53	1.35
	450.0889	C18H27O8Br1	1.9	0.44	1.50
	451.0352	**C15H20O9N2Br1**	**1.2**	**0.60**	**1.33**
	452.0430	**C15H21O9N2Br1**	**3.5**	**0.60**	**1.40**
	452.1046	**C18H29O8Br1**	**2.0**	**0.44**	**1.61**
	452.2177	**C21H37O8Cl1**	**1.0**	**0.38**	**1.76**
	456.0631	**C16H25O10Br1**	**4.4**	**0.63**	**1.56**
	458.0212	C18H19O9Br1	1.3	0.50	1.06
	458.0940	**C20H27O7Br1**	**1.1**	**0.35**	**1.35**
	460.0005	C17H17O10Br1	1.0	0.59	1.00
	460.0369	C18H21O9Br1	1.5	0.50	1.17
	460.0500	**C16H26O8Cl1Br1**	**1.3**	**0.50**	**1.63**
	460.0733	C19H25O8Br1	1.3	0.42	1.32
	460.1097	C20H29O7Br1	1.3	0.35	1.45
	460.1864	**C22H33O8Cl1**	**1.5**	**0.36**	**1.50**
	462.0162	C17H19O10Br1	1.1	0.59	1.12
	462.0525	C18H23O9Br1	1.7	0.50	1.28
	462.0889	**C19H27O8Br1**	**1.2**	**0.42**	**1.42**
	462.1253	**C20H31O7Br1**	**1.5**	**0.35**	**1.55**
	462.2020	**C22H35O8Cl1**	**1.8**	**0.36**	**1.59**
	464.0682	**C18H25O9Br1**	**1.6**	**0.50**	**1.39**
	464.2177	**C22H37O8Cl1**	**1.6**	**0.36**	**1.68**
	468.2126	**C21H37O9Cl1**	**1.1**	**0.43**	**1.76**
	471.9732	**C15H22O7Br2**	**1.0**	**0.47**	**1.47**
	474.0162	C18H19O10Br1	1.0	0.56	1.06
	474.0525	C19H23O9Br1	1.3	0.47	1.21
	474.0889	C20H27O8Br1	1.2	0.40	1.35
	476.0318	C18H21O10Br1	1.1	0.56	1.17
	476.1046	C20H29O8Br1	1.5	0.40	1.45
	478.0475	C18H23O10Br1	1.2	0.56	1.28
	478.0838	**C19H27O9Br1**	**1.0**	**0.47**	**1.42**
	478.1202	**C20H31O8Br1**	**1.4**	**0.40**	**1.55**
	478.1970	**C22H35O9Cl1**	**1.2**	**0.41**	**1.59**
	480.1359	**C20H33O8Br1**	**1.9**	**0.40**	**1.65**

	480.2126	**C22H37O9Cl1**	**1.3**	**0.41**	**1.68**
	486.1253	C22H31O7Br1	1.0	0.32	1.41
	488.1046	**C21H29O8Br1**	**1.0**	**0.38**	**1.38**
	488.1410	**C22H33O7Br1**	**1.9**	**0.32**	**1.50**
	494.1151	**C20H31O9Br1**	**1.1**	**0.45**	**1.55**
	501.0487	**C18H31O6Br2**	**1.0**	**0.33**	**1.72**
	502.1202	C22H31O8Br1	1.1	0.36	1.41
	504.1359	**C22H33O8Br1**	**1.5**	**0.36**	**1.50**
	506.1515	**C22H35O8Br1**	**1.9**	**0.36**	**1.59**
	514.0202	**C18H28O7Br2**	**1.0**	**0.39**	**1.56**
	522.1464	**C22H35O9Br1**	**1.3**	**0.41**	**1.59**
	525.1937	**C22H40O8N1Br1**	**1.4**	**0.36**	**1.82**

Appendix 3

Table A3.1. All brominated DBPs 92 days after electrochlorination of ballast water measured by ultrahigh resolution mass spectrometry with a relative abundance of at least 1%. DBPs are listed in the order of abundance calculated by comparison to the highest DOM *m/z* ion. Possible structures are based on suggestions found in a literature review. Highlighted text identifies previously unreported DBPs.

Possible Structure	Mass (neutral)	Formula (neutral)	Rel. Abundance	O/C	H/C
3,5-Dibromo-4-hydroxybenzoicacid	292.8454	C7H4O3Br2	3.3	0.43	0.57
	336.8351	C8H4O5Br2	2.5	0.63	0.50
2,6-Dibromo-4-nitrophenol	293.8406	C6H3O3N1Br2	2.3	0.50	0.50
	325.9306	**C11H6O6N1Br1**	**2.2**	**0.55**	**0.55**
	403.0033	C15H17O8Br1	2.1	0.53	1.13
	417.0189	C16H19O8Br1	2.0	0.50	1.19
	389.0241	C15H19O7Br1	2.0	0.47	1.27
	405.0190	C15H19O8Br1	2.0	0.53	1.27
	372.9926	C14H15O7Br1	1.9	0.50	1.07
	360.9926	C13H15O7Br1	1.9	0.54	1.15
	388.9877	C14H15O8Br1	1.9	0.57	1.07
	433.0139	C16H19O9Br1	1.9	0.56	1.19
	415.0033	C16H17O8Br1	1.8	0.50	1.06
	302.9144	C9H5O7Br1	1.8	0.78	0.56
	431.0345	C17H21O8Br1	1.8	0.47	1.24
	387.0083	C15H17O7Br1	1.8	0.47	1.13
	391.0032	C14H17O8Br1	1.7	0.57	1.21
	375.0083	C14H17O7Br1	1.7	0.50	1.21
	418.9982	C15H17O9Br1	1.7	0.60	1.13
	400.9876	C15H15O8Br1	1.7	0.53	1.00
	447.0295	C17H21O9Br1	1.7	0.53	1.24

	429.0189	C17H19O8Br1	1.6	0.47	1.12
	430.9982	C16H17O9Br1	1.6	0.56	1.06
	459.0295	C18H21O9Br1	1.6	0.50	1.17
	374.9720	C13H13O8Br1	1.6	0.62	1.00
	386.9721	C14H13O8Br1	1.6	0.57	0.93
	376.9876	C13H15O8Br1	1.6	0.62	1.15
	416.9826	C15H15O9Br1	1.5	0.60	1.00
	401.0240	C16H19O7Br1	1.5	0.44	1.19
	391.0396	C15H21O7Br1	1.5	0.47	1.40
	362.9144	C14H5O7Br1	1.5	0.50	0.36
	445.0502	C18H23O8Br1	1.5	0.44	1.28
	362.9719	C12H13O8Br1	1.5	0.67	1.08
	346.9771	C12H13O7Br1	1.5	0.58	1.08
	443.0345	C18H21O8Br1	1.5	0.44	1.17
	457.0138	C18H19O9Br1	1.5	0.50	1.06
	445.0139	C17H19O9Br1	1.5	0.53	1.12
	461.0089	C17H19O10Br1	1.5	0.59	1.12
	384.9926	C15H15O7Br1	1.4	0.47	1.00
	332.9978	C12H15O6Br1	1.4	0.50	1.25
	403.0397	C16H21O7Br1	1.4	0.44	1.31
	330.9822	C12H13O6Br1	1.4	0.50	1.08
	404.9825	C14H15O9Br1	1.4	0.64	1.07
	375.0449	C15H21O6Br1	1.4	0.40	1.40
	370.9408	C13H9O8Br1	1.4	0.62	0.69
	373.0292	C15H19O6Br1	1.4	0.40	1.27
	461.0451	C18H23O9Br1	1.4	0.50	1.28
	347.0136	C13H17O6Br1	1.4	0.46	1.31
	402.9669	C14H13O9Br1	1.4	0.64	0.93
	359.0133	C14H17O6Br1	1.4	0.43	1.21
	406.9981	C14H17O9Br1	1.4	0.64	1.21
	419.0346	C16H21O8Br1	1.4	0.50	1.31

	281.9406	**C10H6O4N1Br1**	**1.4**	**0.40**	**0.60**
	433.0501	C17H23O8Br1	1.4	0.47	1.35
	421.0138	C15H19O9Br1	1.4	0.60	1.27
	396.9563	C15H11O8Br1	1.4	0.53	0.73
	473.0451	C19H23O9Br1	1.4	0.47	1.21
	442.9983	C17H17O9Br1	1.4	0.53	1.00
	458.9931	C17H17O10Br1	1.4	0.59	1.00
	415.0395	C17H21O7Br1	1.4	0.41	1.24
	428.9825	C16H15O9Br1	1.3	0.56	0.94
	363.0083	C13H17O7Br1	1.3	0.54	1.31
	398.9720	C15H13O8Br1	1.3	0.53	0.87
	446.9933	C16H17O10Br1	1.3	0.63	1.06
	444.9775	C16H15O10Br1	1.3	0.63	0.94
	473.0088	C18H19O10Br1	1.3	0.56	1.06
	370.9771	C14H13O7Br1	1.3	0.50	0.93
	412.9877	C16H15O8Br1	1.3	0.50	0.94
	318.9822	C11H13O6Br1	1.3	0.55	1.18
	342.9457	C12H9O7Br1	1.3	0.58	0.75
	358.9772	C13H13O7Br1	1.3	0.54	1.00
	300.9352	C10H7O6Br1	1.3	0.60	0.70
	258.9247	C8H5O5Br1	1.3	0.63	0.63
	372.9564	C13H11O8Br1	1.3	0.62	0.85
	435.0295	C16H21O9Br1	1.3	0.56	1.31
	376.9302	C15H7O7Br1	1.3	0.47	0.47
	371.0134	C15H17O6Br1	1.3	0.40	1.13
	429.0552	C18H23O7Br1	1.3	0.39	1.28
	348.9927	C12H15O7Br1	1.3	0.58	1.25
	417.0552	C17H23O7Br1	1.3	0.41	1.35
	387.0449	C16H21O6Br1	1.2	0.38	1.31
	475.0246	C18H21O10Br1	1.2	0.56	1.17
	414.9669	C15H13O9Br1	1.2	0.60	0.87

	459.0658	C19H25O8Br1	1.2	0.42	1.32
	430.9618	C15H13O10Br1	1.2	0.67	0.87
	426.9669	C16H13O9Br1	1.2	0.56	0.81
	407.0345	C15H21O8Br1	1.2	0.53	1.40
	432.9776	C15H15O10Br1	1.2	0.67	1.00
	344.9979	C13H15O6Br1	1.2	0.46	1.15
	399.0084	C16H17O7Br1	1.2	0.44	1.06
	377.0241	C14H19O7Br1	1.2	0.50	1.36
	441.0189	C18H19O8Br1	1.2	0.44	1.06
	356.9615	C13H11O7Br1	1.2	0.54	0.85
	352.9302	C13H7O7Br1	1.2	0.54	0.54
	384.9565	C14H11O8Br1	1.2	0.57	0.79
	358.9406	C12H9O8Br1	1.2	0.67	0.75
	471.0294	C19H21O9Br1	1.2	0.47	1.11
	310.9197	C11H5O6Br1	1.2	0.55	0.45
	427.0033	C17H17O8Br1	1.2	0.47	1.00
	413.0240	C17H19O7Br1	1.2	0.41	1.12
	390.9670	C13H13O9Br1	1.2	0.69	1.00
	457.0501	C19H23O8Br1	1.2	0.42	1.21
	487.0245	C19H21O10Br1	1.2	0.53	1.11
	427.0396	C18H21O7Br1	1.1	0.39	1.17
	449.0089	C16H19O10Br1	1.1	0.63	1.19
	359.8510	**C10H5O4N1Br2**	**1.1**	**0.40**	**0.50**
	394.9406	C15H9O8Br1	1.1	0.53	0.60
	382.9407	C14H9O8Br1	1.1	0.57	0.64
	456.9775	C17H15O10Br1	1.1	0.59	0.88
	312.9353	C11H7O6Br1	1.1	0.55	0.64
	361.0293	C14H19O6Br1	1.1	0.43	1.36
	360.9564	C12H11O8Br1	1.1	0.67	0.92
	431.0708	C18H25O7Br1	1.1	0.39	1.39
	389.0604	C16H23O6Br1	1.1	0.38	1.44

	455.0345	C19H21O8Br1	1.1	0.42	1.11
	356.9250	C12H7O8Br1	1.1	0.67	0.58
	282.9247	C10H5O5Br1	1.1	0.50	0.50
	434.9932	C15H17O10Br1	1.1	0.67	1.13
	443.0709	C19H25O7Br1	1.1	0.37	1.32
	487.0607	C20H25O9Br1	1.1	0.45	1.25
	447.0658	C18H25O8Br1	1.1	0.44	1.39
	382.9772	C15H13O7Br1	1.1	0.47	0.87
	385.0292	C16H19O6Br1	1.1	0.38	1.19
	463.0247	C17H21O10Br1	1.1	0.59	1.24
	477.0402	C18H23O10Br1	1.1	0.56	1.28
	332.9614	C11H11O7Br1	1.1	0.64	1.00
	356.9978	C14H15O6Br1	1.1	0.43	1.07
	412.9513	C15H11O9Br1	1.1	0.60	0.73
	379.0033	C13H17O8Br1	1.1	0.62	1.31
	489.0401	C19H23O10Br1	1.1	0.53	1.21
	232.9091	C6H3O5Br1	1.1	0.83	0.50
	410.9355	C15H9O9Br1	1.0	0.60	0.60
	474.9883	C17H17O11Br1	1.0	0.65	1.00
	489.0039	C18H19O11Br1	1.0	0.61	1.06
	342.9823	C13H13O6Br1	1.0	0.46	1.00
	377.9254	**C14H6O7N1Br1**	**1.0**	**0.50**	**0.43**
	440.9826	C17H15O9Br1	1.0	0.53	0.88
	470.9932	C18H17O10Br1	1.0	0.56	0.94
	316.9666	C11H11O6Br1	1.0	0.55	1.00
	400.9511	C14H11O9Br1	1.0	0.64	0.79
	454.9982	C18H17O9Br1	1.0	0.50	0.94
	338.9508	C13H9O6Br1	1.0	0.46	0.69
	328.9301	C11H7O7Br1	1.0	0.64	0.64
	354.9821	C14H13O6Br1	1.0	0.43	0.93
	324.9353	C12H7O6Br1	1.0	0.50	0.58

	404.9250	C16H7O8Br1	1.0	0.50	0.44
	368.9613	C14H11O7Br1	1.0	0.50	0.79
	405.0553	C16H23O7Br1	1.0	0.44	1.44
	335.0134	C12H17O6Br1	1.0	0.50	1.42
	410.9720	C16H13O8Br1	1.0	0.50	0.81

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