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Stable Bromine Isotope Signature of Bromoform from Enzymatic and Abiotic Formation Pathways and its Application in Identifying Sources of Environmental Bromoform in the Damariscotta River

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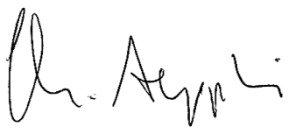
May 21, 2018

A thesis submitted to the faculty of the Environmental Studies Program in partial fulfillment of the graduation requirements for the Degree of Bachelor of Arts with honors in Environmental Studies



Christoph Aeppli, Advisor	Whitney King, Reader	Denise Bruesewitz, Reader

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ABSTRACT

Bromoform is a major source of atmospheric bromine. Most bromoform is produced by marine organisms including macroalgae and phytoplankton, using the enzyme bromoperoxidase (BPO). Bromoform can also be a byproduct of industrial processes such as water disinfection. Identifying sources of environmental bromoform is still a challenge. A novel technique of using quadrupole mass spectrometry coupled to a gas chromatography (GCqMS) was developed and optimized for Br isotope analyses. The study shows that GCqMS in single ion monitoring (SIM) mode can measure δ^{81}Br with precision of around ±0.7‰ (60pmol bromoform injected). This study aims to investigate stable Br isotopes of bromoform produced from different pathways, including macroalgae (*Ascophyllum Nodosum* and *Fucus Vesculosis*) and abiotic bromination with HOBr and phenol. The experimental δ^{81}Br results were then used to interpret of bromoform production in the Damariscotta River estuary. The Br isotope signature of bromoform in the Damariscotta River was -0.6±1.8‰ relative to our isotopic standard. That of bromoform produced by *A. Nodosum*, *F. Vesculosis*, and the abiotic reaction were 1.8±0.7‰, 2.4±2.6‰, and -1.3±1.2‰, respectively. H_2O_2 decreased Br isotopic fractionation of bromoform produced by *A. Nodosum*.

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1. INTRODUCTION

1.1. Environmental brominated organic compounds

Brominated organic compounds (BOCs) are produced by industrial and biological processes in large amounts (Carpenter et al. 1999; Gribble 2003; Howe et al. 2005; Heeb et al. 2014). The major industrial sources of BOCs in the environment include fire retardant production and water disinfection using chlorine (Nokes 1999; Richardson et al. 2003; Guerra et al. 2010). Many biochemical processes in the ocean are also significant sources of BOCs to the planet (Atlas et al. 1993; Carpenter and Reimann et al. 2014; Stemmler et al. 2015). At the same time, the produced BOCs have several sinks in seawater, including photodegradation, biodegradation, and physical processes such as air-sea exchange, lateral physical transportation, and deposition to the seabed (Goodwin et al. 1997b; Zakon et al. 2013; Ziska et al. 2013; Stemmler et al. 2015).

Many industrial sources of BOCs, such as fire retardant production, have been long recognized. However, studies have also found that chlorination during water disinfection gives rise to BOCs in the presence of bromide and certain dissolved organic matters (DOMs) in raw water (Nokes et al. 1999). Besides the human sources, marine ecosystems are predominant sources of certain halocarbons, especially several halomethanes, found in the environment (Butler et al. 2007). A study by Quack and Wallace (2003) suggests that the ocean is the largest global source for volatile brominated and iodinated organic compounds (2003).

1.2. The Sources, sinks, and environmental implications of bromoform as a volatile BOC

Bromoform ($CHBr_3$) is a volatile BOC. According to a study by Butler et al., (2007) bromoform supersaturates in the ocean. The supersaturation indicates that the ocean is the predominant source of the compound. While phytoplankton species are thought to be the major sources of bromoform in the open oceans, the compound found in coastal environments can be attributed to both biological and industrial sources. The biological origins of bromoform in coastal environments include macroalgae (including multiple green algae, brown algae, and red algae species) (Fig.1; Gschwend et al. 1985; Flodin et al. 1999), eukaryotic phytoplankton (including several diatom species) (Hill and Manley 2009), and marine bacteria (such as *synechococcus spp.*) (Moore et al. 1996; Johnson et al. 2011). Among biological sources,

macroalgae species are expected to contribute more to bromoform in coastal environments. Bromoform produced by eukaryotic and prokaryotic phytoplankton in the open ocean is expected to be significant for the global bromine cycle as well, given phytoplankton's major contribution to the global primary production (Johnson et al. 2011). Industrial processes such as water disinfection directly produce bromoform as a byproduct (Nokes et al. 1999). Many other processes release BOCs that can be further brominated by biological processes to form bromoform (Howe et al. 2005). Therefore, both direct HOBr input and industrial BOC emission can also be sources of oceanic bromoform.

The major sinks of oceanic bromoform include physical processes including air-sea exchange, lateral transportation, and vertical transportation, and (bio)chemical reactions including photodegradation and microbial turnover (Fig.1; Goodwin et al. 1997b; Quack and Wallace 2003; Butler et al. 2007; Ziska et al. 2013). According to the study by Stemmler et al. (2015), vertical transportation is only significant in the Southern Ocean, where surface water sinks into the deep ocean due to strong convection. So far, there is no clear estimate of photodegradation as a sink of bromoform in the ocean. According to a study by Goodwin et al. (1997b), microbial turnover, that may be significant in the open ocean, does not act as a major sink of the compound in coastal environments. Air-sea exchange is the most intensely studied and probably the largest sink of bromoform in seawater (Butler et al. 2007; Stemmler et al. 2015). As bromoform enters the atmosphere through air-sea exchange, atmospheric convection can potentially carry it to the stratosphere, especially in the tropical areas (Quack and Wallace 2003).

Bromoform is the largest single source of bromine in the atmosphere (Penkett et al. 1985). It releases bromine radical, a potent ozone depleting agent, under the ultraviolet light in the stratosphere (Fig.1). Although many ozone depleting chemicals such as CFCs have been strictly regulated under the Montreal Protocol, bromoform, as a member of the family of volatile BOCs, still threatens the ozone layer (Papanastasiou et al. 1994). According to estimates of different global bromoform sources, biological sources, including macroalgae production and planktonic production, dominate over anthropogenic sources (Quack and Wallace 2003).

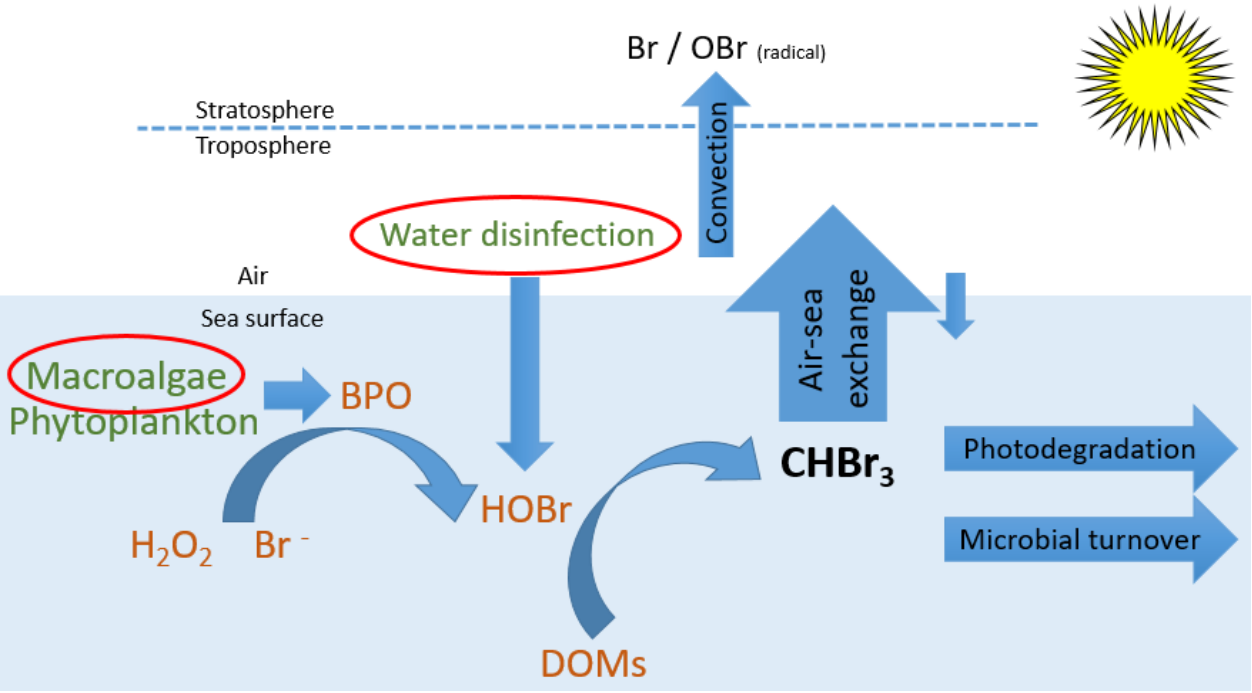

Figure 1: The sources and sinks of bromoform in a coastal ecosystem. DOM refers to dissolved organic matter. BPO refers to bromoperoxidase, a group of enzymes responsible biological bromination in oceans. The sources include water disinfection input, macroalgae production, and phytoplankton production. Phytoplankton consists of both bacteria (prokaryote) and eukaryotic microalgae. The sinks include air-sea exchange, photodegradation, biodegradation, and microbial turnover. Macroalgae and water disinfection are two major sources of bromoform in coastal environment. Both pathways are studied in this project.

1.3. Enzymatic and abiotic pathways of bromoform formation

Haloperoxidase enzymes (HPO) are responsible for halogenated organic compounds (HOCs) production in both terrestrial and marine ecosystems (Moore et al. 1996; Flodin et al. 1999; Butler and Carter-Franklin 2004; Johnson et al. 2011; Reddy et al. 2013; Breider and Hunkeler 2014). Chloroperoxidase (CPO) and bromoperoxidase (BPO) are two of HPOs. H_2O_2 oxidizes heme moiety of CPO or vanadium moiety of BPO. The HPO then oxidizes Br^- to hypobromous acid (HOBr), which then reacts with a wide range of organic moieties, including phenolic groups, β-dicarbonyl compounds, and α-hydroxyl carboxylic acids. Bromoform is one of the final products (Fig.2; Arnold et al. 2008; Dickenson et al. 2008.

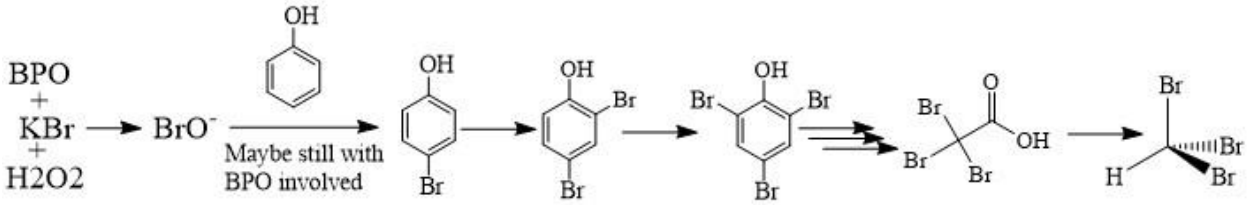

Figure 2: Bromoperoxidase catalyzes the reaction between bromide ion and hydrogen peroxide to form hypobromite. Bromoform is one of the products of the reactions between hypobromite and many DOMs. Phenol is used as the model DOM here.

Bromoperoxidase (BPO) is the enzyme responsible for bromoform production in marine primary producers, including *synechococcus spp.*, some diatom species, and many macroalgae species. Different from CPO, BPO's terrestrial counterpart with an iron active center, BPO has a vanadium active center (Butler et al. 2004). Macroalgae species containing BPO range across green algae, brown algae, and red algae (Moore and Tokarczyk 1993; Reddy et al. 2002; Quack and Wallace 2003; Wever et al. 2009).

Bromination processes in water disinfection procedures are similar to the biological halogenation reactions described above (Arnold et al. 2008). Hypochlorous acid (HOCl) is added to the raw water to kill living organism. HOCl oxidizes a wide groups of organic compounds to chlorinated organic compounds. Chloroform is one of the final products of such reactions. With presence of bromide ion, HOCl is quickly converted to HOBr (Heeb et al. 2014). HOBr formed further oxidizes organic compounds through a similar pathway. In this case bromoform is produced instead of chloroform.

Both carbon and chlorine isotopes have been used to identify chloroform formation processes. Several studies have been done to use carbon isotope effect of the chlorination processes to understand sources of chlorinated organic compound pollutants (Hunkeler et al. 2012; Kozell et al. 2015). Arnold et al. (2008) conducted a study on using stable carbon isotope signature to identify precursors of chloroform in lake water. Substrates being studied include resorcinol, phenol, acetylacetone, and acetophenone. Specific stable chlorine isotope signatures ($\delta^{37}Cl$) associated with CPO-catalyzed chlorination reactions are also reported. According to studies on chloroform production in terrestrial ecosystems, chloroform produced by many biological processes has distinctive stable chlorine isotope fingerprint (Reddy et al. 2002; Breider and Hunkeler 2014). A ^{37}Cl depletion resulting in $\delta^{37}Cl$ = -12.06±0.18‰ was observed in chlorination of trimethoxybenzene with CPO from *Caldariomyces fumago* (Reddy et al. 2002).

To the best of our knowledge, however, there is no previous study on bromine isotope shifts ($\delta^{81}Br$) in bromoform produced by BPO. Since both BPO and CPO cause halogenation in similar ways, BOCs, including bromoform, produced by BPO are also expected to have specific $\delta^{81}Br$.

Due to the similar reaction mechanism of chlorination with HOCl and bromination with HOBr, the bromine isotope signature in bromoform is predicted to provide information on its formation pathways. If bromoform from different pathways has distinctive $\delta^{81}Br$, we can learn more about sources and fate of environmental bromoform.

1.4. Application of isotope signatures in determining sources and fates of environmental halogenated organic compounds

Isotope signature has long been a powerful tool for understanding environmental biogeochemical processes. Many studies use stable carbon isotopes to study mechanisms of formation and transformation of halogenated organic compounds in the environment and track their flow (Arnold et al. 2008; Hunkeler et al. 2012; Breider and Hunkeler 2014). Recently, multiple studies have explored application of using isotope signatures of multiple elements, i.e. carbon and halogen (usually chlorine and bromine), to obtain more complementary information on these processes (Wiegert et al. 2012; Kozell et al. 2015). A study in 2017 determined both carbon and chlorine isotope fractionation in chloroform transformation processes (Torrento et al. 2017). This encouraging work demonstrates the potential of dual isotope analysis for exploring environmental (bio)chemical processes.

While carbon isotope analysis method is relatively well developed, relatively limited methods exists for bromine isotope analysis (Zakon et al. 2016). Isotope ratio mass spectrometry (IRMS) has been a conventional method for conducting bromine isotope analysis. However, the sample preparation processes are labor intensive since target compounds first need to be converted to CH_3Br (Shouakar-Stash et al. 2005). A method of using multi-collector inductively coupled plasma mass spectrometry coupled to gas chromatography (GC-MC-ICPMS) for compound-specific bromine isotope analysis have been proposed in 2007 and further developed (Sylva et al. 2007; Gelman and Halicz 2009). The study by Gelman and Halicz (2009) shows the capacity of determining bromine isotope shifts with 1.9‰ error with 0.02nmol bromine injection, without correction using strontium. However, the approach of coupling MC-ICPMS to GC is still limited to several laboratories around the world (Zakon et al. 2016).

A method of conducting compound specific chlorine isotope analysis using widely available quadrupole mass spectrometry coupled to gas chromatography (GCqMS) was developed (Aeppli et al. 2010). The method eliminates sample preparation steps required by standard methods such as IRMS. Given the viable method of conducting chlorine isotope analysis using GCqMS, measuring stable Br isotope ratios in BOCs may also have a significant potential. However, kinetic isotope effects are expected to be weaker in (bio)chemical processes involving bromine than in their chlorine counterparts, since the two Br isotopes have smaller relative mass difference (79 and 81) than chlorine isotopes (35 and 37) (Gelmand and Halicz 2009). Therefore, bromine isotope analysis may require higher precision than its chlorine counterpart.

With the Aeppli and co-workers' success of using GCqMS for chlorine isotope analysis on chlorocarbons, Zakon et al. explored application of using the same method for bromine isotope analysis in BOCs (2016). The research measured δ^{81}Br of bromoform with relative instrumental uncertainty of around ±0.2‰ under optimized conditions. However, conducting isotope analysis on environmental bromoform with very low concentration has not yet been done.

1.5. Objectives of the Study

Although we have general knowledge on sources and sinks of bromoform, the global biogeochemical cycle of the compound and its natural precursors is not fully understood. Studying sources and sinks of $CHBr_3$ in marine ecosystems is crucial for understanding the global Br cycle. A viable way of tracking fate of the compound and its precursors is important for better understanding sources and sinks of the compound and the global Br cycle.

This study i) develops and optimizes methods of using GCqMS to conduct Br isotope analysis on bromoform (environmental concentration as low as 0.03nM). The study also ii) develops lab techniques to determine stable Br isotope signatures (δ^{81}Br) of bromoform formed from abiotic and biological pathways, and iii) explores potential application of using δ^{81}Br to explain sources and sinks of environmental bromoform in water body of the Damariscotta River, in the coastal Gulf of Maine.

The biological pathway involves bromoform formation with *Ascophyllum Nodosum*, *Fucus Vesculosis*, and BPO from *Corallina Officinalis*. The abiotic work studies the bromoform forming reaction between HOBr and phenol. Isotope signatures of bromoform from the abiotic

biological pathways are then compared to isotope ratios of environmental bromoform in Damariscotta River. The spatial distribution of bromoform is used to provide insight into sources of bromoform in the study cite. The spatial distribution information is used to supplement information provided by isotope measurements.

The novel analytical method of using GCqMS to measure $\delta^{81}Br$ of bromoform is applied to all isotope analyses in this study. Existence of the new analytical methods using a relatively available instrument with low cost will enable more researchers to conduct further studies on using stable bromine isotopes to understand environmental processes involving BOCs. More exploration on sources and sinks of bromoform may provide more insight in ozone chemistry in the stratosphere, and is essential for better understanding the global bromine cycle.

2. FIELD SAMPLING SITE AND STUDY METHOD

The field study aims to understand the sources of bromoform in the Damariscotta River by measuring both distribution of bromoform concentration and bromoform Br isotope ratios. Therefore, both bromoform concentrations and isotope characteristics were studied in different locations of the estuary. The combination of both sets of data was expected to complement each other to present a general picture about sources of bromoform in the estuary. The field sampling results were also compared to the results from abiotic and *A. Nodosum* incubation experiments.

2.1. Introduction of Gulf of Maine and Damariscotta River

The Damariscotta River is an estuary located on the west coast of the Gulf of Maine. The field study was conducted in the estuary channel and mouth. With limited freshwater input, the major part of the Damariscotta River is filled with seawater from the Gulf of Maine. *A. Nodosum*, *Fucus Vesculosis*, *Ulva Lactuca* and *Laminaria digitata* are several of the major macroalgae species in the Damariscotta River estuary (Bigelow Semester Program, 2016; Maine Sea Grant, 2018). All three species are known to produce bromoform in relatively large amounts (Nightingale et al. 1991). Since there is no clearly identified anthropogenic input of bromoform in the drainage basin, the local macroalgae, especially the species that are known to produce a lot of bromoform, such as *A. Nodosum*, *L. Digitata*, and *U. Lactuca* are expected to be the major sources of bromoform in local seawater.

2.2. Spatial distribution of bromoform

Local algae beds locate within the estuary channel of the Damariscotta River. Therefore, in coastal Gulf of Maine, bromoform concentrations are expected to decrease from the estuary channel of estuary to the open ocean. To confirm the predicted distribution pattern, surface bromoform concentrations at four sampling sites are measured over a two-month period. The four sites locate in the Damariscotta River channel (Station 1 and Station 2), the river mouth (Station 3), and the open ocean (Station 4) (Fig.3).

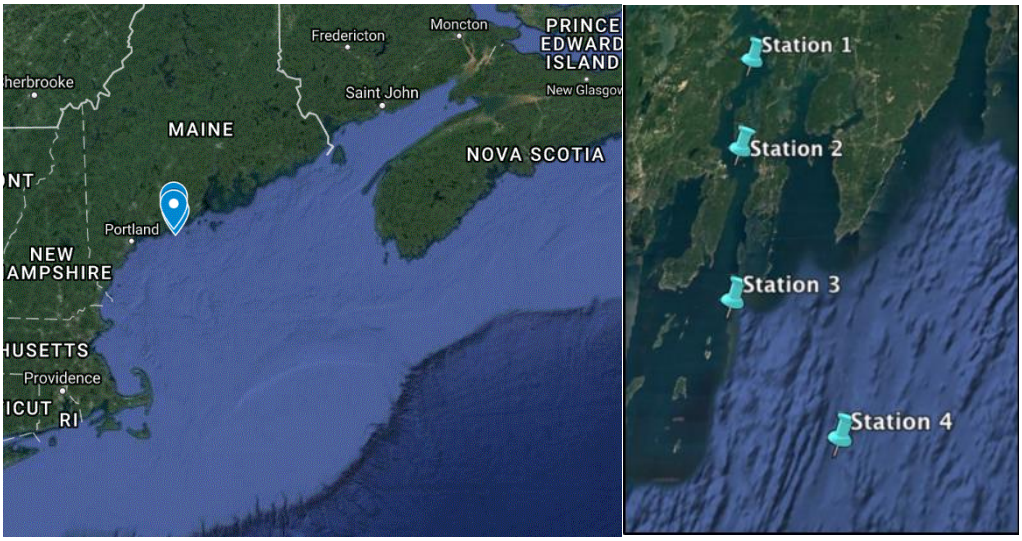

Figure 3: the four sampling sites where field samples were collected to measure surface water bromoform concentration. The coordinates are: 43°53’95’’ N, 69°34’75’’ W (Station 1), 43°51’65’’ N, 69°34’70’’ W (Station 2), 43°48’65’’ N, 69°34’30’’ W (Station 3), and 43°45’04’’ N, 69°30’22’’ W (Station 4). The Colby-Bigelow Changing Ocean Semester Program in 2016 provided all geographical information.

The same field sampling was conducted through four cruises during the Colby-Bigelow Changing Ocean Semester Program in the fall semester of 2016 (Table 1). All seawater samples were collected at 10m depth. 400ml seawater was collected at each sampling site. Seawater was transferred to a gas-tight glass bottle sealed by Teflon cap without headspace. To stop potential depletion or production of bromoform in collected seawater, the pH value was dropped to around 2 by adding 1ml of 6M HCl. The sample was then placed in a dark cooler to avoid photodegradation of bromoform. Dissolved bromoform was extracted from field seawater samples through liquid-liquid extraction within three days and quantified on a gas chromatography-mass spectrometry (GC-MS).

Table 1: the dates when seawater was sampled for bromoform concentration

Sampling Cruise Number	Sampling Date
Cruise 1	Sep 20
Cruise 2	Oct 4
Cruise 3	Oct 19
Cruise 4	Nov 1

2.3. Bromine isotope ratios of bromoform in the surface water

δ^{81}Br of bromoform in the surface seawater from both the open ocean and the estuary were studied. 5L of water was collected to obtain enough bromoform for isotope analysis. Seawater was collected at Station 3 and Bigelow Dock (near Station 2), respectively. Both samples were from the surface. The sample from Station 3 was collected at noon of May 31, 2017, when the tide entered the estuary from the open ocean; that from Bigelow Dock was collected in the morning of May 31, 2017, when seawater flowed from the upper stream towards the ocean. Hence seawater collected at Station 3 was predominantly from the open ocean and that collected at the Bigelow Dock was from the upper stream of the estuary.

2.4. Br isotope ratio measurement

The two isotope analyses were conducted with bromoform extracted from 5 L seawater each. Liquid-liquid extraction with dichloromethane was used to extract bromoform for GC-MS measurements (see *Analytical methods* section).

3. METHODS:

3.1. Material

The abiotic experiment was conducted at Colby College; all remaining experiments and field sample processing were completed at the Bigelow Laboratory for Ocean Sciences. The GC-MS (Agilent 7890A GC, Agilent 5975C MS) at Colby College was used for evaluating bromoform yields from the abiotic processes. The GC-MS (Agilent 7890B GC, Agilent 5977 MS) at the Bigelow Laboratory was used for determining environmental bromoform concentrations, evaluating bromoform yields from enzyme and macroalgae incubations, and measuring isotope signature in bromoform from all experiments and field studies. Detailed information of material used in the study is listed in the appendices (Table 2).

3.2. Bromoform producing experiments

Br isotope analyses were conducted on bromoform produced through two pathways: the abiotic reaction, and macroalgae incubation using *A. Nodosum*. To understand the kinetics of bromoform formation pathway catalyzed by enzyme, bromoform was also produced from BPO enzyme (Sigma Aldrich, extracted from *C. Officinalis*, a tropical red algae species). Since stable isotope analysis needs a reasonably high bromoform concentration, optimal bromoform formation conditions were explored before isotope analyses. Larger scale bromoform formation experiments, if needed, were then designed based on preliminary knowledge on optimal reaction conditions of each pathway. All experiments involving isotope ratio measurements were done in duplicates or triplicates. Desired organic compounds (bromoform in most cases; bromophenols were only studied in the enzyme incubation experiments, to understand the kinetics of bromoform formation) were extracted by liquid-liquid extraction. Concentrations of bromoform were measured by a GC (either at Colby or Aeppli Lab, see *Analytical methods* section). Br isotope analyses were conducted by the GCqMS at the Aeppli Lab.

3.2.1. Abiotic Experiment

The abiotic experiment aimed to mimic the bromination processes in water disinfection procedures involving naturally existing bromide ions. In the lab experiment, phenol was used as the model compound for phenolic moieties, which is usually a significant group of DOMs in many fresh water systems (Arnold et al. 2008). The Arnold group conducted a study on

chloroform formation from HOCl and phenol. This study borrowed many methods from the study by Arnold et al. Similar to its chlorine counterpart in the previous study, bromoform was produced as a product of reaction between phenol and HOBr in this experiment.

At the beginning of reactions, NaOCl (150μM initial concentration) was added into 5mM pH8 phosphate buffer with 840μM KBr. The reaction between NaOCl and KBr rapidly forms HOBr (pH = 8.1, smaller than the pKa of HOBr). Phenol (12μM initial concentration) was then added into the system to start the reaction. The total HOBr was added in excess (around 150μM, assuming all NaOCl is turned into HOBr) to ensure the isotope effects were not influenced by depletion of the bromine source (HOBr, in this case) (Table 3). Two controls were set in this experiment. One had no $^{-}$OCl added, the other had no phenol added, to make sure the reaction systems were not contaminated by either DOMs or HOCl.

Table 3: Reagents used for the abiotic experiment and their concentration. All material is obtained from Colby College. The detailed producer information is listed in the appendix.

Reagent	**Concentration**
pH8 phosphate buffer	5mM (43ml)
Phenol	12μM
KBr	840μM
NaOCl	150μM
HOBr (estimated from the reaction between $^{-}$OCl and Br^{-})	Around 150μM

All reactions were conducted in 43ml gas tight glass vials without head space. The vials were sealed with a Teflon cap. The reactions were ran for 1 minute and 5 minutes in triplicates and 30 minutes in duplicates. Both controls were maintained for 180 minutes (Fig.4).

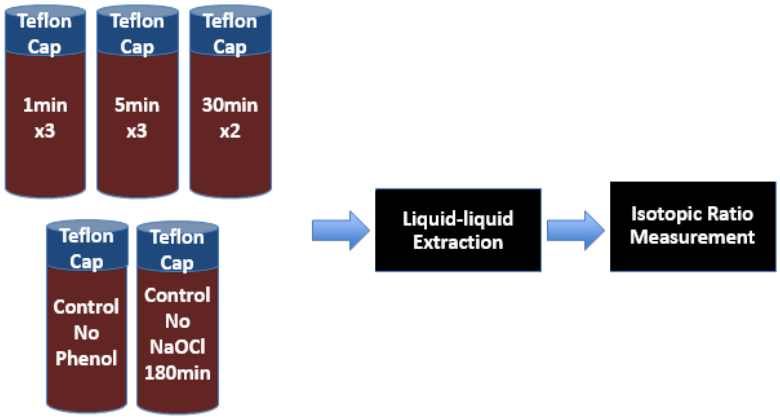

Figure 4: The setup of the abiotic experiment producing bromoform from HOBr and phenol. The 1min and 5min group were done in triplicates while the 30min group was done in duplicates. The reaction time for both controls was 180min. Br isotope analysis was only conducted on the 1min and 30min groups.

A 100µl of mixture of 50g/L $NaSO_3$ and 0.5M HCl was used to stop the reactions and drop the pH values for liquid-liquid extraction. HOBr was quickly depleted as it oxidizes $NaSO_3$ (Srivastava et al. 1980). HCl was added to ensure that the OBr^- was in the form of HOBr. A GC-MS was then used to determine the bromoform concentrations.

$$HOBr + SO_3^- \longrightarrow H^+ + Br^- + SO_4^-$$

3.2.2. Macroalgae Incubation

An incubation experiment was conducted to produce bromoform from *F. Vesculosis* and *A. Nodosum*. Entire plants of *F. Vesculosis* and *A. Nodosum* were incubated in filtered seawater. The Br isotope ratios of bromoform yielded from the incubation experiment were then measured. Each individual plant was incubated in seawater in a 1L glass bottle for six hours. The bottles were sealed with Teflon caps and no head space was left. Temperature was maintained at 16.5 ± 0.5 °C and light intensity was 470 µmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). For the *A. Nodosum* incubation, 0nM, 200nM, and 2mM of H_2O_2 were added to as variables (n=2), despite the naturally occurring H_2O_2 in seawater (original seawater H_2O_2 concentration not measured). No additional H_2O_2 was added for the *F. Vesculosis* duplicates. For the *A. Nodosum* group, a dark control was set up by wrapping the bottle with tin foil.

The macroalgae were harvested in the intertidal zone along the shore by the Bigelow Dock during low tide. For *A. Nodosum*, individual plants around 12cm long and 100g heavy were targeted (wet weight) (See appendices, Table 4). Entire plants including holdfast were harvested to minimize damage to the plants. Seawater used in the experiment was collected from the Bigelow dock and then filtered with 0.2μm filter. To eliminate dissolved bromoform in the seawater, it was bubbled with house air for six hours.

3.2.3. Bromoperoxidase (BPO) Incubation

BPO was used to produce bromoform from phenol, H_2O_2 and KBr in pH=8 phosphate buffer. Conducting incubation experiments with extracted BPO enzyme eliminates many individual variations that using living species can incur. Therefore, pure enzyme offers a relatively easy way to study biological processes. Only the reaction kinetics of the phenol bromination by BPO was studied. The experiment aimed to understand dynamics of precursors of bromoform produced by BPO enzyme activity.

To study the reaction pathway and kinetics of bromoform formation, reactions were conducted for 0, 2, 4, 6, and 8 hours in five separate vials, respectively. The concentrations of phenol, 2-bromophenol, 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol, 2,4,6-tribromophenol, and bromoform from reactions with each time length were measured. The experiment was conducted in 43ml gas tight amber glass vials sealed with Teflon cap with no head space. 10μM phenol, 0.5mM H_2O_2, 840μM KBr, and 0.047U/ml BPO were mixed to produce bromoform (Table 5). The initial reaction conditions were kept the same for all experiments. The H_2O_2 concentrations were regularly monitored using peroxide test stripes. H_2O_2 was added to the systems when it obviously depleted. Stirring bars prevented enzyme from settling throughout the experiment. Quenching the solution into DCM stopped the reactions. Liquid-liquid extraction was conducted right after the quenching process to extract bromoform.

Table 5: A summary of reagents used in the experiment for studying the bromoform forming kinetics with BPO. All material is obtained from Colby College. The detailed producer information is listed in the appendices (Table 2).

Reagent	Concentration
pH8 phosphate buffer	5mM (43ml)
Phenol	10μM
KBr	840μM
BPO	0.047U/ml
H_2O_2	Initial Concentration = 0.5mM, added when obviously depleted during the reaction

3.3. Analytical methods

3.3.1. Bromoform, Bromophenol, and Phenol Concentration Measurement

Liquid-liquid extraction was performed to obtain desired compounds from experiments. Dichloromethane was used as the organic phase. To evaluate the extraction efficiency, 1,3,5-trichlorobenzene was added to the aqueous phase as an internal standard before extraction. Dehydrated sodium sulfate (Na_2SO_4) was used as the drying agent for the organic phase. Extracts were concentrated to around 1ml dichloromethane solution using rotary evaporator.

Dichloromethane was used as the solvent for injection for all measurements conducted at Colby College. For all measurements conducted at the Bigelow Laboratory, dichloromethane was replaced with hexane through a solvent exchange after rotary evaporation. Replacing dichloromethane with hexane aimed to avoid over expansion of solvent in the GC liner. Solvent exchange was conducted on a 40°C heating plate with steady nitrogen flow from the top of vials.

A GC was used to measure concentrations of phenol and BOCs produced (bromoform, 2-bromophenol, 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol, 2,4,6-tribromophenol, and phenol). Slightly different instruments and parameters were used for measurements conducted at Colby and the Aeppli Lab (Table 6; Table 7).

Table 6: Parameters used for measuring bromoform, bromophenol, and phenol concentrations in Aeppli Lab at Bigelow Laboratory.

Target Compound	**Bromoform**	**Bromophenol & Phenol**
Gas Chromatography	Agilent 7890B GC	Agilent 7890B GC
Column	DB-624 30m x 0.25mm iD x 1.4μM film	DB-5 30m x 0.25mm iD x 0.25μm film
Carrier gas	Helium at 1ml/min	Helium at 2ml/min
Solvent delay	6min	6min
Temperature program	Hold at 40°C for 2min, then heat up to 250°C at the rate of 20°C/min, finally hold at 250°C for 10min.	Hold at 40°C for 10min, then heat up to 250°C at the rate of 20°C/min, finally hold at 250°C for 5min.
Injector temperature	260°C	320°C
Split mode	Splitless, 2min	Splitless, 2min
Injection volume	3μl	3μl
Mass spectrometry	5977 MSC	5977 MSC
Ionization method	Electron impact ionization (70eV)	Electron impact ionization (70eV)
Mass trace	SIM mode: 173	SIM mode: 94, 172, 173, 180, 252, and 330
MS source temperature	230°C	230°C
MS quadrupole temperature	150°C	150°C
Dwell time	10ms	10ms

Table 7: Parameters used for measuring bromoform concentrations at Colby College.

Target Compound	**Bromoform**
Gas Chromatography	Agilent 7890A GC
Column	Agilent HP-5ms 30m x 0.25mm
Carrier gas	Helium at 2ml/min
Solvent delay	7min
Temperature program	Hold at 40°C for 8min, then heat up to 250°C at the rate of 20°C/min, finally hold at 250°C for 5min.
Injector temperature	225°C
Split mode	Splitless
Injection volume	3μl
Mass spectrometry	5975C MS
Mass trace	SIM mode: 173
Dwell time	10ms

An instrumental test was conducted for the GC at Colby College prior to bromoform concentration measurements. To test the GC capacity, calibration curves of bromoform with both low and high concentrations were measured. The lower end calibration curve consists of bromoform standards with concentration of 0.05µM, 0.1µM, and 0.2µM; the higher end one consists of concentration of 100µM, 333µM, 667µM, and 1000µM. 10µM 1,3,5-trichlorobenzene was used as the internal standard. The calibration curves shows that the GC was capable of quantifying bromoform between the concentration of 0.05µM and 1000mM, with or without an internal standard (Fig.5).

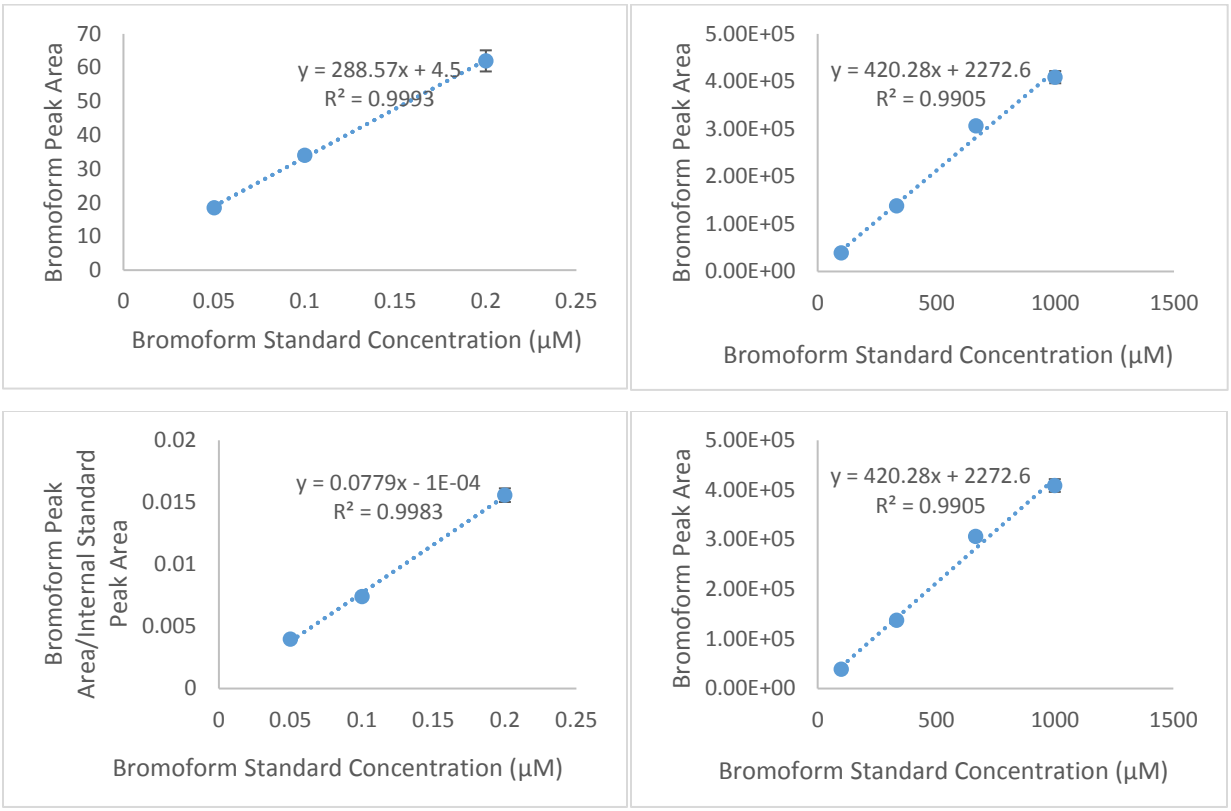

Figure 5: Calibration curves made from bromoform peak area against bromoform concentration at the lower concentrations (left top), ratios between bromoform peak areas and internal standard peak areas at the lower concentrations (right top), bromoform peak area against bromoform concentration at the higher concentrations (left bottom), and bromoform peak area against bromoform concentration at the higher concentrations (right bottom). Each data point is a mean of multiple measurements. Error bars represent the standard deviation (n=10).

3.3.2. Bromine Isotope Ratio Measurement

A GCqMS was used for stable bromine isotope analysis. All isotope analyses were done in the Aeppli Lab of the Bigelow Laboratory for Ocean Sciences. $\delta^{81}Br$ values were calculated by comparing Br isotope ratios of bromoform produced by lab experiments to the ratio of industrially produced bromoform (Sigma Aldrich). Stable bromine isotope ratios of both bromoform standard and that of the sample were measured. The measured ratios of the standards were set as zero. Then Br isotope ratios of the samples were compared to the ratios of the standards. Measurements were conducted in single ion monitor (SIM) mode.

Fragment ion of 171, 173, and 175 are the three major fragments produced from bromoform by an MS (Fig.6). The analytical method of this study is partially borrowed from a previous study by Zakon et al., which adopted m/z 171 and m/z 173 as the targeting mass trace (2016). The m/z 171 and m/z 173 (fragment ion $^{+}CHBr_2$) were chosen as the target mass trace.

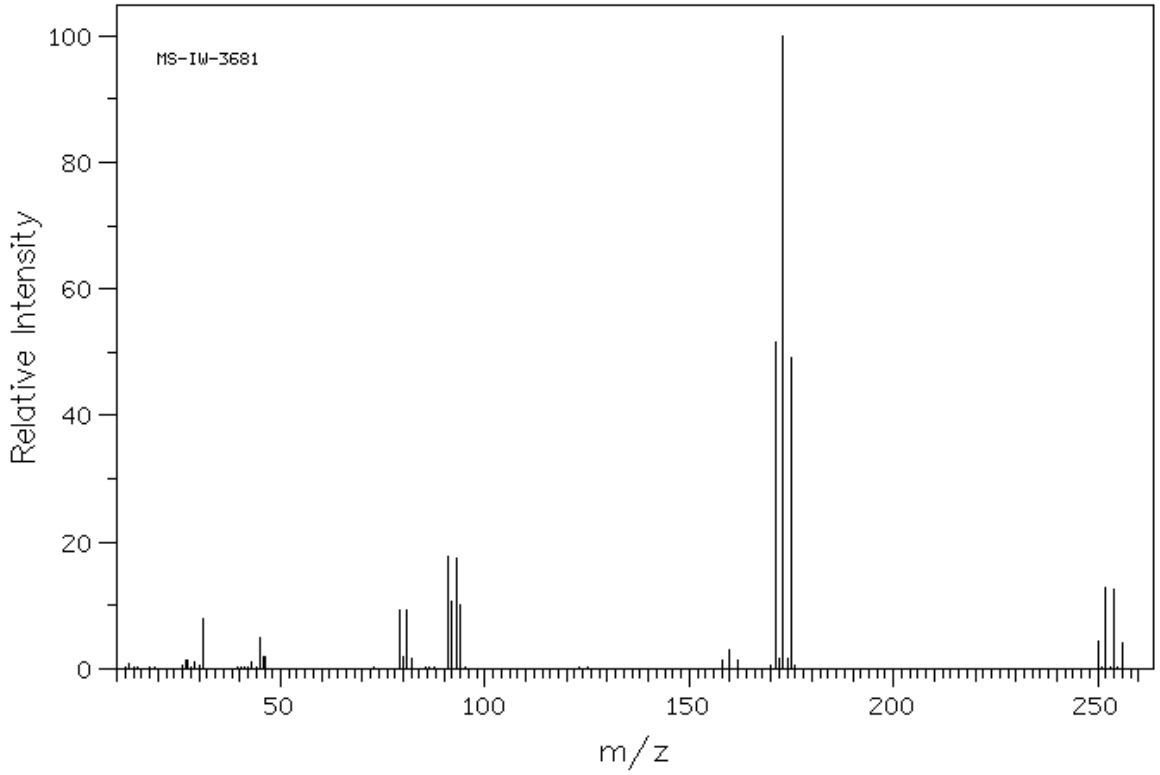

Figure 6: The mass spectrum of bromoform. The spectrum is from the Spectral Database for Organic Compounds SDBS.

Calculation of bromine isotope ratios, R, were performed based on the mass traces of 171 (fragment with two ^{79}Br atoms) and 173 (fragment with a ^{79}Br and an ^{81}Br atom). The Br isotope ratio, R, would be (Aeppli et al. 2010):

$$R = \frac{81Br}{79Br} = \frac{\frac{1}{2} \times I173}{I171} = \frac{1}{2} \times \frac{I173}{I171}$$

Where "I" represents the peak intensity of each mass trace.

^{81}Br (Br isotope signature) was then calculated as:

$$(\frac{R}{R(std)} - 1) \times 1000‰,$$

where R(std) is the isotope ratio of the standard; R is that of the sample.

Br isotope ratios of a sample and its corresponding standard were measured consecutively. Mean and standard deviation values of δ^{81}Br were then calculated (n=6). Each standard was individually prepared so that the difference of standard and sample concentrations is within 10%. Isotope ratios of samples and standards were measured for six times, respectively. Before isotope analysis, concentrations of samples were measured by a GC. D-hexadecane was used as the internal standard.

Preliminary instrumental tests suggest that the MS performs the best at low resolution with a 50ms dwell time (not shown). The MS was hence set to low resolution and 50ms dwell time for all isotope analyses. Detailed instrumental parameters were developed from the previous studies on measuring stable Cl and Br isotope ratios using GCqMS (Aeppli et al. 2010; Zakon et al. 2016; Table 8).

Table 8: Parameters used for stable bromine isotope analysis on bromoform. All bromine isotope analyses were conducted in the Aeppli Lab.

Target Compound	**Bromoform**
Gas Chromatography	Agilent 7890B GC
Column	DB-624 30m x 0.25mm iD x 1.4μM film
Carrier gas	Helium at 1ml/min
Solvent delay	6min
Temperature program	Hold at 40°C for 2min, then heat up to 250°C at the rate of 20°C/min, finally hold at 250°C for 10min.
Injector temperature	260°C
Split mode	Splitless, 2min
Injection volume	3μl
Mass spectrometry	5977 MSC
Ionization method	Electron impact ionization (70eV)
Mass trace	SIM mode: 171, 173
MS source temperature	230°C
MS quadrupole temperature	150°C
Dwell time	50ms
Resolution	Low resolution

3.3.3. Correct for sample-standard concentration ratio

Preliminary instrumental tests suggest that accurate quantifying δ^{81}Br generally requires sample-standard concentration ratios between 0.9 and 1.1 (Fig.7). Sample-standard concentration ratios deviated from 1 negatively impact measured isotope ratio values. In measurements, concentration of a standard does not always precisely matches the concentration of the sample, due to the instrumental shifts and human errors during the concentration determination and standard preparation process. Effects of different sample-standard ratios on measured δ^{81}Br were determined to correct for the real δ^{81}Br values.

The test was conducted by measuring "isotope shift" with samples and standards that have different bromoform concentrations. Both samples and standards are prepared from the same batch of industrially produced bromoform, so that there they have the same actual Br

isotope ratios. Therefore, any measured $\delta^{81}Br$ can be treated as instrumental errors or deviations. The test was set up for sample-standard ratios of 0.8, 0.9, 1, 1.1, and 1.25 (Table 9).

Table 9: The sample-standard ratios used for analytical tests and corresponding sample and standard concentrations of each level. Three measurements were actually conducted. Trial number 4 and 5 were from measurements 2 and 1, respectively, with sample and standard flipped in the data processing process.

No.	Smp/std	Smp Concentration (μM)	Std Concentration (μM)
1	**0.80**	16	20
2	**0.90**	18	20
3	**1.00**	20	20
4	**1.11**	20	18
5	**1.25**	20	16

The generalized equation of y = -1.78x + 1.82 (y is the expected $\delta^{81}Br$; x is the sample-standard ratio) was concluded from the analytical test, i.e. the $\delta^{81}Br$ value is expected to shift by y given a sample-standard ratio (Fig.7). Therefore, the measured $\delta^{81}Br$ minus the measurement shift (y = -1.78x + 1.82) gives the corrected $\delta^{81}Br$ value:

$$\delta^{81}Br = \delta^{81}Br \text{ (measured)} - (1.78x+1.82),$$

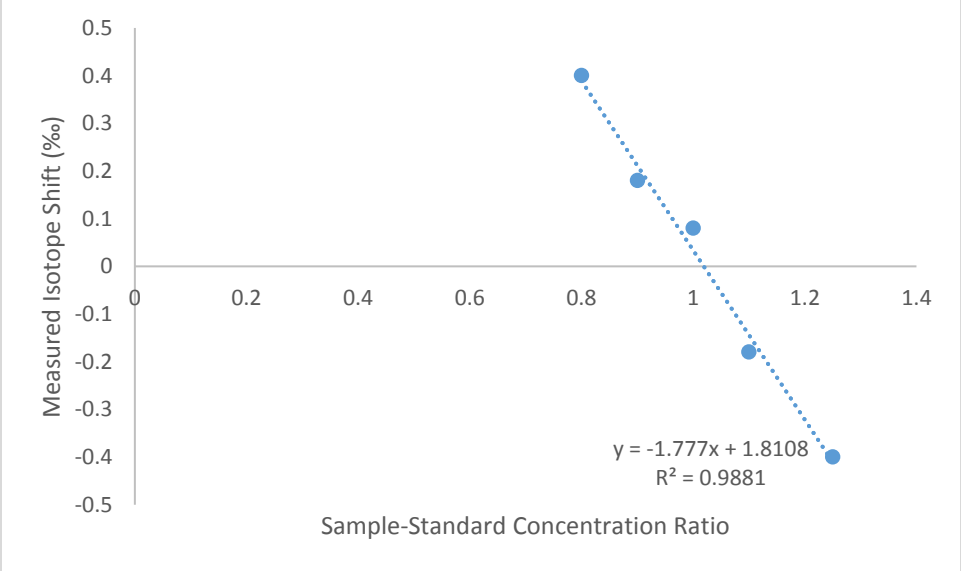

Figure 7: Measured $\delta^{81}Br$ of bromoform against sample-standard ratios (n=1). A linear regression model is fitted to the points. All measurements are done consecutively with GCqMS.

4. RESULTS AND DISCUSSION

4.1. Bromoform yield from lab experiments

4.1.1. Abiotic experiment

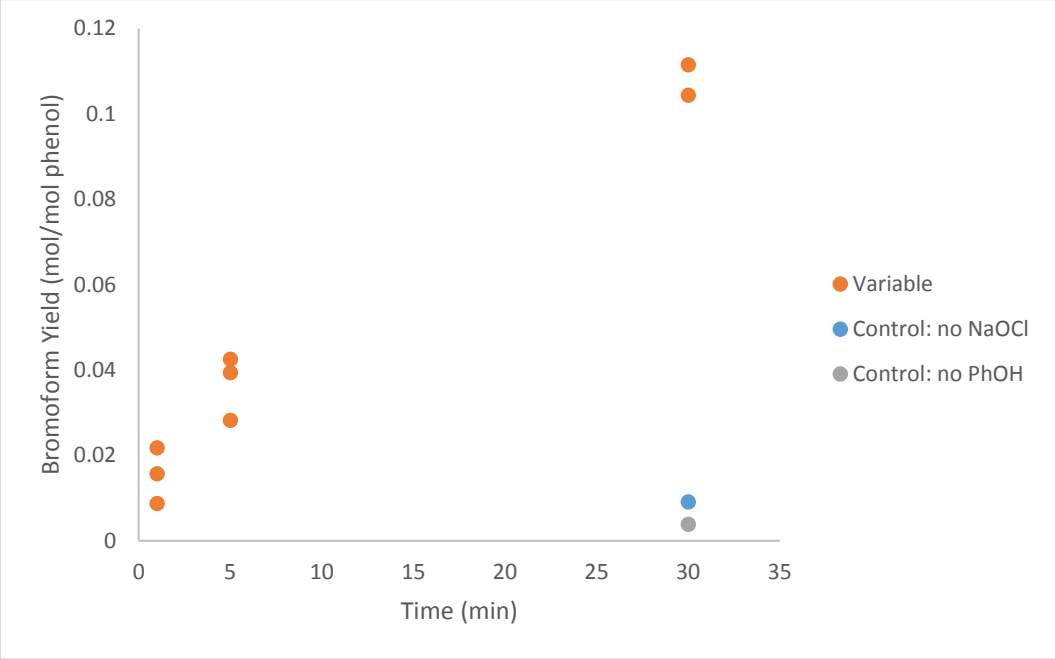

Figure 8: Bromoform yield over time from the abiotic experiment. Each point represents a trial (n=3 for 1min and 5min group, n=2 for the 30min group). Reaction in both controls trials were run for 180min.

The bromoform yield increases with time within the 30min period. The yields of both 30min trials are 0.104mol and 0.112mol bromoform per molar phenol (the original phenol amount), respectively (Fig.8). A study conducted by Arnold et al. measured chloroform formation from phenol, under similar reaction conditions. The Arnold group reported a yield of 0.051mol chloroform per mole phenol (Arnold et al. 2008). The observed highest bromoform yield in this experiment is around two times of the previously reported chloroform yield, but still within the same order of magnitude.

The two potential sources of variation between replicates are i) analytical uncertainties and ii) uncertainties in controlling the reaction time. All measurements were conducted without an internal standard, i.e. bromoform concentrations were calculated directly from GC peak areas. Instrumental tests suggest that the quantification method used in this study has 5% analytical uncertainty (n=10) associated with measuring bromoform concentration (Fig.5). However, this

uncertainty is only from the variability of the GC instrument, not considering possible uncertainties incurred in sampling and extraction processes. The two trials of the 30min group have yield difference of 7.6%. The observed difference is within the expected range of the analytical uncertainty. Variability during sampling and extraction might be more obvious for the two groups with shorter reaction time, especially the 1min group. Since the reaction was started by adding phenol and stopped by adding Na_2SO_3. Relatively slight differences in reagent mixing time even only by seconds can cause proportionally large differences in actual reaction time. Therefore, the proportional variation of yields decreases as reaction time increases, as instrument variation becomes the dominant source of errors (Fig.8).

The amount of bromoform yielded over 180min from the control groups was 0.004mol (control without phenol) and 0.009mol/mol phenol (control without NaOCl), respectively (Fig.8). Observing bromoform from both controls suggest that the system was slightly contaminated by certain DOM and oxidizing agent that can brominate phenol. Background concentration caused by DOM contamination was also observed in other studies (Liu et al. 2015). However, the bromoform yields in the control groups are relatively low comparing to the 30min group. Hence the contamination should not significantly influence the predominant source of bromoform produced in the experiment, especially that of the 30min group. Its effect on Br isotope analysis is also expected to be minor.

Both control groups were allowed to react for 180min. The reagent causing bromoform production in the control groups may not be the reagents used in the variables (phenol and HOBr). The specific substrate type probably influences the bromoform formation rate. The previous study conducted by Arnold et al. indicates that types of substrate significantly influences bromination rates. For example, the rate constant of chloroform formation from resorcinol is three orders of magnitude higher than that from phenol, and per molar chloroform formed from per molar resorcinol was 17 times more than that formed from per molar phenol (2008). Therefore, there is not enough information for estimating background bromoform yields at 1min, 5min, and 30min time points. The upper bound, i.e. 0.004mol (control without phenol) and 0.009mol (control without NaOCl) are taken as the highest bromoform yield possible at each time point, assuming that the reaction completed instantaneously.

4.1.2. Macroalgae incubation

The macroalgae incubation experiment shows relatively large variability between trials with the same conditions. *A. Nodosum* forms more bromoform than *F. Vesculosis* under the same H_2O_2 concentration (Table 10). A study by Wever et al. suggests that bromination rate of *A. Nodosum* is an order of magnitude higher than that of *F. Vesculosis* (1991). The *A. Nodosum* bromoform yield matches the range suggested by other studies. Nightingale et al. reported that bromoform production rate of *A. Nodosum* is 470ng×g^{-1} (dry weight) ×day^{-1} (Table 11). Since this experiment shows that bromination happens in both light and dark environment within the same order of magnitude (Table 5), unit conversion here crudely assumes that the bromination rate of *A. Nodosum* is constant throughout a day. Then 470ng×g^{-1} (dry weight) ×day^{-1} is equivalent to around 0.46nmol×g^{-1} (dry weight) ×$6h^{-1}$. The converted value matches well the results from the *A. Nodosum* incubation groups without or with 200nM H_2O_2 added.

However, Gshwend et al. reported average bromoform production rate of 4500ng×g^{-1} (dry weight) ×day^{-1} by *A. Nodosum* (ranging from 127 to 12000ng×g^{-1} (dry weight) ×day^{-1}) (Table 11). The large variability reported by Gshwend et al. suggests that seasonal variation of bromoform production by *A. Nodosum* is significant.

A high H_2O_2 concentration significantly increases bromoform production by the algae, by around two orders of magnitude (Table 10). A group with additional 2mM H_2O_2 was intentionally added to enhance bromoform production, in order to conduct more precise isotope analysis. Such a high H_2O_2 concentration does not naturally exist in seawater. In the environment, H_2O_2 that BPO uses as an oxidant can either be from seawater or produced as a byproduct of photosynthesis (Elstner 1987; Wever et al. 1991). Therefore, as long as photosynthesis happens, bromination can happen without external supply of H_2O_2. H_2O_2 that already exists in the algae's tissue probably leads to bromination in the dark control. The difference between bromoform yield from the dark control and the group without additional H_2O_2 represents bromination due to H_2O_2 from photosynthesis.

However, effects of H_2O_2 on different macroalgae species can be very different. While the H_2O_2 concentration positively influences bromoform production rate of *A. Nodosum* within the concentration range explored in this experiment, it may affect other species differently. For example, a study by Manley and Barbero shows that bromoform production by *Ulva lactuca* is positively related to photosynthesis. 100μM H_2O_2 can inhibit photosynthesis, and hence

negatively influence bromoform production rate (2001). Therefore, the pattern cannot be generalized.

Table 10: Yields of macroalgae incubation experiments. The H_2O_2 values represent amount of additional H_2O_2 added to the system. Since Damariscotta River seawater was used in all incubation, background H_2O_2 that was not measured also exist in the system.

Experiment	Bromoform Yield ($nmol \times g^{-1} \times 6h^{-1}$, by dry mass)
***A. Nodosum* w/t H_2O_2**	0.24
***A. Nodosum* w/t H_2O_2**	0.57
***A. Nodosum* 200nM H_2O_2**	0.80
***A. Nodosum* 200nM H_2O_2**	0.62
***A. Nodosum* 2mM H_2O_2**	37.50
***A. Nodosum* 2mM H_2O_2**	40.23
***A. Nodosum* dark control**	0.16
***F. Vesculosis* w/t H_2O_2**	0.04
***F. Vesculosis* w/t H_2O_2**	0.09

4.1.3. Enzyme Experiment

Phenol was converted to 2,4,6-tribromophenol and 6.5×10^{-4}mol bromoform per molar phenol was produced after 8hr incubation. After eight hours, the ratio of bromoform yield to 2,4,6-tribromophenol yield was around 1:3000 (Fig.9). The low bromoform yield and accumulation of 2,4,6-tribromophenol indicate that the ring opening steps that finally leads to bromoform formation is the rate determining. Total phenol concentrations vary between 8μM and 148μM (Fig.9), reflecting the experimental and analytical variabilities of each reaction vial.

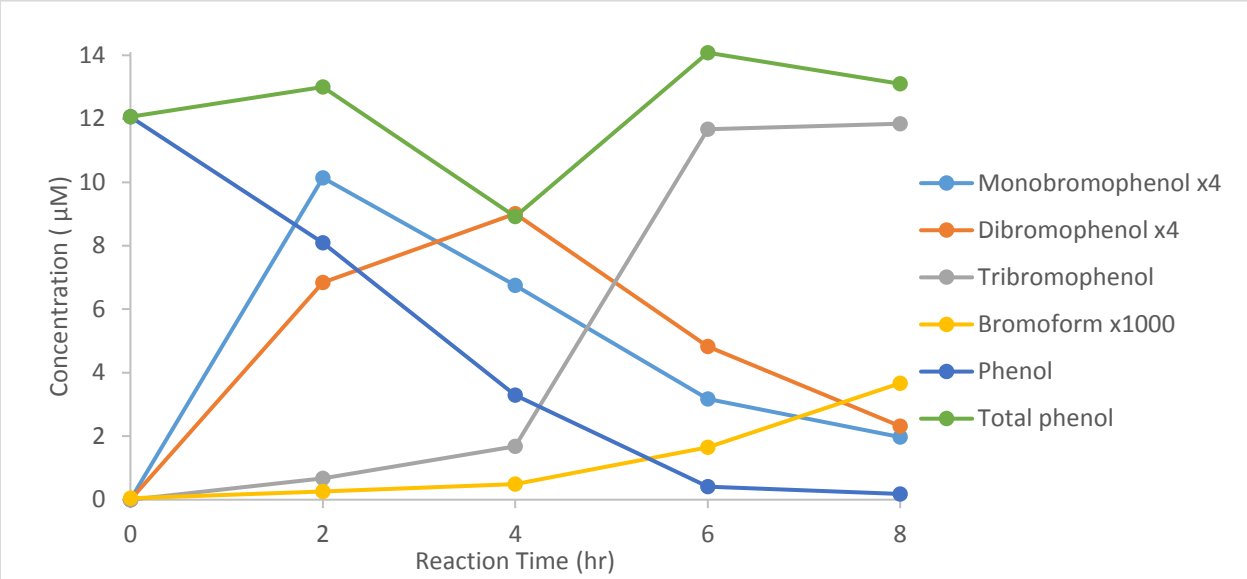

Figure 9: chemical species produced overtime by BPO. Monobromophenol include 2-bromophenol and 4-bromophenol. Dibromophenol include 2,4-dibromophenol and 2,6-dibromophenol. Presented monobromophenol and dibromophenol concentrations are scaled by a factor of 4 and concentration of bromoform scaled by a factor of 1000. Total phenol is the sum of concentration of phenol and all bromophenols measured.

4.2. Spatial distribution of bromoform concentration

In all four sampling dates, bromoform concentration in the Damariscotta River sampling area (depth = 10m) range from 0.1nM to around 0.4nM. Bromoform concentration generally decreases from the estuary channel (Station 1 and 2) to estuary mouth (Station 3) and open ocean (Station 4) (Fig.10). The consistently observed decrease in bromoform concentration from the estuary channel to the open ocean matches conclusions from the previous studies that algae beds are major sources of bromoform in coastal water bodies (Moore and Tokarczyk 1993; Carpenter and Liss 2000; Zhou et al. 2005; Ziska et al. 2013). In this case, the Damariscotta River, where

the local algae beds locate, is probably the major source of bromoform in the area. If the assumption is correct, δ^{81}Br of bromoform in Damariscotta River should reflect the combined effects of bromoform produced from different algae species from local algae beds.

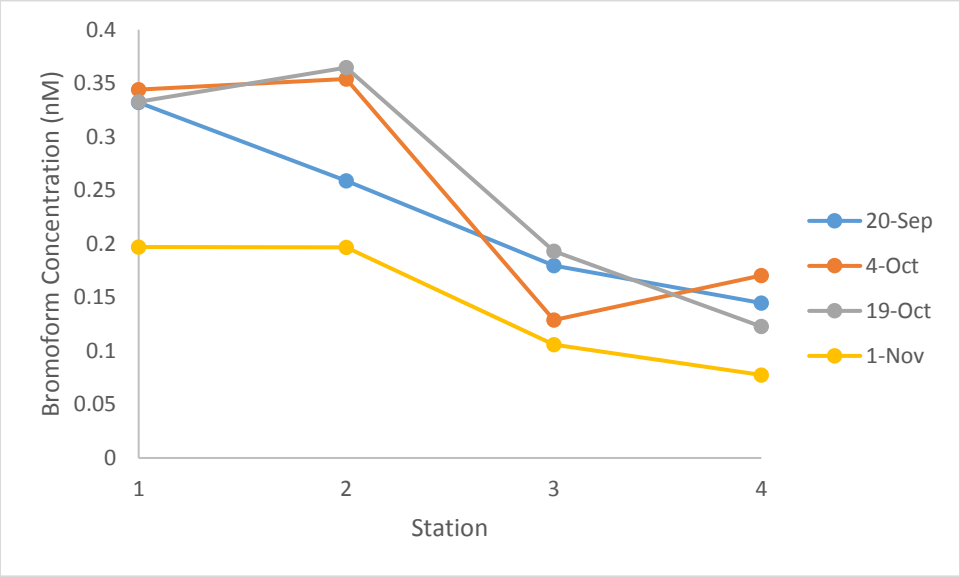

Figure 10: Spatial distribution of seawater bromoform concentration over four dates in 2016. All seawater samples were collected from 10m depth.

4.3. Bromine isotope analysis

4.3.1. Abiotic experiment

There is no statistically significant difference between in δ^{81}Br in bromoform produced by the 1min and 30min reactions (p-value = 0.4255) (Fig.11). The lack of significant difference supports the hypothesis that reaction time does not affect shift in Br isotope ratios in this experiment.

All p-values reported in this experiment are produced by Welch's t-test (see appendices). The reported δ^{81}Br of each trial was the average of six measurements. Two or three replicates (trials) were conducted for each condition. To compare if two conditions give different results, Welch's t-test was conducted by combining values of individual measurements on each of all (both) trials with the same condition. For example, to compare if 1min abiotic reaction gives a result different than 30min abiotic reaction, 6 individual measurement results from trial a and 6 results from trial b are combined (n=12, n=18 for the 30min group). An underlying assumption

of t-test is that all single values are independent to each other. I am aware that placing six measurement values from a single trial in one pool violates the assumption. The p-values from t-test are still reported as a part of data interpretation, acknowledging the potential flaw in the statistical method.

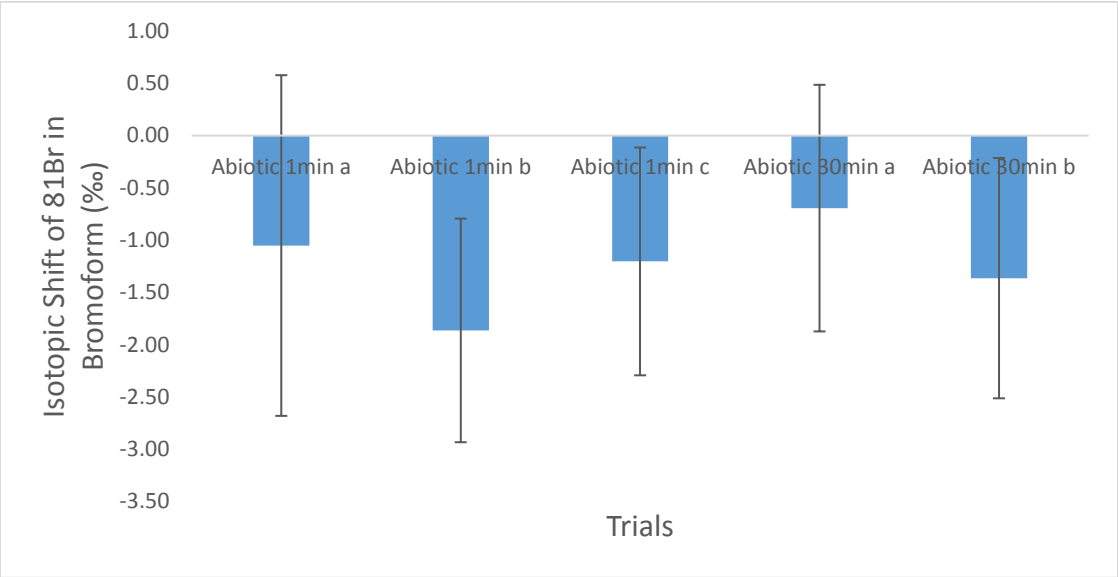

Figure 11: δ^{81}Br of bromoform produced from abiotic reaction with 1min and 30min reaction time. The error bar represents standard deviation of measurements (n=6).

4.3.2. Macroalgae incubation

^{81}Br enrichment decreases with H_2O_2 concentration (Fig.12). *A. Nodosum* incubation without H_2O_2 addition shows greater ^{81}Br enrichment than the group with 200nM H_2O_2 added (p-value = 0.04, n = 12). The group with 200nM H_2O_2 added shows greater ^{81}Br enrichment than the group with 2mM H_2O_2 added (p-value < 0.01, n = 12). No statistically significant difference is observed between ^{81}Br enrichment effects of *A. Nodosum* (without additional H_2O_2) incubation and *F. Vesculosis* incubation (p-value = 0.41, n = 12).

Depletion of bromide ion was not the cause of the small shift in ^{81}Br observed in the *A. Nodosum* incubation with 2mM H_2O_2. Wever et al. conducted an *A. Nodosum* incubation experiment with 2mM H_2O_2 and 100mM Br^- (119 times more concentrated than Br^- in this experiment) (1991; Table 7). The observed bromination rate was 63nmol phenol blue$\times g^{-1}$(wet weight)$\times h^{-1}$. Assuming the same reaction rate, 378nmol HOBr could be consumed in six hours (the incubation length of this experiment). Assuming algae biomass is 100g in each trial, the HOBr consumed should be 37.8μM. In the setup of this experiment, 840μM Br^- in 1L of water

gives 840μmol total Br^-, which exceeds the calculated Br^- consumption by more than twenty times. The bromination rate in this experiment should be lower than the value obtained by Wever et al., since their research shows that increased Br^- concentration drastically increases bromination rate.

As previously discussed, effects of H_2O_2 vary for different types of BPOs. The study conducted by Gelmand et al. suggests that A. Nodosum has two types of BPO (1985). In this case, higher H_2O_2 concentration may significantly increase bromination rate of a certain BPO, while inhibiting or having no effect on the other enzyme. If the BPO whose activity is significantly enhanced by H_2O_2 leads to a smaller apparent kinetic isotope effect (KIE), or a negative KIE, higher H_2O_2 concentration can lead to a smaller overall $\delta^{81}Br$ value.

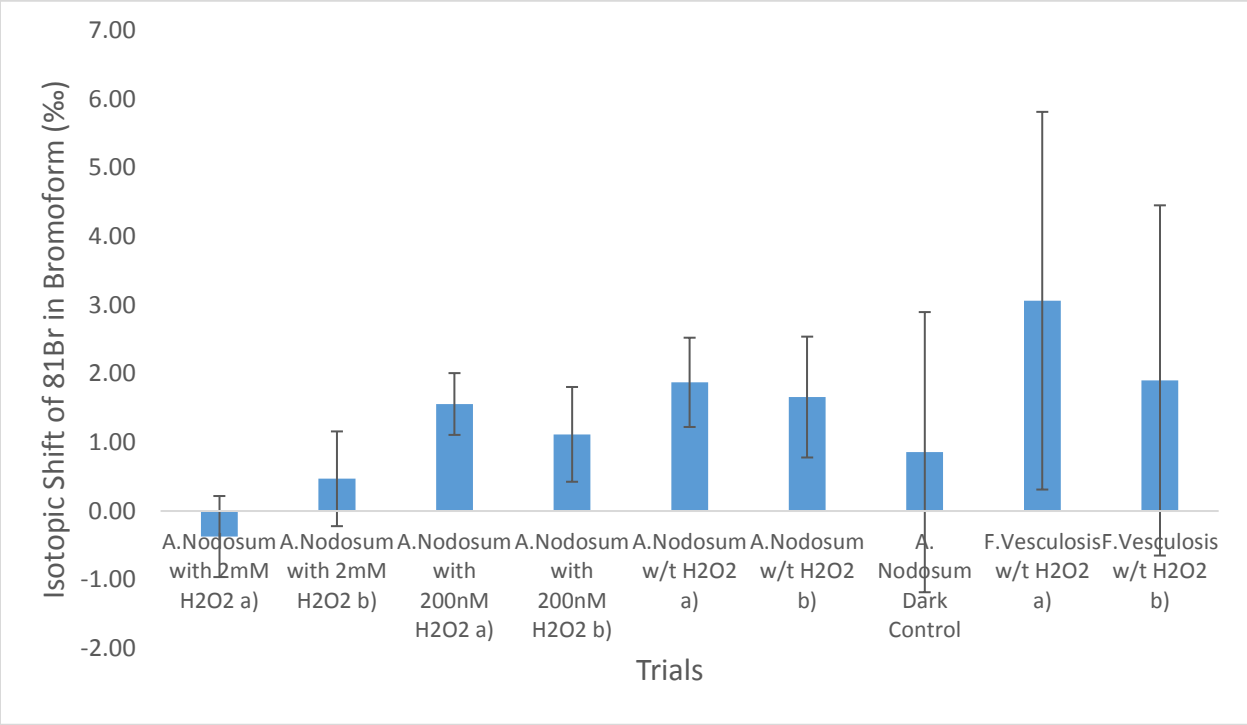

Figure 12: $\delta^{81}Br$ of bromoform produced from *A. Nodosum* and *F. Vesculosis* incubations. The error bar represents standard deviation of measurements (n=6).

4.3.3. Environmental bromoform Br isotope ratio

There is no statistically significant difference in shift of δ^{81}Br in bromoform from the channel and estuary mouth of the Damariscotta River (p-value = 0.82) (Fig.13).

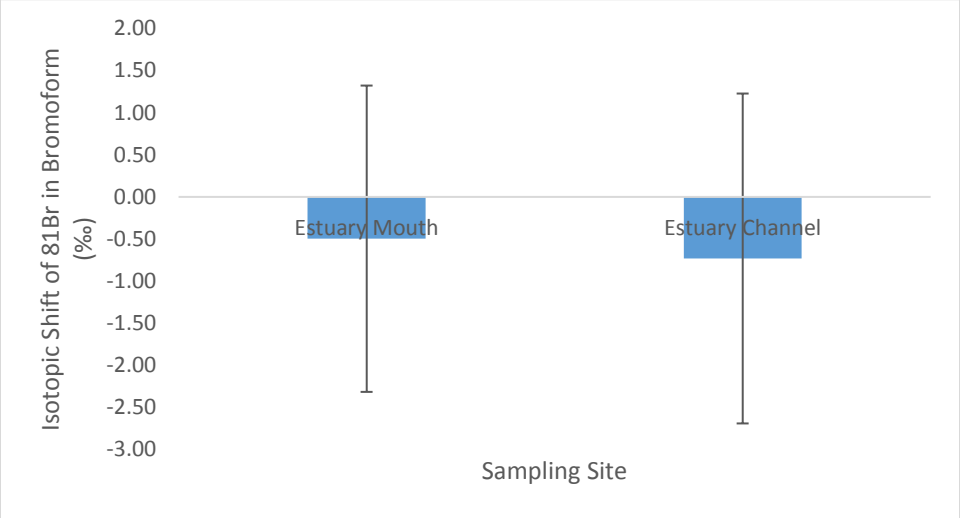

Figure 13: δ^{81}Br of bromoform from the channel and estuary mouth of the Damariscotta River. The error bar represents standard deviation of measurements (n=6).

4.4. Br isotope signature as a potential tool for determining sources of bromoform

There is a statistically significant difference between δ^{81}Br in bromoform produced from *A. Nodosum* and the abiotic experiment (p-value < 0.001). The difference between δ^{81}Br in bromoform from the *A. Nodosum* incubation and the estuary seawater is also statistically different (p-value < 0.001). There is no statistically significant difference between Br isotopic shifts in bromoform from the estuary water and the abiotic experiment (p-value = 0.24) (Fig.14).

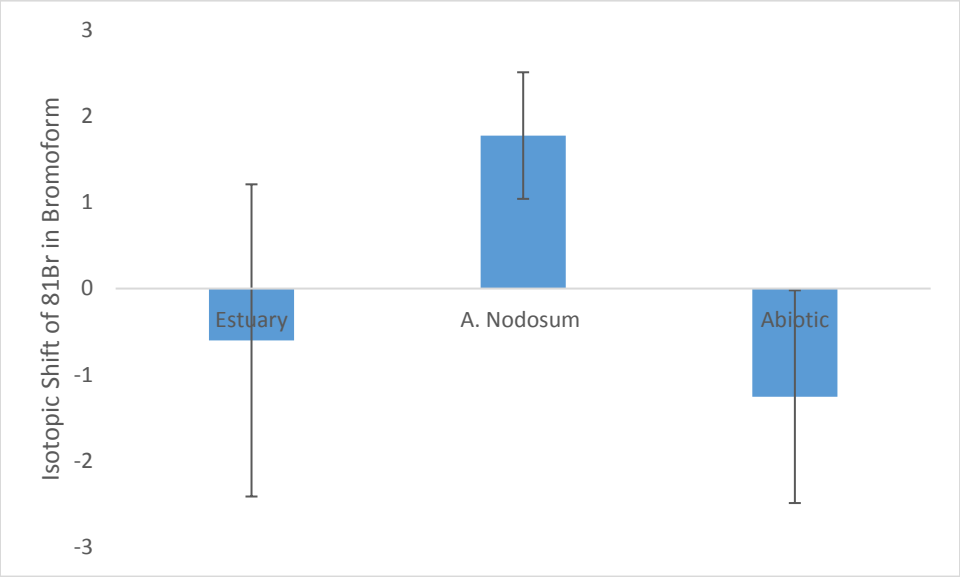

Figure 14: δ^{81}Br of bromoform from the Damariscotta River, the *A. Nodosum* incubation, and the abiotic experiment. The estuary channel and estuary mouth values were combined to produce the estuary value. The *A. Nodosum* value is calculated by combining the results from the two *A. Nodosum* incubation trials without H_2O_2 added. The abiotic value is calculated by combining all five trials (1min and 30min). The error bars represent standard deviation of measurements.

The Br isotope analysis technique using a GCqMS is capable of determining δ^{81}Br with relatively low sample concentration (3µl injection of 20µM sample, i.e. around 60pmol (15ng) injection for bromoform) with standard deviation of around ±1.5‰. The detection limit is similar to that of the existing GC-MC-ICPMS method (Gelman and Halicz 2009). The difference in Br isotopic shifts caused by *A. Nodosum* and the abiotic reaction suggests that measuring δ^{81}Br with GCqMS has a potential in differentiating bromoform with different sources (Fig.14).

The bromoform δ^{81}Br in seawater is expected to reflect the combination of KIE of all sources and sinks of the compound in the system. The observation that the bromoform δ^{81}Br in seawater does not match that of the *A. Nodosum* and *F. Vesculosis* suggests that there are other

sources or sinks in the system influencing the overall value. Other sources of bromoform include other macroalgae and phytoplankton (Table 12). To the best of my knowledge, there is no water disinfection facility along the Damariscotta River, hence the anthropogenic sources cannot contribute to the environmental bromoform in the sampling sites. The several sinks include air-sea exchange, photodegradation, and microbial turnover (Table 12).

Bromoform production by other macroalgae may influence the Br isotope ratio (Table 12). Both sugar kelp (*L. Digitata*) and sea lettuce (*U. Lactuca*) are other two major macroalgae species in the Damariscotta River. According to the study by Nightingale et al., both species show higher bromoform production capacity than *A. Nodosum* (Table 11). Therefore, if bromoform produced by other productive macroalgae has different isotopic shifts, the net bromine isotopic shift can be influenced.

A study by Zakon et al. reported an inverse Br isotope effect up to 5.1‰ associated with C-Br bond cleavage caused by photodegradation of bromophenol, i.e. more ^{79}Br than ^{81}Br is involved in photodegradation (2013). Hence photodegradation would contribute to further enrichment of ^{81}Br in bromoform in seawater. The process does not explain the less ^{81}Br isotopic shift of bromoform in the seawater than that from the *A. Nodosum* and *F. Vesculosis* incubation (Table 12).

The overall effect of phytoplankton is relatively unclear (Table 12). A study on bromoform distribution in an upwelling zone near Iberian Peninsula reported a positive correlation between chlorophyll a (a proxy for phytoplankton) and bromoform concentration (Raimund et al. 2011). The correlation suggests that phytoplankton can be a major bromoform producer in a water body. The highest reported chlorophyll-related bromoform concentration was 0.03nM, which suggests that bromoform production from phytoplankton might lead to 0.03nM bromoform in a coastal environment. The observed bromoform concentrations from field sampling of this study range from 0.1nM to 0.35nM, 0.03nM can be a relatively significant proportion (Fig.10). Therefore, the upper bound from the Raimund et al. (2011) study is a relatively significant proportion. However, while chlorophyll a represents phytoplankton abundance, only certain phytoplankton species produce bromoform. Therefore, the specific species in the local area and their relative abundance matter. To better understand the potential effect of phytoplankton, a specific local study is needed.

A study on brominated phenol suggests that bacterial degradation causes depletion of remaining ^{81}Br (Bernstein et al. 2012). Hence the effect of bacterial degradation can qualitatively explain the observed low ^{81}Br enrichment factor (Table 12). However, the process is unlikely to influence the overall Br isotopic composition in the coastal environment, due to the relatively small biomass of bacteria. Goodwin et al. estimated the contribution of bacterial degradation on eliminating bromoform produced by macroalgae in the coastal environment in California (1997b). They concluded that microbial turnover can eliminate at most 1/100 of bromoform produced by giant kelp (*Macrocystis pyrifera*). The reported bromoform formation rate of giant kelp is 171ng bromoform×g^{-1} (wet weight) ×day-1 (versus 430ng bromoform×g^{-1} (dry weight) ×day^{-1} of A. Nodosum) (Table 11). Therefore, the relative effect of microbial turnover is probably even lower in the Damariscotta River, where more productive species, such as *A. Nodosum*, *L. Digitata*, and *U. Lactuca* are dominant.

Table 11: Bromination and bromoform production rate of different macroalgae species. All data are collected from published peer reviewed studies. Units are not corrected due to the nature of different studies.

Species	Bromination or bromoform production rate	Condition
A. Nodosum **(Wever et al. 1991)**	63nmol phenol blue×g^{-1}×h^{-1} (wet weight)	2mM H_2O_2 and 100mM Br^-
A. nodosum **(Gshwend et al. 1985)**	4500ng (127ng – 12000ng) bromoform×g^{-1}×day^{-1} (dry weight)	Seawater incubation
A. nodosum **(Nightingale et al. 1991)**	470ng bromoform×g^{-1}×day^{-1} (dry weight)	Seawater incubation, west coast of Scotland
F. vesiculosis **(Wever et al. 1991)**	6 nmol phenol blue ×g^{-1}×h^{-1} (wet weight)	2mM H_2O_2 and 100mM Br^-
L. digitata **(Nightingale et al. 1991)**	780 and 1100 ng ×g^{-1}×day^{-1} (dry weight, two experiments conducted)	Seawater incubation, west coast of Scotland
L. digitata **(Wever et al. 1991)**	123 nmol phenol blue ×g^{-1}×h^{-1} (wet weight)	2mM H_2O_2 and 100mM Br^-
L. saccharina **(Wever et al. 1991)**	41 nmol phenol blue ×g^{-1}×h^{-1} (wet weight)	2mM H_2O_2 and 100mM Br^-
U. lactuca **(Nightingale et al. 1991)**	1100 ng ×g^{-1}×day^{-1} (dry weight)	Seawater incubation, west coast of Scotland
Macrocystis pyrifera **(Goodwin et al. 1997)**	171ng bromoform×g^{-1}×day^{-1} (wet weight)	

Table 8: Potential sources and sinks of bromoform in the Damariscotta River, their contribution to the overall seawater bromoform $\delta^{81}Br$, and the qualitative bromoform production (for sources) and depletion (for sinks) rates. The explanation power is assessed by evaluating how possible each source and sink can contribute to the observed difference between the Br isotope ratios of bromoform in the ocean and from *A. Nodosum*. The isotope effect does not mean kinetic isotope effect in this case. For example, ^{81}Br photodegrades more slowly than ^{79}Br (inverse KIE), but the relatively low photodegradation rate of ^{81}Br would mean enrichment (+) of ^{81}Br in remaining bromoform.

Sources and Sinks	Isotope Effect	Production/Depletion Rate	Explanation Power
L. Digitata	Unclear	High (Nightingale et al. 1991)	May explain the observation
U. Lactuca	Unclear	High (Nightingale et al. 1991)	May explain the observation
Phytoplankton production	Unclear	Unclear (Raimund et al. 2011)	May explain the observation
Air-sea exchange	- (Preliminary pure bromoform evaporation tests)	Unclear	May explain the observation
Water disinfection	- (This study)	N/A	Does not explain the observation
Photodegradation	+ (Zakon et al. 2013)	Unclear	Does not explain the observation
Bacterial degradation	- (Bernstein et al. 2012)	Low (Goodwin et al. 1997)	Does not explain the observation

Based on the current studies that I am aware of, no conclusive explanation can be made on the observed difference between the Br isotope ratio of bromoform in seawater and that from *A. Nodosum*. The effect of remaining biological sources, including both macroalgae and phytoplankton need to be studied. Most studies suggest that macroalgae are the predominant source of bromoform in coastal environments. Therefore, effects of other macroalgae in Damariscotta River need more intensive studies.

4.5. Limitation and future studies

Low bromoform concentration in seawater (0.3nM) leads to relatively large uncertainty in measured seawater bromoform isotope ratio. 5L of seawater was extracted for Br isotope

analysis, and 15pmol (3.8ng bromoform) of bromoform was injected into the GCqMS. Assuming the same seawater bromoform concentration, increasing extraction volume by four times (20L seawater) would increase amount of bromoform injected to 60pmol, reducing the uncertainty to around ±1‰. Preliminary analytical tests (not presented) suggest that injecting 300pmol (100L seawater given the same bromoform concentration) of bromoform would further reduce the uncertainty to around ±0.5‰. For future attempts, a new bromoform extraction technique is required. Developing a purge and trap system that operates in a scale of 100L will greatly improve measurement precision.

Kinetic isotope effects (KIE) of other sources and sinks of bromoform in Damariscotta River need to be studied. Macroalgae such as *Laminaria Digitata, Ulva Lactuca*, and bromoform-producing phytoplankton, and air-sea exchange can be major contributors to seawater bromoform isotope ratio. Further study on phytoplankton should include i) identification of the bromoform-producing species, ii) abundance dynamics, and iii) the KIE that these species incur.

The potential effect of H_2O_2 concentration on the overall isotope signature of bromoform produced by *A. Nodosum* is worth attention. A change in H_2O_2 by 200nM (the scale of change in this experiment) is likely in coastal environment in different location, depth, and weather conditions. The specific effects of H_2O_2 on the KIE of bromoform production by *A. Nodosum* and other species, as well as the mechanism for the different KIE, are a potential topic for further exploration. A better understanding on the effects of H_2O_2 may provide information on activity of certain types of BPO. At the same time, the effects of H_2O_2 may also influence environmental isotope ratios.

This study only examined bromoform isotope signature in Seawater on May 31, 2017. Several studies indicate that bromoform production by macroalgae has obvious seasonal pattern (Gshwend et al. 1985). Therefore, bromoform isotope ratio in seawater may change as the macroalgae input differs. With further understanding of kinetic isotope effect of different processes in Damariscotta River, studying seasonal patterns of Br isotope ratio in seawater bromoform may help us better understand the seasonal change of predominant sources and sinks of bromoform in the local environment.

CONCLUSION

First, this study demonstrates the capacity of measuring stable Br isotope ratio of bromoform using a GCqMS with injection amount of 60pmol bromoform and standard deviation of around ±1‰. Second, this method can successfully measure δ^{81}Br of bromoform produced in lab experiments. *A. Nodosum*, *F. Vesculosis*, and bromination reaction between HOBr and phenol all produce bromoform with Br isotope shift that is measurable by GCqMS. Under the same incubation conditions, Br isotope signatures of bromoform produced by *A. Nodosum* (1.8±0.7‰) and *F. Vesculosis* (2.4±2.6‰) are not statistically different. The bromination reaction between HOBr and phenol leads to depletion of ^{81}Br (-1.3±1.2‰). Third, we were able to compare bromoform δ^{81}Br from these incubation experiments with values measured in seawater, where bromoform concentrations are only 0.03nM. The measured Br isotope shift in Damariscotta River is -0.6±1.8‰.

The spatial pattern of bromoform distribution suggests that macroalgae in the estuary channel are the major sources of bromoform in the study area. Effect of several other sources and sinks of isotope ratio of bromoform are not studied. Examples include macroalgae species such as *L. Digitata* and *U. Lactuca* and sinks such as photodegradation and air-sea exchange. It is worth noticing that H_2O_2 may influence enzyme productivity of certain BPO and the overall isotope signature of bromoform produced.

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APPENDICES

Table 2: A list of material used in experiments of this project and their product information

Material	Product Information
ANALYTICAL STANDARDS	
Bromoform	Aldrich 99%
2-bromophenol	Fluck 99.5%
4-bromophenol	Aldrich 99%
2,4-dibromophenol	Acros 99%
2,6-dibromophenol	Aldrich 99%
2,4,6-tribromphenol	Supelco 99%
1,3,5-Trichlorobenzene	Supelco 99.9%
SOLVENTS	
Dichloromethane	BDH HyperSolv ChromaNorm
Hexane	BDH HyperSolv ChromaNorm
Methanol	BDH HyperSolv ChromaNorm
REAGENTS	
H_2O_2	Sigma-Aldrich 30%
Bromoperoxidase	From Corallina officinalis, lyophilized powder, >100u/mg protein
Phenol	Sigma-Aldrich >99.5%
Sodium sulfite	Sigma 98%
HCl	BHD 34-37%
KH_2PO_4	Sigma-Aldrich >99%
K_2HPO_4	Sigma-Aldrich ACS reagent >98%
OTHER MATERIAL	
H_2O_2 test stripe	Merck MQuant Peroxide Test 0-25 mg/L H2O2
Na_2SO_4 (drying agent)	VWR ACS reagent grade

Table 4: Weight of individual macroalgae used for incubation. Both dry weight and wet weight are reported.

Experiment	Dry Weight (g)	Wet Weight (g)
***A. Nodosum* w/t H_2O_2**	47.1	158.2
***A. Nodosum* w/t H_2O_2**	37.5	125.1
***A. Nodosum* 200nM H_2O_2**	25.1	93.3
***A. Nodosum* 200nM H_2O_2**	20.3	74.4
***A. Nodosum* 2mM H_2O_2**	24.6	94.6
***A. Nodosum* 2mM H_2O_2**	26.1	97.3
***A. Nodosum* dark control**	29.6	98.7
***F. Vesculosis* w/t H_2O_2**	19.8	92.4
***F. Vesculosis* w/t H_2O_2**	14.9	111.9

Statistical Test

I conducted Welch's t-test with the "t.test" function of R (version 3.4.3). The script used for the t-test was:

1. To compare if results from two groups were different:

t.test(variable1, variable2, alt = "two.sided")

2. To compare if one result (variable 1) is larger than the other (variable 2):

t.test(variable1, variable2, alt = "greater")

Citation:

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.