HALOGENATED 1'-METHYL-1,2'-BIPYRROLES (MBPs) IN THE NORTHWESTERN ATLANTIC

By

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Submitted to the MIT/WHOI Joint Program in Oceanography on June 26, 2009, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the field of Chemical Oceanography

THESIS ABSTRACT

Halogenated 1′-methyl-1,2′-bipyrroles (MBPs) are a distinctive class of marine organic compounds. They are naturally produced, they have a unique carbon structure, they are highly halogenated, and they bioaccumulate in upper trophic levels. MBPs share many characteristics with persistent organic pollutants (POPs), and may prove to be useful natural analogues for these anthropogenic compounds. Further, their unique structure suggests that their biosynthetic organism(s) may have new genes to add to current knowledge of biosynthetic chemistry. The objectives of this dissertation were to further clarify the environmental distribution of MBPs, to examine whether MBPs biomagnify, and to investigate possible origins of these compounds through their stable nitrogen isotopic signatures.

Results from these investigations have shown that over 40 highly brominated MBP congeners are present in marine mammals, fish, and squid from the Northwestern Atlantic Ocean. The most abundant MBPs do appear to biomagnify through the food web to reach the concentrations observed in marine mammals. This additional evidence affords greater confidence in the use of MBPs as natural analogues for POPs. However, differences in the environmental chemistry of MBPs and anthropogenic compounds are also evident, and may be due to these compounds' different origins, or to the capacity of degradative enzymes to act upon them. Finally, compound-specific nitrogen isotope analyses on MBPs isolated from dolphin blubber show that these compounds are dramatically enriched in ^{15}N relative to other biosynthetic organic compounds. This enrichment is likely a signal imparted during biosynthesis, and may assist in elucidating the organism(s) and mechanism(s) responsible for the biosynthesis of MBPs.

To my grandmother,

Rachel B. Watkins,

whose strength, independence, and
courage have always inspired me.

And, to my parents,

Diane and Robert Smith,

whose love and support make all
things possible.

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CHAPTER 1

An introduction to halogenated 1′-methyl-1,2′-bipyrroles (MBPs)

History

Ten years ago, *Environmental Science & Technology* published a research article suggesting that a highly halogenated contaminant isolated from seabird eggs, tetrabromodichloro-1,1′-dimethyl-2,2′-bipyrrole, was naturally produced [1]. The natural origin of this compound was confirmed in 2004 [2], and provided the first evidence that halogenated natural products (HNPs) bioaccumulate in upper trophic levels in the same manner as anthropogenic contaminants. Since then, a few other classes of HNPs have been shown to bioaccumulate in these higher trophic level organisms. One such class is the halogenated 1′-methyl-1,2′-bipyrroles (MBPs) [3, 4].

Investigations into MBPs began in 1999 when an unknown contaminant with the molecular formula $C_9H_3Cl_7N_2$, initially called Q1, was detected in marine samples [3]. Shortly thereafter it was determined that Q1 was identical to compounds detected but unidentified in samples dating back to the 1980s [5]. When the structure was determined in 2002, as an *N,C1*-linked bipyrrole with chlorine atoms occupying all ring carbons (Figure 1) [6], it had been detected in a variety of samples worldwide, including seabird eggs, marine mammal blubber,

human breast milk (from women with diets rich in blubber) and Antarctic air [3, 7-10]. No compounds with this structure had ever been commercially produced, and it was only found in marine samples; thus, MBPs were believed to be naturally produced in marine ecosystems. The organism (or organisms) responsible for MBP biosynthesis has yet to be identified.

Although the perchlorinated MBP congener was detected in 1999, brominated congeners were not detected until 2006 [4]. The highly brominated heptabromo-, hexabromochloro-, hexabromo-, and pentabromochloro-1′-methyl-1,2′-bipyrroles (MBP-Br_7, MBP-Br_6Cl, MBP-Br_6, and MBP-Br_5Cl, respectively) were found to be more abundant than the perchlorinated congener, Q1, in the blubber of marine mammals from the Northwestern Atlantic [4, 11]. The lag between the detection of Q1 and the brominated congeners likely resulted from a combination of the geographic distribution and the chemical stability of the different congeners. The procedure for the initial isolation of Q1 used sulfuric acid to remove the lipid matrix [3], conditions under which the highly brominated congeners were unstable [4]. Further, much of the work on Q1 was accomplished in the Pacific and Southern Oceans, locations where highly chlorinated MBPs dominate the MBP distribution [12]. With the detection of the brominated congeners in the Atlantic, it became clear that MBPs consist of a family of compounds that have the same carbon backbone but different halogenation patterns; it was not yet proven that they were produced naturally.

As of 2006, the evidence for MBPs as natural products consisted of a number of observations [4, 5]. MBPs had no known industrial source, either intended or inadvertent (e.g., dioxins are an unintended byproduct of industrial processes [13]). MBPs have only been found in marine samples; a critical point, since all anthropogenic compounds ultimately have terrestrial sources. MBPs shared structural similarities to other marine HNPs, specifically the bipyrrole structure. As mentioned previously, the natural product found to bioaccumulate in higher trophic level organisms was a halogenated bipyrrole [1]. A marine bacteria, *Pseudoalteromonas luteoviolacea* (originally classified as a *Chromobacterium*), is known to produce a perbrominated bipyrrole [14, 15]. A natural source for MBPs was confirmed through their detection in whale oil archived prior to the onset of industrial halogenation [16].

Structure and Properties

MBP structure consists of two nitrogen-containing, aromatic, five-membered rings, with up to seven halogens (either bromine or chlorine) on the ring carbons (Figure 1). This structure results in two properties of MBPs that make them environmentally relevant: persistence and lipophilicity. MBPs have high octanol-water partition coefficients (K_{ow}), as estimated from their molecular structure (log K_{ow} = 6.6-8.3 [16]). Kow values use octanol as a representative of organic matrices to predict the partitioning of chemicals between an organic

phase and water. MBPs are aromatic, and relatively non-polar and non-volatile, characteristics that result in MBPs concentrating in organic matrices, like blubber, rather than aqueous reservoirs or air. The persistence of these molecules partially stems from their high K_{ow} values. When partitioned into storage tissues, such as blubber, there is limited opportunity for metabolism [17]. Their persistence is also due to their high degree of halogenation. Halogens form strong bonds with carbon because of their high electronegativity. Additionally, the lipophilicity of organic molecules tend to increase with the number of halogens. This is due to halogens' large size and their low affinity for forming hydrogen bonds with water [18]. Thus MBPs, which are concentrated in blubber and highly halogenated, have little opportunity for degradation and are likely difficult to metabolize.

Relevance

When initially discovered, MBPs were considered remarkable for two reasons: their high degree of halogenation and the unusual *N,C1*-linkage of the pyrrole moieties. They remain unusual in these characteristics among known natural products today. These characteristics are particularly relevant to researchers studying the cycling and fate of persistent organic pollutants and those developing new pharmaceutical therapeutics.

Relevance: Natural Analogues to POPs

Persistent organic pollutants (POPs) are a group of chemicals defined (and regulated) under the United Nations Environment Program's Stockholm Convention on Persistent Organic Pollutants (2001) as compounds that "…possess toxic properties, resist degradation, bioaccumulate and are transported, through air, water and migratory species, across international boundaries and deposited far from their place of release, where they accumulate in terrestrial and aquatic ecosystems…" [13] Two chemical characteristics that define POPs, halogenation and lipophilicity, are shared by MBPs. These similarities are potentially useful when considering the long term cycling of halogenated compounds. As natural products, MBPs likely evolved with their ecosystem, and have presumably been 'in production' for a very long time. Anthropogenic POPs have only been produced and emitted into the environment in recent decades, and thus their long-term fate is unclear. By using MBPs and other HNPs as model compounds, we can more clearly understand the routes and reservoirs important to the fate of POPs in the environment. For example, by recognizing which structural moieties are enzymatically degraded in natural products, and which are removed through abiotic processes (such as photodegradation or surface-mediated catalysis), we can better predict the fate of pollutants containing the same or similar structures. Additionally, HNPs that only have a marine source may help gauge the seafood-based contribution to

human uptake of POPs. This pathway is a concern, but its relative contribution is difficult to assess, as POPs are present in both terrestrial and marine food sources. Transfers of marine HNPs into human tissues have previously been demonstrated [9] and may offer a method for disentangling the two POP vectors.

The utility of MBPs to act as natural analogues for POPs depends on a thorough understanding of the environmental distribution of MBPs and the controls on their cycling and fate. An important difference between these types of compound is their geographic origin. Although POPs have only been produced on land, POPs are ubiquitous pollutants and are now present in both terrestrial and marine ecosystems [13]. Thus, they have multiple inputs to the marine ecosystem, e.g., rivers, dust, aerosols, and freely diffusible gasses. MBPs are natural products that have only been detected in marine samples or samples closely tied to the marine food web [5, 12, 19]. It is likely that they are made by a specific organism or group of organisms, and thus their source is much more geographically limited than that of the POPs. From this source difference, it is likely that MBPs and POPs enter the marine food web differently. This may impact the availability of the compounds for bioaccumulation, biomagnification, and biotic and/or abiotic degradation. Therefore, their sources may ultimately result in different distributions in marine biota.

For use as natural analogues to POPs, it is also necessary that the mechanisms known to be important in POP cycling are also relevant to MBPs.

An important control on POP cycling is biomagnfication, which is the increase of the lipid-normalized concentration of a contaminant with trophic level [20]. Previous work has established that biomagnification can occur for hydrophobic compounds with octanol/water partition coefficients exceeding 10^4 [21]. Additionally, biomagnfiying compounds must be persistent to avoid any appreciable abiotic or enzymatic degradation. Contaminant concentrations increase in each trophic level (Figure 2) because persistent compounds are not significantly metabolized during digestion and contaminant fugacities increase as prey is digested [22]. This increase in fugacity during digestion explains how contaminants are transferred against the apparent thermodynamic fugacity gradient.

It is important to note that biomagnification and bioaccumulation are different concepts and are not interchangeable. Bioaccumulation is equilibrium partitioning between biota and the surrounding environment, and can occur through inhalation, ingestion, and/or absorption [18]. Biomagnification acts through ingestion and results in top predators with contaminant concentrations that are much higher than what would be expected based on equilibrium partitioning between the surrounding environment (i.e., water, soil, or sediment) and biotic tissues [18].

Biomagnification is a concern for POPs. Indeed, the high concentrations of POPs in top predators are partially responsible for the concerns and strict

regulation of these compounds [13]. As of 2006, when this study began, it was unclear whether the high concentrations of MBPs found in marine mammal blubber resulted from biomagnification. Based on the concentrations observed, the chemical properties of MBPs, and the detection of MBPs in one sample of a marine mammal prey species [16], biomagnfication was suspected. In order to show that biomagnification occurs, however, the lipid-normalized concentration increase with trophic level must be demonstrated.

Relevance: Pharmaceutical Therapeutics

The development of pharmaceutical agents began with the isolation of bioactive compounds from environmental samples. For example, quinine was 'discovered' by western medicine by noting that indigenous populations of South America used cinchona bark (a source of quinine) to treat fevers [23]. Although drug development turned away from natural products in recent decades, there is a renewed interest in naturally produced compounds for novel therapeutic agents [24]. This interest has focused significant attention on marine sources. Marine sources were, until recently, largely ignored, and in this brief period of renewed interest they have already proven a rich source of novel bioactive compounds [25]. One route of recent drug development is the use of biosynthetic pathways to produce or to assist in the production of complex chemical structures [26]. The novel pyrrole linkage and the highly halogenated

nature of MBPs suggest that the organism(s) responsible for their biosynthesis may contribute new mechanisms to our growing 'library' of biosynthetic chemistry. Additionally, many organisms that produce secondary metabolites (compounds not required for primary metabolism, but which benefit the producing organism) synthesize more than one type of compound [27, 28]; for example, the marine bacterium that produces 3,3′,4,4′,5,5′-hexabromo-2,2′-bipyrrole also produces other brominated pyrrole structures and biosynthetically-unrelated polysaccharides [14, 29]. Identification of the producing organisms of MBPs may result in the discovery of yet more novel bioactive compounds. In order to fully realize the therapeutic potential of MBPs, we must identify their biosynthetic origins.

Although the producer(s) of MBPs has yet to be identified, there are organisms known to produce structurally similar compounds. The *N,C1*-linkage in MBPs has not been previously observed linking two pyrrole rings. However, there are a few biosynthetic examples of the *N,C1*-linkage between other five-membered, nitrogen-containing, bicyclic ring systems (Figure 3). Two lily species are known to produce bicyclic systems with this linkage, *Lilium hasnonii* [30] and *Lilium candidum* [31]; *Wasabi japonica* produces a bi-indole system linked through its 5-membered rings [32]. In a broader search of all chemical literature, the motif is also present in *N*-fused porphyrins [33], azofullerenes [34],

pyrrolodiazines [35], compounds for drug development [36], and pyrrollic sensors [37].

Two additional bipyrrole types have been described in the literature that have *C1,C1*- and *N,C2*-linkages (Figure 3). The *C1,C1*-linked bipyrroles include another class of biomagnifying marine natural products, halogenated 1,1′-dimethyl-2,2′-bipyrroles [38]. Although their origin is unknown, a halogenated bipyrrole of similar structure (the aforementioned 3,3′,4,4′,5,5′-hexabromo-2,2′-bipyrrole) has been isolated from a marine bacterium [14] now identified as *Pseudoalteromonas luteoviolacea* [15]. A group of gram-positive bacteria commonly known as actinomycetes also produce bipyrroles: the *C1,C1*-linked prodigiosin series of natural products are produced by *Streptomyces* species [39, 40], and marine actinomycete strain CNQ-418 produces the *N,C2*-linked marinopyrroles [41].

In addition to the unusual carbon backbone, MBPs are halogenated, and their halogenation appears geographically dependent: chlorinated congeners dominate the MBP distributions in the Pacific and Southern Oceans {Vetter, 2007 #422}, while highly brominated congeners dominate in the Northwestern Atlantic {Teuten, 2006 #35;Pangallo, 2008 #454}. (Thus far no iodinated MBPs have been reported in any environmental samples.) This halide-specificity suggests that the halogenases involved in MBP biosynthesis may also have specific geographic distributions. Of the three classes of known halogenases that

target aromatic rings, vanadium-dependent haloperoxidases have shown the greatest halide specificity {Blasiak, 2009 #873}. However, both heme-dependent haloperoxidases and flavin-dependent halogenases are also capable of both brominating and chlorinating aromatic rings, and many questions remain regarding the halide specificity of the halogenase enzymes {Blasiak, 2009 #873}.

Summary

When the research for this dissertation was initiated, in the summer of 2006, we knew of 7 MBP congeners that were present in the blubber of marine mammals [5, 16]: the perchlorinated Q1, the perbrominated MBP-Br_7, one congener of MBP-Br_6Cl, one congener of MBP-Br_6, and three congeners of MBP-Br_5Cl. We knew that these compounds were natural by virtue of their presence in whale oil archived prior to the onset of industrial halogenation [16]. Q1 was the most abundant MBP detected in samples from Australia and the Southern Ocean, while the highly brominated congeners dominated the MBP distribution in samples from the Northwestern Atlantic [4, 11, 16]. MBPs had also been identified in squid [4] and the breast milk of women who had diets rich in blubber [9], suggesting that MBPs could undergo trophic transfer. However, it was not yet certain whether they biomagnified.

To facilitate discussion of the MBPs, Vetter et al. [12, 42] suggested a numbering system for the MBPs based on the system developed for the

anthropogenic polychlorinated biphenyls (PCBs) [43]. In this system the perchlorinated Q1 is given the acronym MBP-79, and the perbrominated MBP-Br_7 is termed Br_7-MBP-79. This is the nomenclature that will be used for these two compounds for Chapters 4-6. Chapters 2 and 3 are already published [19, 44], and use MBP-Cl_7 and MBP-Br_7 for MBP-79 and Br_7-MBP-79, respectively. For further details on nomenclature please see Chapter 3.

Objectives

The objectives of this dissertation were to further clarify the environmental distribution of MBPs, to determine whether MBPs biomagnify, and to investigate possible origins of these compounds through their stable nitrogen isotopic signatures. The following four chapters each delve into one or more of these areas. Chapter 2 dramatically expands the number of MBPs present in blubber, most of which were detected at trace abundances. A comparison of analytical techniques for MBP analysis (gas chromatography/electron capture negative ion mass spectrometry and comprehensive two-dimensional gas chromatography/time of flight mass spectrometry) is also presented in this chapter. To further explore the environmental distribution of MBPs, Chapter 3 describes the MBP content of blubber and liver from a wide variety of marine mammals. (Additional information regarding the lipid content of the samples from this chapter is

included in the Appendix to the thesis.) This chapter presents strong evidence for MBP biomagnification by comparing MBP distribution patterns with those of a PCB known to biomagnify. The comparisons also illustrate differences in the cycling of the natural MBPs and anthropogenic PCB. These themes are further explored in Chapter 4, which demonstrates that lipid-normalized MBP concentrations do indeed increase with trophic level. Finally, Chapter 5 presents the measurement of compound-specific nitrogen isotope ratios for four individual MBPs and discusses the implications of the remarkable enrichment that was found. Chapter 6 summarizes the results and suggests future directions for research on MBPs.

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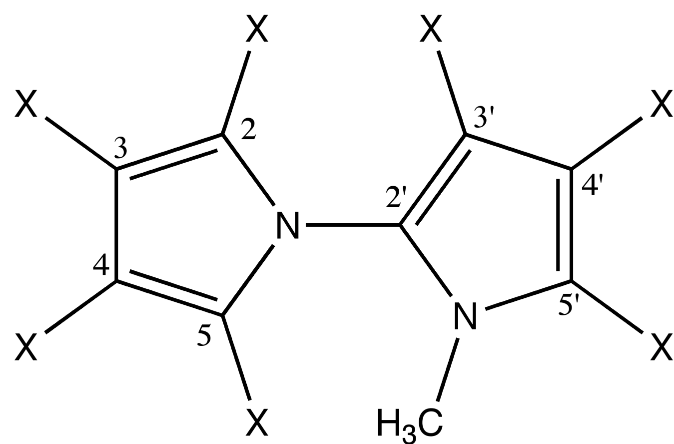

Figure 1. The general structure of MBPs, where X represents Br, Cl or H.

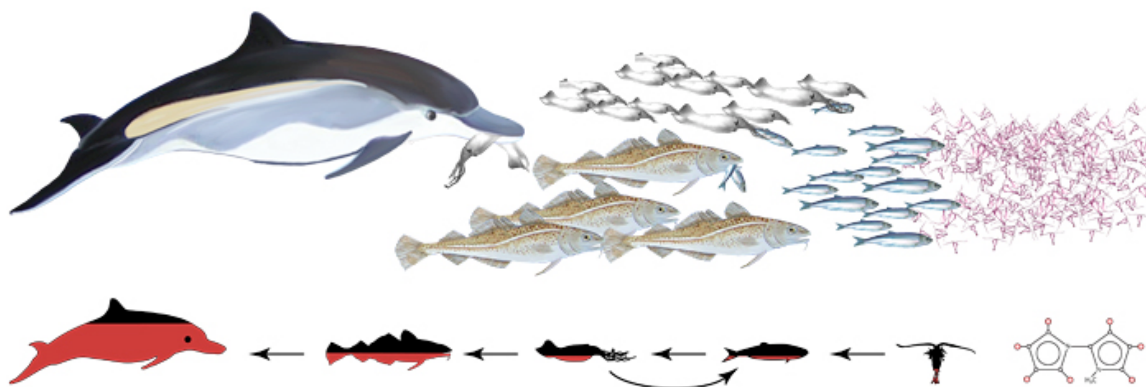

Figure 2. A visual representation of biomagnification of MBPs. The concentration of MBPs in each trophic level is represented in red. (E. Paul Oberlander, WHOI)

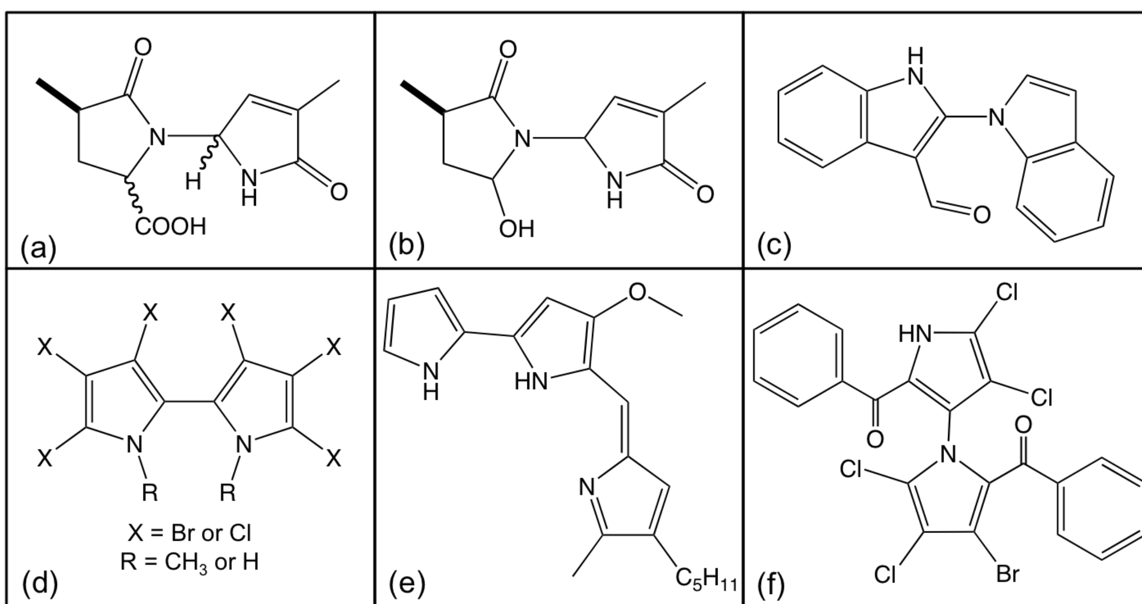

Figure 3. Structures of compounds similar to the MBPs: (a) 1-(2,5-dihydro-4-methyl-5-oxo-1H-pyrrol-2-yl)-4-methyl-5-oxo-proline isolated from *Lilium hansonii* [30]; (b) 1,5-dihydro-5-hydroxy-3,4'-dimethyl-[1,2'-Bi-2H-pyrrole]-2,5'(1'H)-dione isolated from *Lilium candidum* [31]; (c) [1,2'-bi-1H-indole]-3'-carboxaldehyde isolated from *Wasabi japonica* [32]; (d) X = Br or Cl, R = CH_3: halogenated 2,2′-dimethyl-1,1′-bipyrrole [45]; X = Br, R = H: 3,3′,4,4′,5,5′-hexabromo-1,1′-bipyrrole [14]; (e) Prodigiosin [40]; (f) (-)-marinopyrrole B [41].

CHAPTER 2

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Expanding the range of halogenated 1′-methyl-1,2′-bipyrroles (MBPs) using GC/ECNI-MS and GC×GC/TOF-MS

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Byron E. Pedler and Christopher M. Reddy

Abstract

Halogenated 1′methyl-1,2′-bipyrroles (MBPs) have been identified worldwide in marine mammals. Here we present the tentative identification of previously undetected MBP congeners in *Delpinus delphis* blubber using gas chromatography/electron capture negative ion mass spectrometry (GC/ECNI-MS) and comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GC×GC/TOF-MS). This is the first report of 26 isomers, only two of which are perhalogenated, one with bromine and one with chlorine. The presence of numerous partially halogenated congeners suggests that they are either biosynthesized concomitantly with their perhalogenated counterparts or that their dehalogenation products can also bioaccumulate. The newly found compounds fit the geographic trend that has been previously noted. That is,

samples from the Atlantic Ocean are dominated by the more brominated congeners while those from the Pacific are dominated by the more chlorinated congeners.

1. Introduction

Halogenated natural products (HNPs) are a class of secondary metabolites found in environmental matrices [1]. Most of the HNPs currently known have been identified in organisms that produce high quantities of secondary metabolites or that concentrate the bioactive compounds of their prey in their own tissues [2]. However, a select subset of HNPs bioaccumulates in the tissues of higher trophic-level organisms at trace concentrations in similar patterns to persistent organic pollutants (POPs). Several lines of evidence support the classification of these compounds as natural products, for example, radiocarbon dating [3, 4] and their presence in pre-industrial, archived whale oil [5]. Classes of HNPs shown to bioaccumulate include the polybrominated phenoxyanisoles [4, 6], polybrominated dibenzo-*p*-dioxins [7], 1,1′-dimethyl-2,2′-bipyrroles (DMBPs) [8] and halogenated 1′-methyl-1,2′-bipyrroles (MBPs) [9-11]. These compounds have been thoroughly reviewed in recent publications by Covaci et al. [12] and Vetter [1]. Like the POPs, bioaccumulating HNPs are available for human uptake [13-15] and at least one such compound is present in human breast milk [16].

Organisms responsible for synthesizing most of these compounds have not been identified. Nevertheless, these HNPs are of great interest to environmental chemists and toxicologists since they have similar physical and chemical properties to POPs [17]. In fact, it may be that the degradative mechanisms used in POP remediation originally evolved to degrade HNPs [18]. Hence, they can be used as models to help understand the cycling and environmental fate of POPs [3, 19].

Recently published research has shown that bioaccumulative MBPs (Figure 1) include more than 20 congeners of mixed halogenation (containing bromine and chlorine) in addition to the perchlorinated and perbrominated MBPs [10, 11]. Unlike POPs, these compounds appear to have a specific geographic distribution: the more highly-chlorinated derivatives tend to dominate in the South Pacific [11] and the more highly-brominated derivatives tend to dominate in the North Atlantic [10, 20]. Of the more than 20 MBPs described, most are perhalogenated (i.e., 7 halogens). Four hexahalogenated MBP derivatives of mixed halogenation have been measured in blubber from animals stranded along the North American coast [10], as well as in archived whale oil from the 1920s [5]. Six hexachlorinated MBPs have been detected as photolytic transformation products of MBP-Cl_7 (formerly referred to as Q1) [21], of which 5 were detected in marine biota samples that contain large amounts of MBP-Cl_7 [21, 22].

Here we report the presence of additional MBP congeners in the blubber of a common dolphin (*Delphinus delphis*) stranded along the east coast of North America. Out of 43 MBP congeners that we report here, 26 have not been detected previously. Possible implications of these compounds are also discussed.

2. Methods

2.1 Samples

The blubber of a *D. delphis* fatally stranded in January 2006 in Orleans, MA was obtained from the Cape Cod Stranding Network (identification number: CCSN06-013Dd).

2.2 Sample extraction and purification

The blubber was homogenized in a blender and the lipids were obtained by filtration through a glass fiber filter with a nominal pore size of 0.7 μm. To quantify the MBPs, the cetacean oil was spiked with an internal standard, 3,3′,4,4′,5,5′-hexachloro-1,1′-dimethyl-2,2′-bipyrrole (DMBP-Cl_6), which is not found in environmental samples and was synthesized for us by Drs. David Blake and Gordon Gribble of Dartmouth College [23]. The majority of lipids were removed by gel permeation chromatography (GPC) on an OI Analytical GPC Autoprep 2000. The 700-mm glass column was packed with 60 g of Envirobeads SX-3 in 1:1 hexane:dichloromethane. One gram of lipid was solvated to 10 mL

with 1:1 hexane:dichloromethane, and 5 mL of this solution was injected into the GPC. The column was eluted with 1:1 hexane:dichloromethane at 5 mL min^{-1}, and MBPs were collected between 16 and 55 minutes. Column chromatography was then used to isolate MBPs from other organic molecules and remove any residual lipids. Fully-activated, 100-200 mesh, Fisher silica gel (8 g) was packed in hexane and topped with fully-activated, 20-325 mesh, Fisher neutral alumnia (5 g) in a 1-cm i.d. column. The column was eluted with 100 mL hexane and then 100 mL of 95:5 hexane:dichloromethane. The MBPs were collected between 50-200 mL of the total eluent. For reference, polychlorobiphenyls (PCBs) were collected in the first 50 mL.

2.3 Production of an MBP standard solution

Due to the lack of synthetic standards, we isolated the four previously identified [10] MBP isomers from *D. delphis* blubber to use as reference and calibration standards. They were purified by preparative capillary gas chromatography (PCGC) [24]. Briefly, after ~70 injections onto a PCGC using a CP-Sil 5CM column (60 m × 0.25 mm i.d. 0.25 μm film thickness), the individual MBPs were rinsed from their respective U-tubes with dichloromethane. The solvent was removed and the mass of each collected MBP was determined. The purity of the isolated compounds was verified by gas chromatography coupled to a flame ionization detector (>99%). These compounds were analyzed by gas chromatography high-resolution mass spectrometry (GC-HRMS) [5, 10] and

nuclear magnetic resonance (NMR) spectroscopy [20]. The mass of the collected compounds was determined, and a solution was prepared containing 6 ng μL^{-1} of each MBP and 4 ng μL^{-1} of the synthetic standard, DMBP-Cl_6.

2.4 GC/ECNI-MS procedures

Compounds were tentatively identified by gas chromatography mass spectrometry using electron capture negative ion mass spectrometry (GC/ECNI-MS) with an Agilent 6890N series GC interfaced to an Agilent 5973 network mass selective detector. Extracts were injected in splitless mode and separated by a J & W Scientific DB-XLB column (60 m × 0.25 mm i.d., 0.25 µm film thickness) using He as a carrier gas at a constant flow rate of 1.1 mL min^{-1}. The oven program was: initial temperature of 50 °C, followed by a temperature increase at 20 °C min^{-1} to 115 °C and held for 10 minutes, then a 2 °C min^{-1} increase to 320 °C, which was held for 15 minutes. Methane was used as a reagent gas, and the source and transfer lines were maintained at 150 °C. Spectra were acquired in full scan mode, and the data was analyzed for the mass-to-charge (m/z) ratios representative of the possible MBP congeners. The molecular ions, fragmentation patterns, and relative retention times were used in tandem to detect MBPs congeners. The tentative identification was verified by comparison of the isotope ratios in the molecular ion clusters to theoretical ratios. Due to differences in response factors [21], only the MBPs present in the standard solution (described previously) were quantified. We used two methods to report

the amount of each MBP not quantified. For isomers of the MBPs in the standard solution, we used the response factors of the quantified MBPs to estimate the concentrations of their isomers in the blubber extract. Response factors have been shown to vary within an order of magnitude for the hexachlorinated MBPs [21], and thus only one significant figure is reported. For the remaining MBPs, their abundance was reported relative to that of MBP-Br_7. As a method blank, vegetable oil was extracted and analyzed for MBPs; none were detected.

2.5. GC×GC/TOF-MS procedures

Each extract was analyzed on a GC×GC/TOF-MS system that employed a dual stage cryogenic modulator (Leco, Saint Joseph, Michigan) installed in an Agilent 6890N gas chromatograph configured with a 7683B series split/splitless auto-injector, two capillary gas chromatography columns, and a time of flight mass spectrometeric detection system. Each extract was injected in splitless mode and the purge vent was opened at 0.5 minutes. The inlet temperature was 300 °C. The first-dimension column and the dual stage cryogenic modulator reside in the main oven of the GC. The second-dimension column is housed in a separate oven installed within the main GC oven. The first-dimension column was a nonpolar Restek Rtx-5 Crossbond, (10 m × 0.18 mm i.d., 0.2 μm film thickness) that was programmed to remain isothermal at 95 °C for 5 minutes and then ramped from 95 to 250 °C at 1.25 °C min^{-1}. Compounds eluting from the first dimension column were cryogenically modulated on deactivated fused silica

(0.5 m × 0.11 mm i.d.). The modulator cold-jet gas was dry N_2 that was chilled with liquid N_2. The thermal modulator hot-jet air was set to be 75 °C above the temperature of the main GC oven. The hot jet was pulsed for 1.5 seconds every 15 seconds with a 6-second cooling period between stages. Second-dimension separations were performed on a 50% phenyl polysilphenylene-siloxane column (SGE BPX50, 0.70 m × 0.10 mm i.d., 0.1 μm film thickness) that was programmed to remain isothermal at 120 °C for 5 minutes and then ramped from 120 to 275 °C at 1.25 °C min^{-1}. The carrier gas was He at a constant flow rate of 1.1 mL min^{-1}. The TOF-MS detector signal was sampled at 50 spectra sec^{-1}. The transfer line from the second oven to the TOF-MS was deactivated fused silica (0.5 m length, 0.18 mm i.d.), which was held at a constant temperature of 295 °C. The TOF-MS source temperature was 230 °C and the detector voltage was 1575 V.

3. Results and Discussion

3.1 Tentative identification of halogenated 1′-methyl-1,2′-bipyrroles by GC/ECNI-MS

A variety of compounds derived from the 1′-methyl-1,2′-bipyrrole backbone (Figure 1) have been measured at trace concentrations in the tissues of marine mammals [10, 11, 20, 25]. These compounds have been analyzed by GC-HRMS [10], NMR spectroscopy [20, 21], and, for the perchlorinated congener, comparison with a synthetic standard [26]. These congeners span the full range

of possible halogenation patterns, from perchlorinated [26] to perbrominated [10]. Previous to this effort, ten partially halogenated congeners have been tentatively identified: three isomers of pentabromochloro-1′-methyl-1,2′-bipyrrole (MBP-HBr_5Cl) and one isomer of hexabromo-1′-methyl-1,2′-bipyrrole (MBP-HBr_6) [5, 10], and six isomers of hexachloro-1′-methyl-1,2′-bipyrrole (MBP-HCl_6) [21, 22].[1]

We hypothesized that there were additional partially halogenated MBPs present in *D. delphis* blubber. To guide us in searching for these compounds, we used the MBP standard solution that was isolated by PCGC. The latter was first injected on our GC/ECNI-MS. Briefly, the four MBP congeners in our standard solution elute in the order expected based on their masses; the smaller, partially halogenated compounds elute first and are followed by the more massive perhalogenated MBPs (Figure 2a, Table 1). Also, the MBPs containing chlorine elute prior to MBPs that have the same degree of halogenation, but contain solely bromine. The mass spectrum of every MBP is dominated in the higher mass range (>350 m/z) by the sequential loss of either a bromine or chlorine atom. In our standard solution, the mass spectra of the two hexahalogenated isomers are very similar to the perhalogenated MBP standards (example: Figure 3a and 3b);

[1] Saint-Louis and Pelletier (2005) published evidence for partially halogenated 1'-methyl-1,2'-bipyrroles in marine mammal liver tissue, but identified them as halogenated naphthols. More recently, Teuten et al. (2006c) presented evidence in collaboration with Saint-Louis and Pelletier that these compounds had been misidentified and were tentatively identified MBPs.

they have the same fragmentation patterns, but their molecular ions are lower corresponding to their decreased molecular weight. In this way, predicting the molecular ions and the fragmentation patterns of partially halogenated derivatives is straightforward.

Employing the knowledge gained from analyzing the MBP standard solution, we found 43 MBP isomers within the blubber of this *D. delphis*, 28 of which were partially halogenated isomers (Figure 2b, Table 1). In addition to using mass spectral evidence, we had the added confidence that all of these compounds eluted in our expected silica gel fraction (and not with less polar compounds like PCBs).

This is the first report of MBP-HBr_4Cl_2, MBP-HBr_3Cl_3, MBP-H_2Br_5, MBP-H_2Br_4Cl, MBP-$H_2Br_3Cl_2$, MBP-H_3Br_4 and MBP-H_3Br_3Cl isomers (Table 1). It is also the first finding of polychlorinated MBP congeners in samples from the North Atlantic, which may be amongst those previously identified by Vetter et al. (2007). Additionally, this is the first detection of two more isomers of MBP-Br_6Cl, previously only one isomer had been detected [10, 11]. The partially halogenated compounds display the same fragmentation pattern as the MBPs in the standard solution (Figure 3) and have the correct molecular ions for the proposed molecular formulas. The molecular and fragment ions in the mass spectra also display the distinct halogen isotope pattern expected for their molecular formula. For example, Figure 3 a-c shows the isotopic patterns for the

molecular ions of MBP-Br_7, MBP-HBr_6 and MBP-H_2Br_5. Finally, these compounds follow the elution order established by previously identified MBPs; the less halogenated and more chlorinated derivatives elute earlier (Figure 2, Table 1).

Using calibration solutions of the MBP standards (MBP-Br_7, MBP-Br_6Cl, MBP-HBr_6 and MBP-HBr_5Cl), the concentrations of these four isomers within the blubber extract were quantified (Table 1). Due to the lack of quantification standards, the other MBPs were not quantified but were estimated (section 2.4). The previously characterized isomer of MBP-Br_6Cl is the most abundant congener. However, the sum of the MBP-HBr_5Cl isomers exceeds that of the MBP-Br_6Cl isomers. The novel MBPs that we detected were all present at low abundances relative to the previously characterized congeners. The MBP concentrations reported here are similar to those observed in other samples of *D. delphis* blubber from Cape Cod [10].

3.2 Further evidence of partially halogenated congeners of the halogenated 1′-methyl-1,2′-bipyrroles by GC×GC/TOF-MS

GC×GC is an analytical technique that separates complex mixtures based on both their volatility and, most often, their polarity. Hence, GC×GC has often been used to resolve compounds that co-elute in one-dimensional gas chromatography (in which elution is based primarily on a compound's volatility). However, we drew upon a different property of GC×GC for the

tentative identification of MBP isomers, that structurally similar compounds tend to elute along lines in GC×GC space. This pattern has been observed for many chemical classes, such as long chain ketones [27] and PCBs [28]. Moreover, this empirical observation is corroborated by established chemical concepts: a theoretical analysis shows that a compound family (i.e., a set of compounds that have a common parent structure, but which have varying numbers/patterns of a certain substituent group) exhibits a linear free energy relationship in the GC×GC two-dimensional retention index space [29]. Consequently, a compound family will also elute along a line or a monotonic curve in the GC×GC retention time space. This tendency has been exploited to find previously unidentified compounds of the same class in complex mixtures. For example, Xu et al. (2001) showed that long chain ketones (C_{35-39}) eluted along two distinct lines in GC×GC space due solely to the position of the carbonyl group; methyl ketones aligned on one diagonal and ethyl ketones aligned on a parallel line just below the methyl ketones. Additionally, by following the elution lines of the known long chain ketones, previously unknown ketones were identified [27]. Consequently we expected MBPs to elute in a line in GC×GC space. If an unidentified peak falls on the elution line of the set of known MBP standards, this constitutes important corroborative evidence that the unidentified peak is also an MBP.

To expand our search for MBPs, we analyzed the MBP standard solution and the dolphin extract via GCxGC/TOF-MS. Before introducing any GCxGC

data, it is important to understand how to interpret these chromatograms. Briefly, elution along the x-axis is volatility-based, mimicking retention patterns of one-dimensional gas chromatography. Elution along the y-axis is polarity-based, with the more highly polar components eluting later in the chromatogram. We first analyzed the standard solution in order to determine where the MBPs elute in GC×GC space (Table 1, Figure 4a). Clearly, the four MBPs of the calibration solution exhibit a diagonal trend, with the more halogenated compounds eluting later along both axes.

Using a similar approach to that which we employed with GC/ECNI-MS, we also detected numerous MBPs by GC×GC/TOF-MS in the blubber extract (Figures 3d-f and 4b, Table 1). MBPs were recognized by the fragmentation patterns and isotope ratios in their mass spectra, and that they aligned in GC×GC space with the MBPs in the standard solution (with the exception of two isomers, described below). A comparison of mass spectra for the sample compounds with ECNI-MS and TOF-MS is shown (Figure 3); as expected, the electron impact of TOF-MS (Figure 3d-f) displays greater fragmentation than the softer chemical ionization of ECNI-MS (Figure 3a-c). The molecular ion clusters and the major fragment ions are otherwise unchanged.

The diagonal elution pattern in GC×GC space that was established by the standard solution extends to the newly found derivatives. Importantly, MBP-Br_7 and its brominated congeners align on one diagonal, and MBP-Br_6Cl and

congeners containing one chlorine align along a second, lower, parallel diagonal. This provides strong supporting evidence for the tentative identification of these new compounds as MBPs [27, 29]. There are two exceptions to the diagonal alignment; one isomer each of MBP-HBr_5Cl and MBP-H_2Br_4Cl elute later (higher) in the second dimension than would be expected from the general trend. In GC×GC analysis of PCBs, it has been noted that non-*ortho* and mono-*ortho* PCBs elute later in the second dimension than PCBs fully halogenated in the *ortho* position [28]. Upon applying the observations of PCB elution in GC×GC space, it is likely that these two outlying MBPs are partially halogenated MBP congeners with at least one hydrogen atom in position 2, 3′, 5 or 5′.

The presence of partially halogenated MBPs that do not contain a halogen in the *ortho* position is important because they may be more toxic then other MBPs containing *ortho*-halogens. Previous research has shown that the perchlorinated congener binds the aryl hydrocarbon receptor and induces cytochrome P450 activity [30]. This receptor has been shown to bond effectively with planar molecules, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [31]. Thus, understanding the non- and mono-*ortho* MBP congeners, which can approach a more planar configuration, is critical in evaluating MBP toxicity.

Many of the congeners detected in this sample by GC/ECNI-MS were not detected with GC×GC/TOF-MS (Table 1). These congeners were present at extremely low abundances when detected by GC/ECNI-MS. The selectivity of

ECNI-MS may allow for a higher signal-to-noise ratio, thus facilitating identification of minute quantities of these compounds. However, GC×GC is superior to one-dimensional GC with respect to resolving co-eluting compounds and providing structural evidence. Thus, combining GC×GC-TOF/MS with GC-ECNI/MS allows for detecting novel congeners and quantifying known compounds for many types of compounds classes.

4. Conclusions

By using GC/ECNI-MS in tandem with GC×GC/TOF-MS, we have tentatively identified 26 new partially halogenated MBP congeners. The novel MBP congeners were highly brominated and detected in the blubber of a *D. delphis* fatally stranded on Cape Cod, MA, USA. The concentration of many of the less halogenated isomers is very small, which may explain why these compounds have been previously overlooked. GC×GC/TOF-MS analysis proved very useful in providing support for our tentative identification of the new congeners by GC/ECNI-MS. In two-dimensional space it is clear that newly characterized compounds elute along the same set of lines as previously identified MBPs. Although this identification is still tentative, the presence of such a large number of isomers is notable. Structural confirmation of the tentatively identified compounds will require isolation of the individual

compounds, full structural elucidation by NMR spectroscopy and/or x-ray crystallography, and, ideally, comparison with a synthetic standard.

Although the task is challenging, full identification of the MBP congeners present in environmental matrices will be important in attempts to understand their toxicity, their biosynthetic pathway, and their cycling in the environment. As of yet we do not know the identity of the producing organism or why we do not detect the many other possible MBP isomers. For example, although there are 24 possible MBP-HBr_5Cl isomers, only six have been reported to date. Putting constraints on the factors that determine which MBPs are present in the environment can provide insight into the production, transport, and fate of all halogenated organic compounds in marine ecosystems. For example, the presence of such a large number of diverse isomers may be evidence for dehalogenation as a degradative mechanism for these bioaccumulating natural products. Further work must be accomplished to confirm this theory, but the data presented herein is compelling.

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Table 1. MBPs identified in 0.5 g of lipid from the blubber of a common dolphin (*D. delphis*). Congeners in bold are previously detected MBPs and were components of the standard solution. The listed ion (±0.5) was used in finding the MBPs and for quantification. Only MBPs that were part of the standard solution (in bold) were quantified, concentrations of their isomers were estimated. For MBPs identified but for which no isomer was quantified the abundance relative to that of MBP-Br_7 is presented: (+++) for a relative abundance of >10%, (++) for <10% and (+) for <1%. Congeners not detected by GC×GC/TOF-MS are indicated by a (-).

Compound	(*x*-1′-methyl-1,2′-bipyrrole) *x* =	Ion (m/z)	GC-ECNI/MS Retention Time (minutes)	Lipid Conc. (ng/g lipid)	GCxGC Retention Time 1 (seconds)	GCxGC Retention Time 2 (seconds)
MBP-Br_7	**heptabromo-**	**697.7**	**97.643**	**2550**	**5795**	**9.99**
MBP-Br_6Cl	hexabromochloro-	653.7	93.426	40	-	-
MBP-Br_6Cl	hexabromochloro-	653.7	93.648	40	-	-
MBP-Br_6Cl	**hexabromochloro-**	**653.7**	**93.849**	**3480**	**5495**	**9.09**
MBP-HBr_6	hexabromo-	619.7	85.945	80	-	-
MBP-HBr_6 [d]	**hexabromo-**	**619.7**	**86.554**	**1260**	**4820**	**8.58**
MBP-HBr_6	hexabromo-	619.7	91.118	90	-	-
MBP-HBr_6	hexabromo-	619.7	94.044	200	-	-
MBP-HBr_5Cl [c]	pentabromochloro-	575.7	81.860	400	4430	7.60
MBP-HBr_5Cl	pentabromochloro-	575.7	82.085	70	4505	7.88
MBP-HBr_5Cl	pentabromochloro-	575.7	82.370	30	-	-
MBP-HBr_5Cl [a]	**pentabromochloro-**	**575.7**	**82.668**	**2360**	**4490**	**7.94**
MBP-HBr_5Cl [b]	pentabromochloro-	575.7	87.174	500	4850	9.51
MBP-HBr_5Cl	pentabromochloro-	575.7	90.378	500	5120	8.90
MBP-Br_5Cl_2	pentabromodichloro-	609.8	89.321	+	-	-
MBP-Br_5Cl_2	pentabromodichloro-	609.8	89.550	++	-	-
MBP-Br_5Cl_2	pentabromodichloro-	609.8	89.776	++	-	-
MBP-Br_4Cl_3	tetrabromotrichloro-	565.6	85.368	++	-	-
MBP-Br_4Cl_3	tetrabromotrichloro-	565.6	85.621	+	-	-
MBP-H_2Br_5	pentabromo-	539.8	73.870	+++	3695	7.12
MBP-H_2Br_5	pentabromo-	539.8	78.844	++	-	-
MBP-H_2Br_5	pentabromo-	539.8	79.331	++	-	-
MBP-H_2Br_5	pentabromo-	539.8	81.157	+++	4310	8.00
MBP-HBr_4Cl_2	tetrabromodichloro-	531.8	77.785	+	-	-
MBP-HBr_4Cl_2	tetrabromodichloro-	531.8	78.100	+++	-	-
MBP-HBr_4Cl_2	tetrabromodichloro-	531.8	78.394	++	-	-
MBP-	tetrabromochloro-	495.8	69.623	++	3365	6.39

HBr_4Cl						
MBP-HBr_4Cl	tetrabromochloro-	495.8	69.750	+++	-	-
MBP-HBr_4Cl	tetrabromochloro-	495.8	74.705	++	-	-
MBP-HBr_4Cl	tetrabromochloro-	495.8	75.232	+++	3815	8.17
MBP-HBr_4Cl	tetrabromochloro-	495.8	77.174	+++	4010	7.17
MBP-HBr_4Cl	tetrabromochloro-	495.8	78.021	+	-	-
MBP-HBr_3Cl_3	tribromotrichloro-	485.7	73.751	++	-	-
MBP-Br_2Cl_5	dibromopentachloro-	475.7	72.158	+	-	-
MBP-Br_2Cl_5	dibromopentachloro-	475.7	76.770	+	-	-
MBP-H_3Br_4	tetrabromo-	461.7	68.025	+	-	-
MBP-$H_2Br_3Cl_2$	tribromodichloro-	451.9	64.790	+	-	-
MBP-$H_2Br_3Cl_2$	tribromodichloro-	451.9	65.519	+	-	-
MBP-$BrCl_6$	bromohexachloro-	431.8	72.371	+	-	-
MBP-$BrCl_6$	bromohexachloro-	431.8	72.959	+	-	-
MBP-$BrCl_6$	bromohexachloro-	431.8	73.078	+	-	-
MBP-H_3Br_3Cl	tribromochloro-	417.7	63.793	+	-	-
MBP-Cl_7	heptachloro-	385.9	68.420	+++	-	-

[a] Previously identified as MBP-HBr_5Cl Isomer A, Teuten and Reddy (2007).
[b] Previously identified as MBP-HBr_5Cl Isomer B, Teuten and Reddy (2007).
[c] Previously identified as MBP-HBr_5Cl Isomer C, Teuten and Reddy (2007).
[d] Previously identified isomer of MBP-HBr_6, Teuten and Reddy (2007).

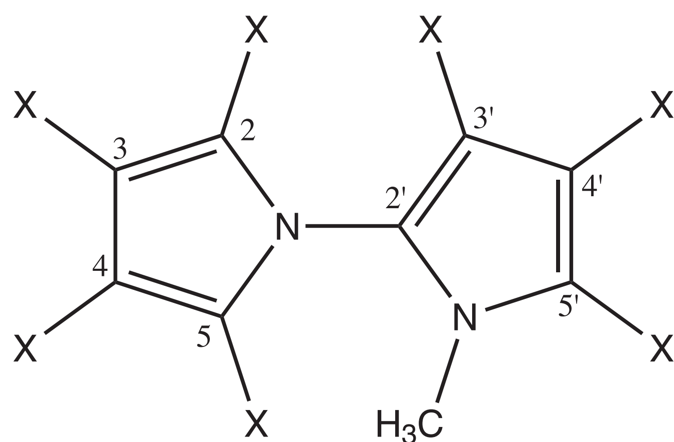

Figure 1. The structure of the halogenated 1′-methyl-1,2′-bipyrroles is shown with the carbon atoms numbered. The atoms labeled X can be H, Br or Cl. The nomenclature system used in this paper will indicate the number of H, Br and Cl on the ring system by subscripts. For example, the heptachlorinated congener is referred to as MBP-Cl_7, and the congener containing a hydrogen, five bromines and a chlorine on the ring system is called MBP-HBr_5Cl.

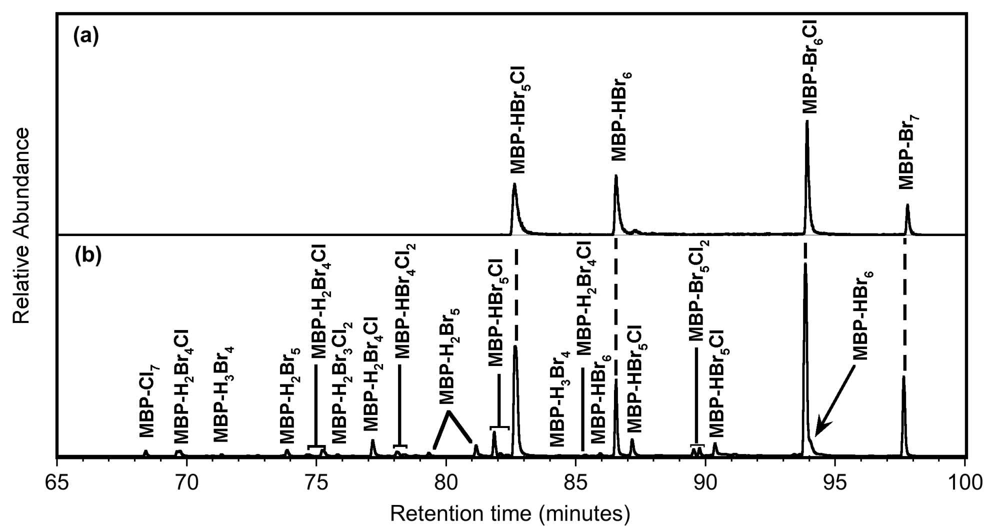

Figure 2. The summed ion chromatograms of the (a) MBP standard solution and the (b) blubber extract from CCSN06-013Dd are presented. Peaks that are visible at this scale are identified. The summed chromatograms result from summation of the following ions (±0.5): 697.7, 653.7, 619.7 and 575.7 (MBP standard solution); 697.7, 653.7, 619.7, 609.8, 575.7, 565.6, 539.8, 531.8, 495.8, 485.7, 461.7, 451.7, 431.8, 417.7 and 385.9 (blubber extract).

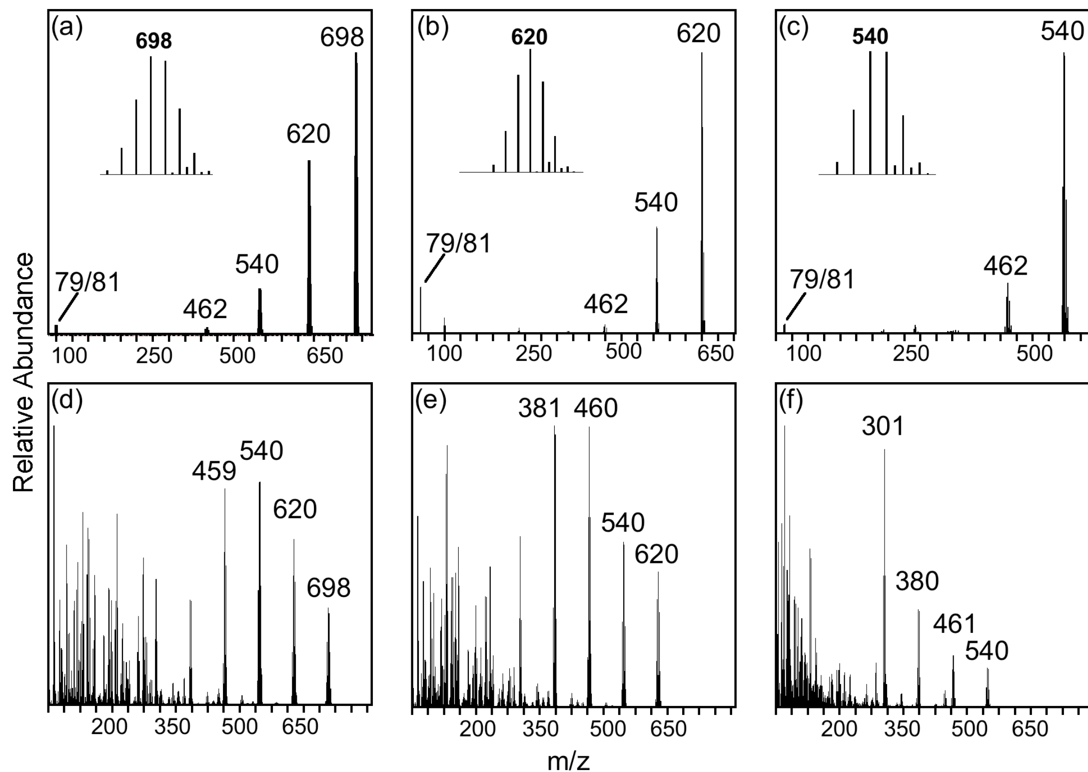

Figure 3. The mass spectra of MBP-Br_7, MBP-HBr_6 and MBP-H_2Br_5 from both ECNI-MS (a, b and c, respectively) and TOF-MS (d, e and f, respectively) are shown. Inset in panels (a), (b) and (c) are enlargements of the molecular ion cluster. The isotope pattern of the molecular ion cluster for MBP-Br_7: m/z 698 (100%), 700 (95.9%), 696 (63.4%), 702 (55.5%), 694 (22.5%), and 704 (18.1%); for MBP-HBr_6: m/z 620 (100%), 618 (79.1%), 622 (73.1%), 616 (33.2%), 624 (28.9%), and 614 (5.8%); for MBP-H_2Br_5: m/z 536 (10.3%), 538 (51.5%), 540 (100%), 542 (98.6%), 544 (47.7%), and 546 (9.8%).

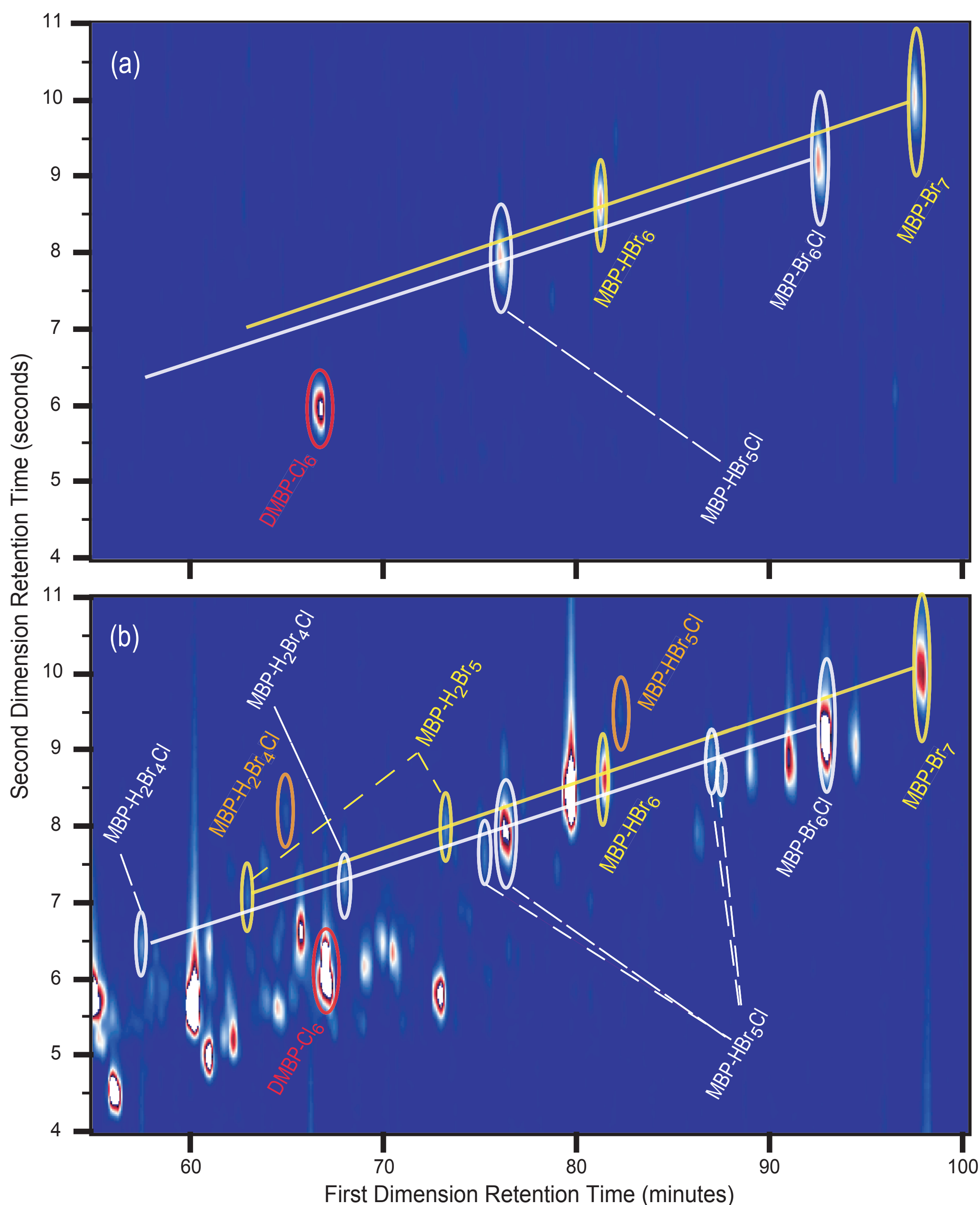

Figure 4. Partial GC×GC chromatogram of (a) the total ion chromatogram of the MBP standard solution and (b) the MBPs in the *D. delphis* blubber extract resulting from the summation of the following ions (±0.5): 496, 540, 575, 620, 654 and 698. Visible peaks that correspond to MBPs are circled in yellow, white or orange, and the internal standard, DMBP-Cl_6 is circled in red. We have drawn lines representing the MBPs containing only bromine (top, yellow) and MBPs containing one chlorine (bottom, white). The MBPs that elute above these lines, and are proposed to be non- or mono-*ortho*, are circled in orange. PCBs and chlorinated pesticides elute earlier than the MBPs in the second dimension, and can be seen beneath the MBPs in the lower left corner in the GC×GC chromatogram of the blubber extract in (b).

CHAPTER 3

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Distribution patterns suggest biomagnification of halogenated 1′-methyl-1,2′-bipyrroles (MBPs)

Kristin C. Pangallo and Christopher M. Reddy

Abstract

The halogenated 1′-methyl-1,2′-bipyrroles (MBPs) are a suite of marine natural products that have been detected in marine mammals worldwide. Although their concentrations are similar to persistent organic pollutants that biomagnify, such as 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153), it is not yet clear that these natural products also biomagnify. Here we analyze MBPs and CB-153 isolated from the blubber and liver of marine mammals stranded on the eastern coast of Massachusetts. Four odontocete species (*Delphinus delphis, Lagenorhynchus acutus, Phocoena phocoena* and *Globicephala melas*) and two pinniped species (*Halichoerus grypus* and *Phoca groenlandica*) were sampled. MBPs were present in all odontocetes, but not detected in pinnipeds; CB-153 was detected in every species. MBP patterns indicative of biomagnification were

found, including age-dependent concentration increases and reduced concentrations in adult females. Also explored are the similarities and differences with CB-153, the effects of nutritional state on contaminant distribution, and the maternal transfer of blubber-based organic contaminants.

1. Introduction

The halogenated 1′-methyl-1,2′-bipyrroles (MBPs) are a suite of chlorinated and/or brominated congeners (Figure S1) and there is overwhelming evidence that they are natural products (1). The organism or organisms that produces them has yet to be identified, but is likely marine, as these compounds have been found solely in marine samples (2). The first congener found, the perchlorinated congener (MBP-Cl_7), was detected in marine mammals in 1999 (3), and its structure was confirmed in 2002 (4). Thus far, 29 primarily chlorinated congeners have been detected in samples from the Pacific (5) and 43 primarily brominated congeners in samples from the Atlantic (6). MBPs have been found in a variety of trophic levels from marine food webs (5-7), as well as in human breast milk (8) and products for human consumption (9,10). Despite their presence in these reservoirs, it is uncertain whether MBPs biomagnify.

Biomagnification is a major concern for many anthropogenic compounds that are classified as persistent organic pollutants (POPs) (11), as well as for some halogenated natural products (HNPs), such as the halogenated 1,1′-dimethyl-2,2′-bipyrroles (DMBPs) (12). Man-made halogenated organic compounds

(HOCs) that biomagnify have been shown to have negative impacts on both human and environmental health (13,14). Some of these health affects can occur at the concentrations detected in environmental samples (15). Despite strict environmental controls that have been placed on the production, use and release of POPs (11), these compounds continue to be measured in an enormous variety of samples from all over the globe and in almost every ecosystem (15). Due to their longevity, the ultimate fate of these compounds has yet to be determined. However, by studying HNPs that share many of the same physical and chemical properties as POPs, we can start to understand and predict their transport and metabolism. Prior to this use, the similarities and differences between individual HNPs and their anthropogenic counterparts must be determined. Ideally, biomagnification should occur in HNPs in order for them to prove useful in elucidating mechanisms that control the environmental concentrations of POPs.

HNPs that have only a marine source, like the MBPs, may also prove useful in tracing the flux of HOCs from marine to terrestrial ecosystems. This pathway is potentially a concern for human consumption of seafood. Indeed, transfers of marine HNPs into human tissues has previously been demonstrated (8). Therefore, marine HNPs may help gauge the seafood-based contribution to human uptake of POPs.

Here we show the presence of MBPs in blubber and liver samples from North Atlantic marine mammals. The MBP patterns are consistent with

biomagnification, which is not unexpected since MBPs are hydrophobic, as are the POPs, such as polychlorinated biphenyls (PCBs), that biomagnify. For comparison, we also report on the abundance and distribution of the PCB 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153). While the MBPs and CB-153 share some of the same patterns, there are intriguing differences in their distributions and this may be reflective of their different origins.

2. Experimental

2.1 Sampling

The 25 animals analyzed in this study include both odontocetes (toothed whales) and pinnipeds (earless seals). The odontocete samples consisted of liver and blubber tissue from thirteen common dolphins (*Delphinus delphis*), three Atlantic white-sided dolphins (*Lagenorhynchus acutus*), one harbor porpoise (*Phocoena phocoena*) and one long-finned pilot whale (*Globicephala melas*). The pinniped samples consisted of liver and blubber tissue from two grey seals (*Halichoerus grypus*) and five harp seals (*Phoca groenlandica*). The samples included males and females, adult and youth specimens, and spanned a range of nutritional states from robust to emaciated (Tables 1, S1). All the samples were acquired through the Cape Cod Stranding Network, 23 were fatally stranded on Cape Cod from 2005-2007 and 2 were caught in fishing gear (the harbor porpoise and one grey seal) just south of New England.

2.2 HOC extraction and purification

Blubber samples were extracted and HOCs purified following a previously published method (6). Liver samples were kept frozen until ready for use. Upon thawing, liver tissues were chopped into ~1 cm^3 pieces and rinsed under cold water to remove excess blood. The samples were freeze-dried, homogenized with mortar and pestle, and extracted using a Dionex ASE200 Accelerated Solvent Extractor (ASE). An ASE cell was packed with 2.5 g of homogenized, dry tissue and spiked with 25 µL of our internal standard, a 50.8 ng μL^{-1} solution of 3,3′,4,4′,5,5′-hexachloro-1,1′-dimethyl-2,2′-bipyrrole (DMBP-Cl_6). The ASE program extracted each cell three times at 100 °C and 1000 psi with a dichloromethane/methanol (90:10) solution. The solvent was removed (Zymark TurboVap LV Evaporator) and the mass of the oily residue recorded as the total lipid extract (TLE). Ten-mL of dichloromethane/hexane (50:50) was added to dissolve the TLE and the samples were then centrifuged at 2000 rpm for 10 minutes. The clear solution (10 mL) was decanted from the insoluble material. The lipids were removed using gel permeation chromatography and the HOCs were purified from the residual lipids with column chromatography as for blubber samples (6).

2.3 Detection and quantification by GC/ECNI-MS

Compounds were tentatively identified by gas chromatography mass spectrometry using electron capture negative ion mass spectrometry (GC/ECNI-MS) in full scan mode with a previously published method (6). Briefly, on an

Agilent 6890N series GC interfaced to an Agilent 5973 network mass selective detector, extracts were injected in splitless mode and separated by a J & W Scientific DB-XLB column (60 m × 0.25 mm i.d., 0.25 μm film thickness) using He as a carrier gas at a constant flow rate of 1.1 mL min^{-1}. The oven program was: initial temperature of 50 °C, followed by a temperature increase at 20 °C min^{-1} to 115 °C and held for 10 minutes, then a 2 °C min^{-1} increase to 320 °C, which was held for 15 minutes. Methane was used as a reagent gas, and the source and transfer lines were maintained at 150 °C.

Each MBP was recognized by its relative retention time and congeners within a homolog series were designated by their halogen content plus a lower case letter (see Table 2 for nomenclature). This nomenclature system is used because the full identification of these congeners has yet to be completed due to the lack of synthetic standards. At this time we have a synthetic quantification standard for only the heptabrominated congener (MBP-Br_7). It was provided by Gordon Gribble (Dartmouth College) (16), along with our internal standard that was used to determine recovery percentages, DMBP-Cl_6. Congeners that are unavailable synthetically must be isolated in large quantity from an environmental matrix; we used this technique to isolate MBP-Br_6Cl-b, MBP-HBr_6-b and MBP-HBr_5Cl-d for use as quantification standards (6). Recent work has established that the response factors of MBP homologs vary within an order of magnitude (17), which allows us to estimate concentrations (to one significant

figure) for homologs of the four MBPs for which we have standards. The presence of MBPs not quantified is noted in Tables S4 and S6.

In addition to the MBPs, we also analyzed our samples for the PCB CB-153. CB-153 was chosen because it has similar chemical and physical properties to the MBPs; importantly, both have large octanol-water partition coefficients, $10^{6.9}$ for CB-153 and a range of $10^{7.2}$ to $10^{8.3}$ for MBPs (1). Additionally, CB-153 is known to biomagnify and is relatively abundant in environmental samples (18). It has been recommended for monitoring by various international organizations (19). Thus, it has been very well studied in many environmental samples, and has been used previously for comparison with marine halogenated natural products (20). Therefore, it is quite useful for comparison with our relatively newly-discovered MBPs.

The extraction methods and quantification were tested by running blank samples, determining method detection limits for each MBP and CB-153, and by replicate analyses to determine the overall method errors. Details on these protocols can be found in the Supplemental Information.

3. Results and Discussion

This study examined six species of marine mammals stranded along the northeast coast of North America for the presence of halogenated MBPs in blubber and liver tissues. To our knowledge, this is the first study to examine MBPs in *G. melas* and the first to analyze MBPs in liver samples from *D. delphis,*

P. phocoena, H. grypus and *P. groenlandica*. MBPs have been previously detected in liver tissues from *L. acutus* (21,22). Twenty-seven MBPs were detected, out of which four were quantified and concentrations were estimated for nine (Table S2). From the MBPs measured, MBP-Br_6Cl-b (Table 2) was chosen for direct comparison with CB-153 because it was the most abundant MBP measured in every sample, and it is representative of the MBP and ΣMBP (sum of MBPs for which concentrations were quantified or estimated, Table 2) patterns in our data set. MBPs in odontocete tissues were present at concentrations similar to that of CB-153, suggesting that MBPs may biomagnify throughout the North Atlantic food web. The abundance of MBPs in odontocete blubber relative to CB-153 is orders of magnitude greater than what has been observed for another suite of natural halogenated bipyrroles, the DMBPs (20).

MBPs were present in all odontocetes, though concentrations between individuals varied widely (Tables S3-S6). MBP-Br_6Cl-b was always the most abundant MBP out of those for which concentrations were quantified or estimated; the other MBPs detected (Tables S4 and S6) had very small peaks and, presumably, abundances. MBP individual congener profiles did show a consistent pattern, four congeners dominated the profiles of every sample: MBP-Br_7, MBP-Br_6Cl-b, MBP-HBr_6-b and MBP-HBr_5Cl-d. MBPs were present in similar concentrations (ΣMBP ~ 2.0 μg/g lipid) in the blubber of all odontocete species investigated (Table 2) and individual variation (Table S3) exceeded the

variation between species averages (Table 2). There was more variation in the liver concentrations of MBPs (Table 2); the average dolphin ΣMBP concentration (~2.6 μg/g lipid) far exceeded that of the harbor porpoise and pilot whale (0.08 μg/g lipid and 0.18 μg/g lipid, respectively). The concentration of CB-153 varied between odontocete species, but was present at similar concentrations to MBP-Br_6Cl-b in blubber (~1.0 μg/g lipid) and liver (~2.5 μg/g lipid), with the exception of the liver of the harbor porpoise for which the concentration was below the detection limit (Table 2).

The high concentrations of MBPs in all odontocete samples, and their similarities to the CB-153 concentrations, are consistent with biomagnification, which is the increase of the lipid-normalized concentration of a contaminant with trophic level (23). Previous work has established that biomagnification can occur for hydrophobic compounds with octanol/water partition coefficients exceeding 10^4 (24). Additionally, these compounds must be persistent to avoid any appreciable abiotic or enzymatic degradation. Contaminant concentrations increase in the tissues of each trophic level because the persistent compounds are not significantly metabolized with the rest of the prey and the fugacity of the contaminant increases in the stomach as the prey tissue is digested (25). This increased fugacity during digestion explains how contaminants are transferred against the apparent thermodynamic fugacity gradient.

3.1 *Age dependence of contaminant concentrations*

In addition to high concentrations, the processes that produce biomagnification also result in age-specific patterns within a marine mammal population. (To avoid the complications of interspecies comparison, here we limit our data set to the 13 *D. delphis* samples.) Due to constant exposure, concentrations of biomagnifying compounds increase with age in male mammals. Although the exact age of the individuals was unknown, we are able to categorize them as juvenile or adult, and as male or female. With this information, we can use length as a proxy for age among animals of the same category (juvenile/adult, male/female). Among the *D. delphis* samples, blubber concentrations for both MBP-Br_6Cl-b and CB-153 increase with length in adult males (Figure 1), implying that these concentrations increase with age.

A different pattern is evident in younger juveniles, their concentrations decrease then increase with increasing length (Figure 1). Due to their small size and milk-based diet, which places them at a higher trophic level, nursing calves tend to have relatively high body burdens of HOCs (26-28). As they grow and switch to a non-milk diet, blubber volume increases and dietary concentrations of HOCs decrease. These changes both result in a characteristic growth dilution pattern for this age group (29). In older individuals, growth dilution is overcome as growth rate slows and blubber concentrations increase (29). These patterns not only match experimental evidence on PCBs, but also show the same features as described by numerical biomagnification models (26-29).

There is an interesting difference between the MBPs and CB-153 in the age (length) at which the switch from growth dilution to adult accumulation patterns occurs. The concentration of MBP-Br_6Cl-b decreases with increasing length until just past 180 cm, at which point the concentrations in juvenile and adult male samples increase dramatically. However, in CB-153 there is a sharp reduction in concentration after the smallest juvenile, but concentrations then generally increase with length from 160 cm through the rest of the juvenile and adult male samples. This difference could be due to the limited number of samples, or it may be due to inherent differences between these two types of compounds.

Such differences between natural and anthropogenic compounds may be explained by two different, but not necessarily mutually exclusive, causes. These are their distinctive origins and differences in the ability of degradative enzymes to act upon them. In the first case, we expect that the distribution of natural and anthropogenic compounds in the marine environment to be different. CB-153 is a ubiquitous pollutant, and is present in both terrestrial and marine ecosystems (11); thus, it has multiple inputs to the marine ecosystem. MBPs are natural products that have only been detected in marine samples or samples closely tied to the marine food web (2,5,6). It is likely that they are made by a specific organism or group of organisms, and thus their source is much more limited than that of CB-153. From this source difference, it is likely that MBPs and CB-153 enter and move through the marine food web differently. Unlike the

ubiquitous CB-153, MBPs may have a limited distribution among the species of the North Atlantic food web, though this has yet to be verified. If this were the case it would not be surprising if MBP concentrations were more variable than those of CB-153 among prey species.

Secondly, the enzymes that degrade both types of compounds have evolved in the presence of HNPs (30). Thus, it would not be surprising if these enzymes could more easily degrade MBPs than the newly introduced CB-153. It is well established that the degree of chlorination and substitution patterns affect enzymatic degradation of PCBs. Dehalogenation studies performed using marine mammal hepatic cells suggest that HOCs can undergo cyctochrome P450 metabolism, and congeners with *ortho-meta* unsubstituted sites are more available for metabolism (31). CB-153 has both *ortho-* and *meta-* chlorine substituents, structural aspects that are consistent with its persistence and tendency to biomagnify. It is likely that these degradation patterns will also apply to MBPs, since the same enzyme systems are likely involved in their dehalogenation and degradation.

In the *D. delphis* samples, the difference observed between CB-153 and the MBPs may be explained by these differences between natural and anthropogenic compounds. In milk, the dose of lipophilic compounds, such as CB-153 (26-28), is high relative to that of an adult food source, and it is likely that lipophilic MBPs are also relatively enriched in milk. However, both a limited distribution

of MBPs and an enhanced susceptibility to degradation could lower the dose of MBPs relative to CB-153 for newly weaned dolphins. From this lower dose, we would expect to see a longer transition for MBPs from the growth dilution phase to the age accumulation trend evident in older animals.

3.2 Maternal transfer of contaminants

Adult females of our *D. delphis* data set display significantly reduced MBP blubber concentrations relative to males or to juveniles of similar length (Figure 1). Although females experience similar exposure to males, they have an additional removal process for HOCs. During gestation and lactation, blubber-based contaminants can be mobilized and transferred to offspring (26-28,32). Thus, among adult mammals, females tend to have much lower contaminant body burdens than do males.

Maternal transfer of contaminants can be examined more directly in our data set. An adult female (CCSN06-264Dd) and a male calf (CCSN06-263Dd) *D. delphis* were found stranded together. Based on observational evidence it is possible that the two are a cow-calf pair. The calf was quite young, the female had three follicular scars – morphology consistent with pregnancy (33), no other dolphins were found in the vicinity, and the HOC concentrations in the female's blubber and liver were indicative of gestational/lactational transfer. Regardless of direct kinship, it is highly probably that these two individuals belonged to the

same *D. delphis* population and are representative of young juveniles and adult females from that group.

For our presumed cow-calf pair, the concentration of MBPs and CB-153 were much lower in the adult female than in her presumed offspring, though the MBP congener profiles in the blubber of each animal were very similar (Figure 2). As compared to CB-153, the MBPs have a higher juvenile male/adult female ratio. This is surprising since replacement of chlorine substituents with bromine results in higher K_{ow}s in HOCs (34), so highly brominated compounds would be more likely to remain in the blubber than be transferred to milk, and thus, offspring. In fact, past studies have shown that the less lipophilic compounds are mobilized from maternal blubber and transferred to offspring, and the more lipophilic compounds were retained in the maternal blubber (26,28,35). However, there are a few studies that indicate selective mobilization of brominated compounds compared with chlorinated compounds into blood plasma (28) or milk (36). If this is the case, then once mobilized the more brominated MBPs may partition more strongly into the blubber of the offspring than more chlorinated HOCs, such as PCBs. Although this explains our results, it is dependent upon selective MBP mobilization from maternal blubber.

3.3 Comparison of nutritionally-compromised and healthy individuals

To further understand how the blubber mobilization affects these compound concentrations, we compared the blubber and liver concentrations of

MBPs in all odontocete samples. The presence of MBPs in the liver of the odontocete samples was anticipated based on the relatively high lipid content of the liver relative to other body tissues and the presence of detectable levels of MBPs in the blubber of these individuals. Concentrations in liver samples were generally about the same order of magnitude as for the blubber samples (on a lipid-normalized basis). Sorting by body condition (Table 1) results in higher liver MBP concentrations relative to blubber in nutritionally compromised individuals (Table S1). However upon averaging these values, the pattern is overwhelmed by large differences in the absolute concentrations of MBPs and CB-153 among these samples. When this interference is removed (by normalizing the liver concentrations to the blubber concentrations), individuals with body conditions characterized as robust or good have significantly (Wilcoxon-Mann-Whitney rank sum test, $p = 0.001$) lower MBP-Br_6Cl-b in their blubber as compared to their liver (Figure 3). Interestingly, CB-153 is statistically the same in both groupings ($p = 0.596$), again showing differences between these two types of HOCs (Figure 3).

This significant difference between liver and blubber samples may be explained by the roles of blubber and liver tissues, and their sensitivity to rapid changes in blood concentrations of contaminants. Blubber is a dynamic reservoir, and in times of energetic need its lipids can be mobilized and its mass reduced (37-39). Since blubber is the major reservoir for lipophilic HOCs, lipid

mobilization can intensely affect their blubber concentrations. Contaminants can be mobilized into the bloodstream, where they are more susceptible to metabolism, but, potentially, they are also more available to cause toxicity (40). In studies of other animals, nutritional stress has been shown to redistribute HOCs from lipid-storage reservoirs into other tissues (41,42). Liver is more vascularized than blubber and may better reflect changes on a short time-scale.

The distributions of MBP-Br_6Cl-b and CB-153 in nutritionally compromised animals suggests that either MBP-Br_6Cl-b is more efficiently mobilized during times of nutritional stress, or that odontocetes are better able to rid their liver, and/or the bloodstream, of PCBs than of MBPs. The first explanation is consistent with previous evidence presented regarding the maternal transfer of MBPs, other highly brominated organic compounds, and PCBs. The second possibility is not consistent with other evidence presented here, but it cannot be excluded without further study.

3.4 Interspecies differences

Finally, there is also a major difference between CB-153 and MBPs in the seal samples. MBP concentrations fell below the detection limit for all pinniped samples (Table 2). However, average concentrations of CB-153 in pinniped blubber (~0.11 μg/g lipid) were in the same range, though at the low end, as concentrations in odontocete blubber. The average CB-153 concentration in

pinniped liver samples (~1.1 μg/g lipid) was higher than pinniped blubber samples, but that average reflects a very large range in values.

These results are consistent with previous studies on other bioaccumulating HNPs. Past work on a set of biomagnifying halogenated bipyrroles has shown that ringed seals (*Phoca hispida*) had lower blubber concentrations than would be expected based on their trophic position (12). Additionally, harbor seals (*P. phocoena*) were shown to have lower blubber concentrations of MBP-Cl_7 than do harbor porpoises from the same geographic region, despite sharing comparable concentrations of the POP *trans*-nonachlor (43). Either differences in diet or in metabolic capabilities could explain the low HNP load of seals as compared with the odontocetes. The presence of CB-153 in the seal samples may indicate that seals share dietary exposure to HOCs but selectively degrade MBPs, or it could indicate different distributions between anthropogenic and natural HOCs in the food web. In either case, there is a distinct difference between the natural MBPs and anthropogenic CB-153 in the seal samples.

The results presented herein suggest that MBPs biomagnify in marine food webs. Our results support previous evidence that there are subtle, but potentially important, differences between anthropogenic and naturally produced compounds that biomagnify in these ecosystems (12,43). In order to prove the biomagnification of MBPs, a full trophic level analysis of these

compounds must be accomplished. This would also serve to further elucidate the differences found between POPs and HNPs within food webs, particularly clarifying whether prey preference is responsible for the lack of HNPs within seals. In conjunction with the results that we have presented here, future studies will help determine whether MBPs can be high-quality models for understanding the future of POPs.

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Supplemental Information

In addition to the data presented here, we have provided a supplemental section that contains the concentrations of each MBP quantified or estimated for each of the 25 mammals, as well as the detection of MBPs for which no concentration can be estimated. Also included are the procedures and calculations performed for quality control, and the general structure of MBPs.

This information is available free of charge via the Internet at http://pubs.acs.org.

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Table 1. Species, gender and maturity distributions for the 25 samples analyzed. Age was determined as the average length at which that species becomes sexually mature. The body condition of the animals was determined during necropsy as robust, good, thin or emaciated. Here we divide the animals into two groupings. A robust classification includes animals stated as in robust and good condition. A compromised classification includes animals in a thin or emaciated condition. Species accompanied by asterisks (*) are pinnipeds (earless seals), the remaining species are odontocetes (toothed whales).

	Gender		Age		Body Condition	
Species	# male	# female	# mature	# immature	# robust	# compromised
D. delphis	9	4	9	4	6	7
L. acutus	1	2	1	2	2	1
*H. grypus**	2	0	0	2	0	2
*P. groenlandica**	4	1	1	4	0	5*
P. phocoena	1	0	0	1	1	0
G. melas	1	0	0	1	0	1

Table 2. Lipid-normalized concentrations (ng g^{-1} lipid) of individual MBP congeners and CB-153 in the blubber and liver of 6 marine mammal species. Data is grouped by species for the odontocetes; due to similarities the two pinniped species (*H. grypus* and *P. groenlandica*) were grouped togther. For each compound the mean and a range is given. In categories where n>1 the range reflects the variability within the group as the 90% Confidence Interval. In categories for which *n* = 1, the compound-specific standard error is stated. The method measurement error for each compound is lower than 10% for CB-153 and all MBPs in the blubber (except for MBP-HBr_6-c and MBP-HBr_6-d, which were 29% and 11%, respectively, likely due to their low concentrations). The errors for all MBPs in the liver range from 19%-31%, and again are likely high due to the small samples. Compounds for which we have quantification standards are reported to 2 significant figures and are in bold. Concentrations for homologes are estimated to one significant figure.

Congener	Rel. RT*	Detection Limit	*D. delphis* *n* = 13	*L. acutus* *n* = 3	*P. phocoena* *n* = 1	*G. melas* *n* = 1	*Seals* *n* = 7
BLUBBER							
MBP-Br_7	**1.754**	**130**	**380±140**	**350±180**	**470±20**	**250±10**	**< d.l**
MBP-Br_6Cl-a	1.682	0.7	9±6	5±2	7±0.4	4±0.2	< d.l
MBP-Br_6Cl-b	**1.685**	**150**	**1000±300**	**1100±500**	**1100±100**	**490±40**	**< d.l**
MBP-HBr_6-a	1.543	3	20±10	6±2	9±1	8±1	< d.l
MBP-HBr_6-b	**1.554**	**59**	**350±130**	**250±100**	**280±20**	**170±10**	**< d.l**
MBP-HBr_6-c	1.637	3	10±10	6±6	20±5	10±3	< d.l
MBP-HBr_6-d	1.690	2	60±40	30±10	60±6	80±9	< d.l
MBP-HBr_5Cl-a	1.469	8	80±30	50±20	70±3	40±2	< d.l
MBP-HBr_5Cl-b	1.473	2	100±200	5±2	6±0.4	4±0.3	< d.l
MBP-HBr_5Cl-c	1.478	1	9±6	2±1	2±0.2	2±0.1	< d.l
MBP-HBr_5Cl-d	**1.483**	**73**	**520±180**	**560±180**	**650±40**	**310±20**	**< d.l**
MBP-HBr_5Cl-e	1.565	40	70±50	60±50	100±40	80±20	< d.l
MBP-HBr_5Cl-f	1.625	10	100±100	30±10	90±5	200±10	< d.l
ΣMBP			3000±1000	2000±1000	3000±200	2000±100	< d.l
CB-153	**1.380**	**62**	**1000±400**	**1400±500**	**1200±100**	**740±70**	**150±70**

(continued on next page)

Table 2. (continued)

LIVER							
MBP-Br$_7$	**1.754**	**100**	**260±210**	**570±610**	**< d.l.**	**140±30**	**< d.l.**
MBP-Br$_6$Cl-b	**1.685**	**1100**	**1500±1000**	**1600±1400**	**< d.l.**	**< d.l.**	**< d.l.**
MBP-HBr$_6$-b	**1.554**	**200**	**250±190**	**220±220**	**< d.l.**	**< d.l.**	**< d.l.**
MBP-HBr$_5$Cl-d	**1.483**	**800**	**<d.l.**	**<d.l.**	**< d.l.**	**< d.l.**	**< d.l.**
MBP-HBr$_5$Cl-e	1.565	40	50±50	100±100	80±20	40±10	< d.l.
ΣMBP			3000±2000	3000±3000	80±20	200±40	< d.l.
CB-153	**1.380**	**53**	**2400±1600**	**1500±500**	**< d.l.**	**1400±300**	**1100±1500**

* The relative retention times (Rel. RT) were determined under the conditions described in Experimental section 2.3, and were calculated relative to 2,2′,4,6-tetrachlorobiphenyl (CB-50).

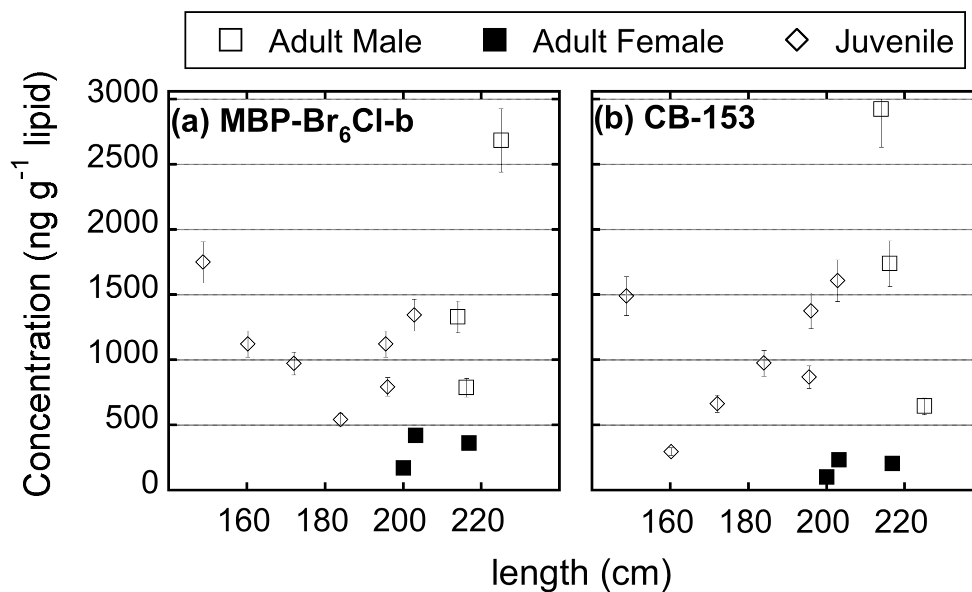

Figure 1. Lipid-normalized concentrations of (a) MBP-Br_6Cl-b and (b) CB-153 in the blubber of 13 common dolphins (*D. delphis*). Adult females are plotted with solid squares, adult males with open squares, and juveniles of both genders are plotted with open diamonds. Length (cm) is plotted on the x-axis as a proxy for the age of the individuals. Note that there is overlap between adult females and juvenile males on the x-axis. This is a function of both sexual dimorphism (the average length of mature females is smaller than that of mature males) and that females attain sexual maturity at a younger age, and therefore shorter length.

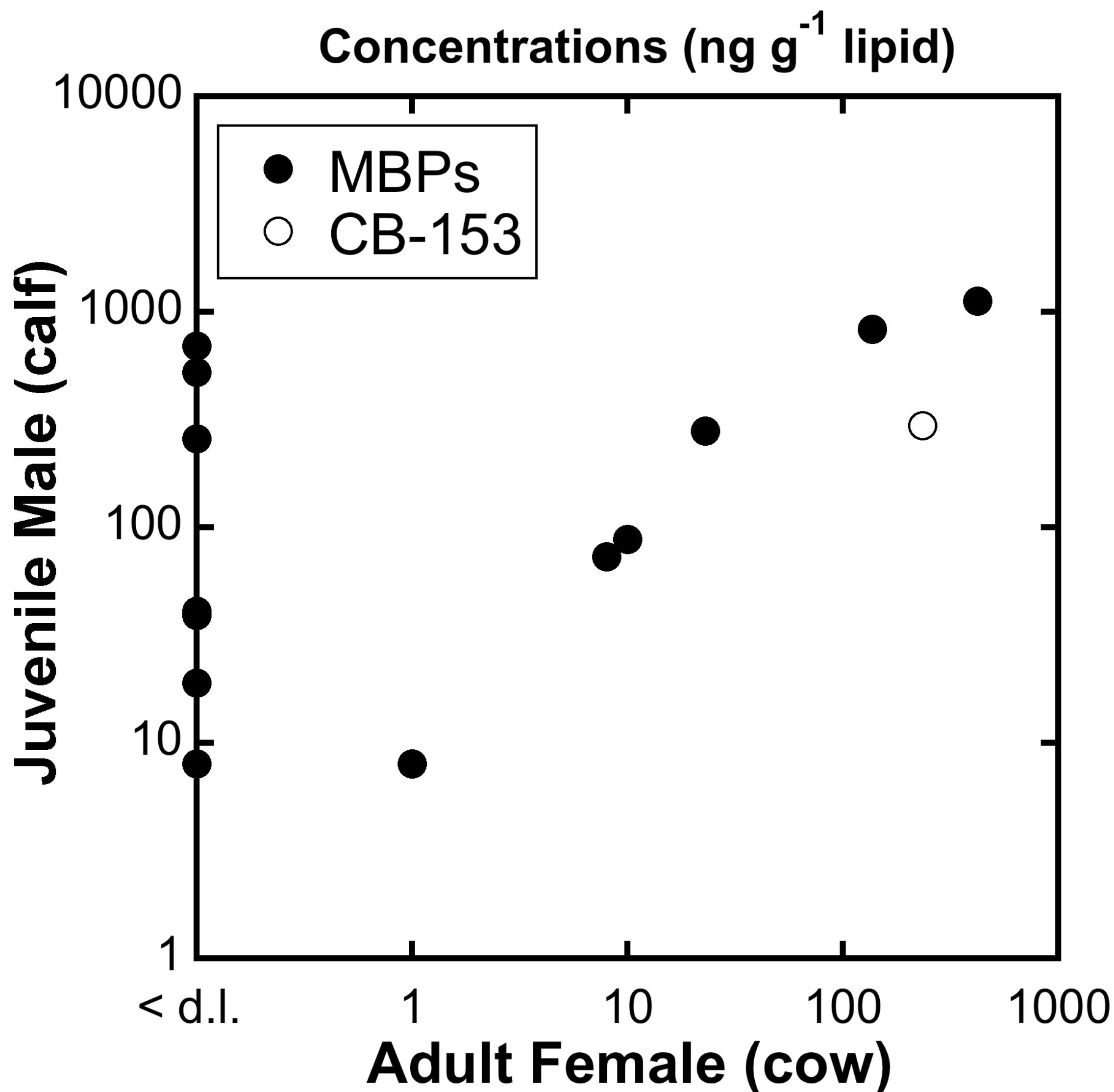

Figure 2. Lipid normalized concentrations of MBPs and CB-153 in the blubber of a pair of common dolphins (*D. delphis*) found stranded together. MBPs are plotted with solid circles and CB-153 is plotted with an open circle. Error bars are not visible as they are smaller than the circles. Many MBPs were below the detection limit (< d.l.) in the adult female (the presumed cow). However, the detection limits for these compounds fall along the line formed by the measured MBPs, and it is possible that the MBPs below the detection limit continue the trend established by the measured MBPs.

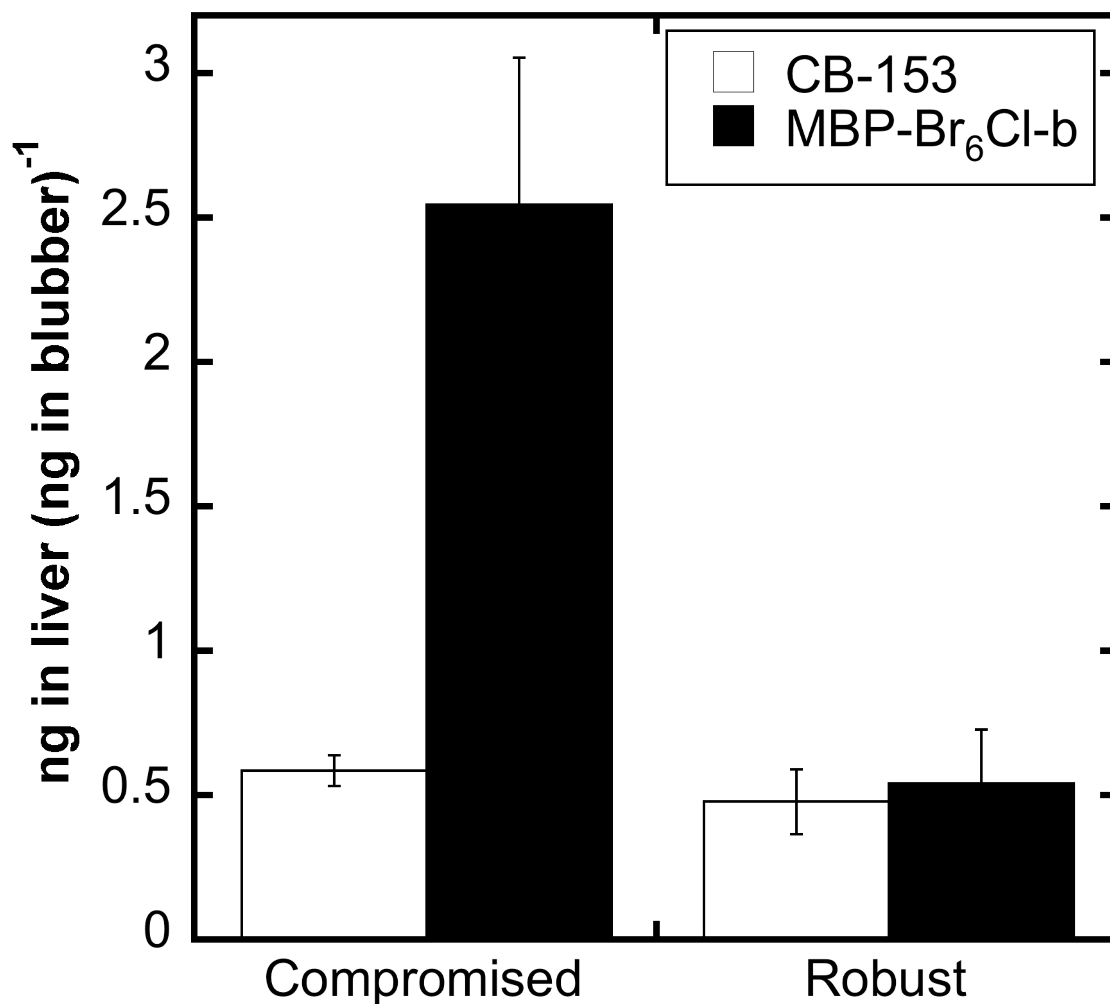

Figure 3. The mean (18 odontocetes) of liver concentrations relative to blubber concentrations (both lipid-normalized) of MBP-Br_6Cl (shaded bar) and CB-153 (open bar). Odontocetes are divided into two groups based on their state of health, those in a nutritionally-compromised state and those of robust health (Table 1).

BRIEF

Halogenated 1′-methyl-1,2′-bipyrroles were analyzed in liver and blubber tissues of 25 marine mammals; the patterns exhibited are consistent with biomagnification.

Supplemental Information (SI)

Paper title:	Distribution patterns suggest biomagnification of halogenated 1′-methyl-1,2′-bipyrroles (MBPs)
Paper authors:	Kristin C. Pangallo Christopher M. Reddy
Pages:	12
Tables:	S1-S6
Figures:	S1

Quality Controls: blanks, detection limit and error

Blanks were analyzed for both the blubber and liver methods, no MBPs or PCBs were detected in the blanks. The blubber blank consisted of vegetable oil and the liver blank was made of an ASE cell packed with combusted sand. Both types of blanks were spiked with the same internal standard in order to ensure full extraction.

The detection limit of the blubber and liver methods were determined separately. Seven replicates of 10% of a normal sample (0.10 g blubber oil and 0.25 g dried liver) were extracted and analyzed from a common dolphin (Cape Cod Stranding Network accession number: CCNS06-013Dd). The detection limit was calculated as three times the standard deviation of these seven replicates (1), thus it was positively correlated with the abundance of each congener in this sample. The detection limit had a range of 0.7-150 ng g^{-1} lipid for blubber samples and 40-1100 ng g^{-1} for liver samples (Table 1). For compounds that were not quantified, the detection limit calculation was performed on the relative abundance of the compound compared to the recovery standard, 2,2',4,6-tetrachlorobiphenyl (CB-50).

The error associated with the analysis was determined by running replicate samples from five mammals. For each individual mammal, three blubber replicates and two liver replicates were analyzed. The standard error of the mean relative to the mean concentration was calculated for each compound

of interest in each set of replicates. This relative standard error for each set of replicates was then averaged to give a generalized error for each compound of interest (Tables S3 and S5).

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Table S1. Descriptions of the individual mammals sampled in this study are presented. "NA" indicates that a data point was not acquired.

CCSN ID number	*Genus*	*species*	Common name	gender	age	length (cm)	weight (kg)	condition
CCSN05-316Dd	*Delphinus*	*delphis*	Common dolphin	M	NA	216.2	100	Robust
CCSN06-013Dd	*Delphinus*	*delphis*	Common dolphin	M	NA	195.5	NA	Robust
CCSN06-022La	*Lagenorhynchus*	*acutus*	Atlantic white-sided dolphin	F	Adult	205.0	135	Robust
CCSN06-024Dd	*Delphinus*	*delphis*	Common dolphin	F	Adult	216.8	110	NA
CCSN06-029Dd	*Delphinus*	*delphis*	Common dolphin	F	Adult	200.0	105	Robust
CCSN06-096Dd	*Delphinus*	*delphis*	Common dolphin	M	Subadult	184.0	60	Thin
CCSN06-119La	*Lagenorhynchus*	*acutus*	Atlantic white-sided dolphin	M	Subadult	193.0	100	Good
CCSN06-133Pg	*Phoca*	*groenlandica*	Harp seal	M	Male	160.0	91	Thin
CCSN06-137Dd	*Delphinus*	*delphis*	Common dolphin	M	NA	196.0	90	Thin
CCSN06-263Dd	*Delphinus*	*delphis*	Common dolphin	M	Yearling	160.2	46	Robust
CCSN06-264Dd	*Delphinus*	*delphis*	Common dolphin	F	Adult	203.2	72	Emaciated
CCSN07-022Gm	*Globicephala*	*melas*	Long-finned pilot whale	M	Subadult	352.0	560	Thin
CCSN07-023La	*Lagenorhynchus*	*acutus*	Atlantic white-sided dolphin	F	Subadult	182.5	64	Emaciated
CCSN07-036Dd	*Delphinus*	*delphis*	Common dolphin	M	Adult	214.0	135	Thin
CCSN07-040Dd	*Delphinus*	*delphis*	Common dolphin	M	NA	225.0	130	Robust
CCSN07-041Dd	*Delphinus*	*delphis*	Common dolphin	M	Adult	202.8	NA	Thin
CCSN07-063Pg	*Phoca*	*groenlandica*	Harp seal	F	Yearling	101.2	25	Thin
CCSN07-080Pg	*Phoca*	*groenlandica*	Harp seal	M	Juvenille	101.5	29	Thin
CCSN07-084Pg	*Phoca*	*groenlandica*	Harp seal	M	Yearling	101.0	22	Thin
CCSN07-109Dd	*Delphinus*	*delphis*	Common dolphin	M	Subadult	172.0	55	Emaciated
CCSN07-115Dd	*Delphinus*	*delphis*	Common dolphin	F	Subadult	148.8	NA	Emaciated
CCSN07-116Pg	*Phoca*	*groenlandica*	Harp seal	M	Yearling	98.5	25	Thin
CCSN07-129Hg	*Halichoerus*	*grypus*	Gray seal	M	Yearling	104.5	142	Thin
D07041	*Phocoena*	*phocoena*	Harbor porpoise	M	Subadult	126.0	35.0	Robust
D08742	*Halichoerus*	*grypus*	Gray seal	M	Juvenille	105.0	25.5	Thin

Table S2. The GC/ECNI-MS relative retention times and quantification ions of the compounds that are discussed in this study. These times are relative to CB-50 under the conditions described in the methods section (section 2.3). Concentrations of compounds in the upper section were quantified or estimated (Tables S3 and S5), the detection of those in the lower section is noted in Tables S4 and S5.

Compound	Relative Retention Time	Quantification Ion
CB-50	1.000	257.0
MBP-Br_7	1.754	697.7
MBP-Br_6Cl-a	1.682	653.7
MBP-Br_6Cl-b	1.685	653.7
MBP-HBr_6-a	1.543	619.7
MBP-HBr_6-b	1.554	619.7
MBP-HBr_6-c	1.637	619.7
MBP-HBr_6-d	1.690	619.7
MBP-HBr_5Cl-a	1.469	575.7
MBP-HBr_5Cl-b	1.473	575.7
MBP-HBr_5Cl-c	1.478	575.7
MBP-HBr_5Cl-d	1.483	575.7
MBP-HBr_5Cl-e	1.565	575.7
MBP-HBr_5Cl-f	1.625	575.7
CB-153	1.380	360.0
MBP-Br_5Cl_2-a	1.608	609.8
MBP-Br_5Cl_2-b	1.612	609.8
MBP-H_2Br_5-a	1.324	539.8
MBP-H_2Br_5-b	1.421	539.8
MBP-H_2Br_5-c	1.424	539.8
MBP-H_2Br_5-d	1.458	539.8
MBP-HBr_4Cl_2-a	1.393	531.8
MBP-HBr_4Cl_2-b	1.401	531.8
MBP-HBr_4Cl_2-c	1.405	531.8
MBP-H_2Br_4Cl-a	1.248	495.8
MBP-H_2Br_4Cl-b	1.339	495.8
MBP-H_2Br_4Cl-c	1.349	495.8
MBP-H_2Br_4Cl-d	1.385	495.8
MBP-Cl_7	1.225	385.7

Table S3. Individual concentrations (ng g^{-1} lipid) of CB-153 and the MBPs quantified or estimated from blubber are presented. Compounds for which a quantification standard is available were quantified and are given to 2 significant figures. Homologes of these compounds were estimated to one significant figure, as described in section 2.3. The method error for the measurement of each compound is given as ±X%, and the calculation is described on page S1.

CCSN ID number	MBP-Br_7 ±4%	MBP-Br_6Cl-a ±6%	MBP-Br_6Cl-b ±9%	MBP-HBr_6-a ±6%	MBP-HBr_6-b ±5%	MBP-HBr_6-c ±29%	MBP-HBr_6-d ±11%
CCSN05-316Dd	200	2	790	3	200	5	10
CCSN06-013Dd	520	9	1100	10	390	10	50
CCSN06-022La	550	7	1700	8	360	10	40
CCSN06-024Dd	<d.l.	1	360	<d.l.	82	<d.l.	9
CCSN06-029Dd	<d.l.	<d.l.	170	<d.l.	<d.l.	<d.l.	3
CCSN06-096Dd	130	2	540	5	190	3	20
CCSN06-119La	330	4	1000	6	230	4	30
CCSN06-133Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN06-137Dd	250	5	790	10	290	<d.l.	30
CCSN06-263Dd	700	8	1100	40	520	40	70
CCSN06-264Dd	<d.l.	1	420	<d.l.	<d.l.	<d.l.	9
CCSN07-022Gm	250	4	490	8	170	10	80
CCSN07-023La	170	2	650	3	150	<d.l.	20
CCSN07-036Dd	690	10	1300	20	520	<d.l.	40
CCSN07-040Dd	560	50	2700	100	1000	60	50
CCSN07-041Dd	690	10	1300	10	450	4	50
CCSN07-063Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN07-080Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN07-084Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN07-109Dd	260	4	970	10	250	5	70
CCSN07-115Dd	900	20	1800	40	570	40	300
CCSN07-116Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN07-129Hg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
D07041	470	7	1100	9	280	20	60
D08742	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.

Table S3. (continued)

CCSN ID number	MBP-HBr$_5$Cl-a ±4%	MBP-HBr$_5$Cl-b ±7%	MBP-HBr$_5$Cl-c ±7%	MBP-HBr$_5$Cl-d ±6%	MBP-HBr$_5$Cl-e ±24%	MBP-HBr$_5$Cl-f ±6%	CB-153 ±10%
CCSN05-316Dd	20	5	2	370	40	20	170
CCSN06-013Dd	90	10	4	710	70	70	870
CCSN06-022La	60	7	2	760	100	40	1100
CCSN06-024Dd	10	3	<d.l.	190	<d.l.	10	210
CCSN06-029Dd	<d.l.	<d.l.	<d.l.	70	<d.l.	<d.l.	100
CCSN06-096Dd	30	5	2	390	<d.l.	50	980
CCSN06-119La	50	5	2	560	70	40	1100
CCSN06-133Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	100
CCSN06-137Dd	60	10	40	62	<d.l.	60	1400
CCSN06-263Dd	90	20	8	830	300	300	300
CCSN06-264Dd	10	<d.l.	<d.l.	140	<d.l.	20	240
CCSN07-022Gm	40	4	2	310	80	200	740
CCSN07-023La	30	3	<d.l.	380	<d.l.	20	2100
CCSN07-036Dd	100	20	6	930	40	60	2900
CCSN07-040Dd	200	1000	40	340	50	400	650
CCSN07-041Dd	90	10	4	810	50	70	1600
CCSN07-063Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	250
CCSN07-080Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN07-084Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	130
CCSN07-109Dd	80	9	4	700	80	200	670
CCSN07-115Dd	200	30	10	1300	400	600	1500
CCSN07-116Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN07-129Hg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	270
D07041	70	6	2	650	100	90	1200
D08742	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.

Table S4. The detection of compounds in blubber for which concentrations were not quantified or estimated. Compounds above the detection limit (as described in page S1) are indicated with a (+), compounds below the detection limit are unmarked.

CCSN ID number	MBP-Br_5Cl_2-a	MBP-Br_5Cl_2-b	MBP-H_2Br_5-a	MBP-H_2Br_5-b	MBP-H_2Br_5-c	MBP-H_2Br_5-d
CCSN05-316Dd	+	+	+		+	+
CCSN06-013Dd	+	+	+	+	+	+
CCSN06-022La	+	+	+	+	+	+
CCSN06-024Dd	+		+	+	+	+
CCSN06-029Dd			+		+	
CCSN06-096Dd	+	+	+	+	+	+
CCSN06-119La	+	+	+	+	+	+
CCSN06-133Pg						
CCSN06-137Dd	+	+	+	+	+	+
CCSN06-263Dd	+	+	+	+	+	+
CCSN06-264Dd	+		+		+	
CCSN07-022Gm	+	+	+	+	+	+
CCSN07-023La	+	+	+		+	+
CCSN07-036Dd	+	+	+	+	+	+
CCSN07-040Dd	+	+	+	+	+	+
CCSN07-041Dd	+	+	+	+	+	+
CCSN07-063Pg						
CCSN07-080Pg						
CCSN07-084Pg						
CCSN07-109Dd	+	+	+	+	+	+
CCSN07-115Dd	+	+	+	+	+	+
CCSN07-116Pg						
CCSN07-129Hg						
D07041	+	+	+	+	+	+
D08742						

Table S4. (continued)

CCSN ID number	**MBP-HBr$_4$Cl$_2$-a**	**MBP-HBr$_4$Cl$_2$-b**	**MBP-HBr$_4$Cl$_2$-c**
CCSN05-316Dd		+	+
CCSN06-013Dd	+	+	+
CCSN06-022La	+	+	+
CCSN06-024Dd	+	+	+
CCSN06-029Dd			
CCSN06-096Dd	+	+	+
CCSN06-119La	+	+	+
CCSN06-133Pg			
CCSN06-137Dd	+	+	+
CCSN06-263Dd		+	+
CCSN06-264Dd		+	+
CCSN07-022Gm	+	+	+
CCSN07-023La	+	+	+
CCSN07-036Dd	+	+	+
CCSN07-040Dd	+	+	+
CCSN07-041Dd	+	+	+
CCSN07-063Pg			
CCSN07-080Pg			
CCSN07-084Pg			
CCSN07-109Dd	+	+	+
CCSN07-115Dd	+	+	+
CCSN07-116Pg			
CCSN07-129Hg			
D07041	+	+	+
D08742			

Table S4. (continued)

CCSN ID number	MBP-H_2Br_4Cl-a	MBP-H_2Br_4Cl-b	MBP-H_2Br_4Cl-c	MBP-H_2Br_4Cl-d	MBP-Cl_7
CCSN05-316Dd	+		+	+	+
CCSN06-013Dd	+	+	+	+	+
CCSN06-022La	+	+	+	+	+
CCSN06-024Dd	+	+	+	+	+
CCSN06-029Dd			+		
CCSN06-096Dd	+	+	+	+	+
CCSN06-119La	+	+	+	+	+
CCSN06-133Pg					
CCSN06-137Dd	+	+	+	+	+
CCSN06-263Dd	+	+	+	+	+
CCSN06-264Dd	+		+	+	+
CCSN07-022Gm	+	+	+	+	+
CCSN07-023La	+	+	+	+	+
CCSN07-036Dd	+	+	+	+	+
CCSN07-040Dd	+	+	+	+	+
CCSN07-041Dd	+	+	+	+	+
CCSN07-063Pg					
CCSN07-080Pg					
CCSN07-084Pg					
CCSN07-109Dd	+	+	+	+	+
CCSN07-115Dd	+	+	+	+	+
CCSN07-116Pg					
CCSN07-129Hg					
D07041	+	+	+	+	+
D08742					

Table S5. Individual concentrations (ng g^{-1} lipid) of CB-153 and the MBPs quantified or estimated from liver are presented. Compounds for which a quantification standard is available were quantified and are given to 2 significant figures. Homologes of these compounds were estimated to one significant figure, as described in section 2.3. The method error for the measurement of each compound is given as ±X%, and the calculation is described on page S1.

CCSN ID number	MBP-Br_7 ±21%	MBP-Br_6Cl-b ±22%	MBP-HBr_6-b ±22%	MBP-HBr_5Cl-d ±31%	MBP-HBr_5Cl-e ±19%	CB-153 ±10%
CCSN05-316Dd	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	630
CCSN06-013Dd	180	1300	210	<d.l.	<d.l.	1800
CCSN06-022La	100	<d.l.	<d.l.	<d.l.	<d.l.	1000
CCSN06-024Dd	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN06-029Dd	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	93
CCSN06-096Dd	<d.l.	<d.l.	<d.l.	<d.l.	40	820
CCSN06-119La	300	1700	210	<d.l.	80	1500
CCSN06-133Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN06-137Dd	550	3600	640	2000	100	820
CCSN06-263Dd	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	2600
CCSN06-264Dd	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	920
CCSN07-022Gm	140	<d.l.	<d.l.	<d.l.	40	1400
CCSN07-023La	1300	3000	460	940	300	2000
CCSN07-036Dd	860	3600	750	1700	40	13000
CCSN07-040Dd	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	4200
CCSN07-041Dd	310	2100	360	930	40	4400
CCSN07-063Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	6500
CCSN07-080Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	130
CCSN07-084Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	890
CCSN07-109Dd	<d.l.	1100	<d.l.	<d.l.	40	660
CCSN07-115Dd	1500	7300	1300	3700	400	1500
CCSN07-116Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN07-129Hg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	270
D07041	<d.l.	<d.l.	<d.l.	<d.l.	80	<d.l.
D08742	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.

Table S6. The detection of compounds in liver for which concentrations were not quantified or estimated. Compounds above the detection limit (as described in page S1) are indicated with a (+), compounds below the detection limit are unmarked.

CCSN ID number	MBP-H_2Br_5-a	MBP-H_2Br_4Cl-a	MBP-H_2Br_4Cl-c	MBP-H_2Br_4Cl-d	MBP-Cl_7
CCSN05-316Dd	+	+	+	+	+
CCSN06-013Dd	+	+	+	+	+
CCSN06-022La	+	+	+	+	+
CCSN06-024Dd	+	+	+	+	+
CCSN06-029Dd					
CCSN06-096Dd	+	+	+	+	+
CCSN06-119La	+	+	+	+	+
CCSN06-133Pg					
CCSN06-137Dd	+	+	+	+	+
CCSN06-263Dd	+		+		+
CCSN06-264Dd					
CCSN07-022Gm	+	+			
CCSN07-023La	+	+	+	+	+
CCSN07-036Dd	+	+	+	+	+
CCSN07-040Dd	+		+	+	+
CCSN07-041Dd	+	+	+	+	+
CCSN07-063Pg					
CCSN07-080Pg					
CCSN07-084Pg					
CCSN07-109Dd	+	+	+	+	+
CCSN07-115Dd	+	+	+	+	+
CCSN07-116Pg					
CCSN07-129Hg					
D07041	+	+	+	+	+
D08742					

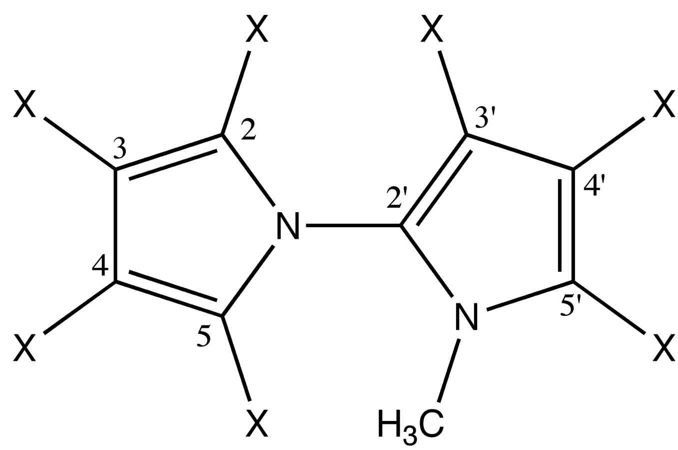

Figure S1. The general structure of the halogenated 1′-methyl-1,2′-bipyrroles. Here, the X substituent represents Br, Cl or H atoms.

CHAPTER 4

Marine natural products, the halogenated 1′-methyl-1,2′-bipyrroles (MBPs), appear to biomagnify in a Northwestern Atlantic food web

Abstract

Halogenated 1′-methyl-1,2′-bipyrroles (MBPs) are putative marine natural products that accumulate in marine mammal blubber in similar concentrations and patterns to biomagnifying pollutants. Here we measure concentrations of MBPs and 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153) in forty samples compromised of eight fish species, two squid species, and six species of marine mammals. To determine their trophic positions, and to further investigate influence of prey preference, we also measured the stable carbon and nitrogen isotopic compositions of all samples. Our results show that lipid-normalized MBP concentrations increase with increasing trophic level, which provides strong evidence that MBPs are another class of biomagnifying marine natural products. The presence of MBPs in pinniped prey and absence in pinniped blubber suggests that these mammals share dietary exposure to MBPs with odontocetes, but have an enhanced ability to metabolize these natural products. Finally, although both MBPs and CB-153 biomagnify in this food web, MBPs do

not display the same coupling to $\delta^{13}C$ as CB-153, further showing their natural origins.

1. Introduction

Halogenated 1′-methyl-1,2′-bipyrroles (MBPs) are marine natural products present in the tissues of marine mammals and other species occupying the highest trophic levels of marine food webs [1-3]. They are unusual among the halogenated natural products (HNPs) in their degree of halogenation, they contain up to seven bromines and/or chlorines on the bipyrrole backbone, and their *N,C1*-linkage, which is not found in other naturally-produced bipyrroles. The MBPs that are the most abundant in environmental samples contain six or seven halogens [2, 3]. Due to the number of halogens and their structure, MBPs have high estimated octanol-water partition coefficients ($K_{ow} = 10^{7.2}$-$10^{8.3}$) [4] and preferentially partition into lipid-rich reservoirs, such as blubber. These characteristics make them very similar to the persistent organic pollutants (POPs), and in this respect they are an untapped resource. By studying the pathways and fate of these HNPs through the environment, we can better understand the ultimate fate of the POPs in the marine environment.

POPs are now highly regulated in most countries due to their negative impacts on human and environmental health [5, 6]. However, due to their recalcitrance, concentrations remain elevated in some reservoirs, especially soil and sediment [7, 8]. Thus, these compounds continue to circulate within food

webs and concentrations remain high in top predators [9]. These high concentrations result from biomagnification, which is an increase in their lipid-normalized contaminant concentrations with increasing trophic level that results from a trophic transfer process [10]. Thus, predators have contaminant concentrations that are higher than what would be expected based on equilibrium partitioning between the surrounding environment (i.e., water, soil, or sediment) and biotic tissues [10]. The mechanisms and time-scales for removal of these contaminants from the biosphere are not yet clear [11].

Due to their structural similarities to POPs, it was suspected that some HNPs biomagnify; this has been confirmed for halogenated 1,1′-dimethyl-2,2′-bipyrroles (DMBPs) [12] and methoxylated polybrominated diphenyl ethers (MeO-PBDEs) [13]. Biomagnifying HNPs have evolved with their ecosystem and, presumably, have established routes of entry (biosynthesis) and exit (degradation and/or physical removal – e.g., burial). In fact, the enzymes found responsible for POP degradation may have evolved to degrade HNPs [14].

There is strong evidence suggesting that MBPs are another type of biomagnifying natural product. MBPs are present in marine mammal blubber in similar concentrations and in similar patterns to POPs known to biomagnify [3]. Additionally, MBPs have been detected in marine mammal prey (squid) [15, 16], fish oil [17], and human breast milk [18], suggesting that these compounds are transferred through food. Here we demonstrate that the lipid-normalized

concentration of MBPs increase with trophic level in the Northwestern Atlantic Ocean. In doing so, we have identified MBPs in nine additional species, many of which are commonly consumed by humans.

2. Methods

2.1 Sampling

The 20 marine mammals analyzed in this study consist of both odontocete cetaceans (toothed whales) and pinnipeds (earless seals). The odontocete samples include seven common dolphins (*Delphinus delphis*), four Atlantic white-sided dolphins (*Lagenorhynchus acutus*), one harbor porpoise (*Phocoena phocoena*) and one long-finned pilot whale (*Globicephala melas*). The pinniped samples are two gray seals (*Halichoerus grypus*) and five harp seals (*Phoca groenlandica*). These samples include males and females, and adult and youth specimens (Table 1 and [3]). All the samples were acquired through the Cape Cod Stranding Network, 18 were fatally stranded on Cape Cod from 2004-2007 and 2 were caught in fishing gear (the harbor porpoise and one grey seal) just south of New England. For additional details on the individual marine mammals, including weight, length, age, and nutritional status, see [3].

The 20 fish and squid analyzed in this study were obtained from the 2007 Fall Bottom Trawl Survey conducted by the National Marine Fisheries Ecosystems Survey Branch (Woods Hole, MA). Species were selected based on their role as marine mammal prey: longfin inshore squid (*Loligo pealeii*), northern

shortfin squid (*Illex illecebrosus*), Atlantic herring (*Clupea harengus*), Atlantic mackerel (*Scomber scombrus*), white hake (*Urophycis tenuis*), red hake (*Urophycis chuss*), silver hake (*Merluccius bilinearis*), Acadian redfish (*Sebastes fasciatus*), Atlantic cod (*Gadus morhua*), and haddock (*Melanogrammus aeglefinus*). The samples were collected opportunistically during the survey, and approximately 1 kg (wet weight) of whole fish was collected per species per tow. Two tows were selected per species for analysis, the tow number is listed after the species name in Table 2. Information regarding the date, location, duration, and depth of each tow is available as a Resource Survey Report [19].

Calanus spp. was collected by a vertical tow from the *R/V Tioga* in Cape Cod Bay on March 30, 2007. The sample was collected in the vicinity of North American Right Whales (*Eubalaena glacialis*), which feed on *Calanus finmarchicus*. *C. finmarcicus* is abundant in this region [20] and it is probable that it made up the majority of our sample. A 0.150 μm mesh net was used, and the tow lasted 2.5 minutes (water depth of 30 ft).

All samples were received and stored frozen. Prior to sub-sampling or initial homogenization they were thawed at room temperature and/or under lukewarm running tap water.

2.2 HOC extraction and purification

Blubber samples were extracted and HOCs purified following a previously published method [21]. Fish and squid samples consisted of 1 to >20

individual animals; all individuals from a sample were homogenized together (whole bodies) to provide sufficient biomass to acquire the ~10 g of total lipid extract (TLE) necessary to detect MBPs at these trophic levels. The whole bodies were homogenized with a manual, stainless-steel meat-grinder, freeze-dried, and then further homogenized with a mortar and pestle. The dried, homogenized biomass was packed into GreenChem extraction vessels, spiked with 635 ng of 2,2′,3,4,4′,5,5′-heptabromo-1′-methyl-1,3′-bipyrrole (1,3′-MBP-Br_7), then solvent extracted by a CEM MARS Xtraction (with Fiber Optic temperature and pressure control). The microwave program ramped to 100°C over 30 minutes, the power was set to 100% of 1600 W with a maximum pressure of 200 psi. The temperature was held at 100°C for 20 minutes, then the vessels were allowed to cool to approximately 35°C before they were vented. The contents of the vessels were filtered, 0.5% of filtrate was removed for gravimetric analysis to determine the total mass of the extract, and solvent was removed from the remaining extract by rotary evaporation. The masses of the fish sampled (wet and dry weight) and the lipid extracts are presented in the Appendix to this Chapter.

Lipids were removed by gel permeation chromatography (GPC). Our 3cm (o.d.) GPC columns were packed with 100g of SX-8 BioBeads (~45cm, uncompressed). We used a mobile phase of 1:1 dichloromethane:hexane and collected two fractions. The first fraction (0-150mL) that contained ~70% of the lipids was discarded; the second fraction (150-400mL), containing the remaining

lipids and the molecules of interest, was reduced in volume by rotary evaporation. The GPC procedure was repeated until < 0.5g lipids remained in the second fraction. The extract was further purified by silica/alumina column chromatography, as described in previous publications (Pangallo 2008, 2009). All samples were spiked with ^{13}C-labelled 1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene (DDE), and analyzed by gas chromatography/electron capture negative ion mass spectrometry (GC/ECNI-MS).

2.3 Detection and quantification by GC/ECNI-MS

Compounds were identified by gas chromatography mass spectrometry using electron capture negative ion mass spectrometry (GC/ECNI-MS) in full scan mode with a previously published method [21]. Briefly, extracts were injected in splitless mode and separated by a J & W Scientific DB-XLB column (60 m × 0.25 mm i.d., 0.25 μm film thickness) on an Agilent 6890N series GC interfaced to an Agilent 5973 network mass selective detector operated in full scan mode.

The position of halogens with an MBP homolog series has yet to be determined. Thus, congeners within a homolog series were designated by their halogen content plus a lower case letter. Details on our nomenclature system are available in our prior publications [3, 21], and consistency between the publications allows results to be easily compared. One exception is 2,3,3′,4,4′,5,5′-heptabromo-1′-methyl-1,2′-bipyrrole, which will be referred to as

Br_7-MBP-79. This is consistent with the nomenclature system suggested by Vetter et al. [2, 22] for MBPs with known structures.

In addition to the brominated and chlorinated congeners, the presence of iodinated congeners was also investigated. No iodinated MBPs were identified. However, the mass spectrometer scanned between m/z 50-750, thus the molecular ions of any congeners with greater than 4 iodine atoms would not be detected in our analysis. The method was not tested for the stability of iodinated congeners, so the absence from our samples is not conclusive evidence against their presence in this food web.

Four MBPs in our samples were identified and quantified by comparison with a synthetic standard (Br_7-MBP-79) or with MBPs isolated from marine mammal blubber and characterized by high resolution mass spectrometry and nuclear magnetic resonance spectroscopy (MBP-Br_6Cl-*b*, MBP-Br_6-*b*, and MBP-Br_5Cl-*d*). Recent work has established that the response factors of MBP homologs vary within an order of magnitude [23], which allows us to estimate concentrations (to one significant figure) for homologs of the four MBPs for which we have standards. These homologs of these congeners were tentatively identified by their relative retention times and mass spectra. For comparison with the MBPs, 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153) was quantified in our samples by comparison with a synthetic standard. A majority of the blubber concentration data has been published previously [3]; MBP and CB-153

concentrations in the six additional blubber samples (Table 3) and in the fish and squid samples (Table 2) are presented here.

2.4 Quality Control of MBP quantification

The extraction methods and quantification were monitored by running blank samples, determining method detection limits (MDLs) for each MBP and CB-153, and by replicate analyses to determine the overall method errors. No MBPs were detected in blanks and recoveries of 1,3'-MBP-Br_7 were 99±19%. MDLs were calculated by analyzing seven replicate samples at 10% of its original concentration; the MDL is defined as three times the standard deviation of this set of measurements [24]. For details on error calculations, see [3].

2.5 Stable isotope analysis

Homogenized *Calanus*, homogenized whole body tissue (fish and squid), and muscle tissue (marine mammals) were each freeze-dried and ground to a fine powder. Nitrogen isotope analyses were performed on unextracted tissue, but extracted tissue was used for the carbon isotope measurements since our samples included lipid-rich tissues. Lipids are depleted relative to cellular biomass [25], and variability in lipid content can obscure the $\delta^{13}C$ value of lipid-rich tissues [26]. Samples were analyzed in duplicate by isotope ratio mass spectrometry (IRMS) at the Stable Isotope Facility at the University of California at Davis. The stable isotope abundances are expressed in δ notation, which

measures the deviation of the sample from a standard reference material in parts per thousand (‰) according to the equation:

$$\delta^{15}\mathrm{N} \text{ or } \delta^{13}\mathrm{C} = \left[\left(R_{\mathrm{sample}} / R_{\mathrm{standard}}\right) - 1\right] \times 1000$$

where R is the ratio of the more rare isotope (^{15}N or ^{13}C) to the more abundant isotope (^{14}N or ^{12}C, respectively). The standard reference materials measured for $R_{standard}$ were atmospheric N_2 and PeeDee Belemnite for nitrogen and carbon, respectively.

3. Results and Discussion

3.1 MBP concentrations

MBPs were present in far lower concentrations in fish and squid (up to 47 ng g^{-1} lipid, Table 2) than odontocetes (up to 3700 ng g^{-1} lipid; Table 3, and [3]). This is consistent with previous research that found concentrations of 2-7 ng g^{-1} lipid in the squid species *L. pealeii* [4]. MBPs were consistently below the MDL in the pinniped samples, with only one exception (MBP-Br_5Cl-*f* was just above the MDL for one Harp seal). Among the non-mammalian samples, the squid, herring, and mackerel contained detectable levels of MBPs, though orders of magnitude below the concentrations observed in odontocetes. For the most part, MBPs were below the MDL for Acadian redfish, cod and haddock. There was a large amount of variability in MBP content of hake samples, but red hake had

lower MBP content than white and silver hake. In contrast, the abundance of CB-153 was highest in Acadian redfish, cod, haddock, and the hake species, and lowest in the squid, herring, and mackerel species. CB-153 was also present in the odontocete and most pinniped samples, again at abundances far exceeding those of the fish and squid samples.

The non-mammals did display characteristics that were unique from the marine mammals. Three MBP-Br_6Cl isomers (MBP-Br_6Cl-*c*, -*d*, and –*e*) were present in the fish and squid that have not previously been identified in mammals from this region (Figure 1). These isomers were present at extremely small, but consistent, abundances; and they elute after MBP-Br_6Cl-*b* and prior to Br_7-MBP-79. Because these congeners were not included in our MDL calculations, we can only report their presence in non-mammals and absence from mammalian blubber samples. It is also noteworthy that fewer MBP congeners were detected in the non-mammalian samples. This is likely because many of the MBP congeners detected in marine mammal blubber were found at very low levels. Thus, even if present in the environment, congeners at trace levels in higher trophic level samples would be well below the detection limits for the less contaminated lower trophic level samples.

3.2 MBP distributions in non-mammalian samples

The distribution of MBP congeners in non-mammals is similar to that in marine mammals. The most abundant MBP in a majority of the samples was

MBP-Br_6Cl-*b* (Figure 2), which is consistent with previous studies of MBPs in animals from this region [15, 27, 28]. In five of the fish and squid samples MBP-Br_5Cl-*d* was the most abundant congener. Aside from the dominance of these congeners, there is a large degree of variability in the abundances of MBPs congeners between species, but within species the patterns are similar. There is no separation geographically or vertically between species with and without MBPs (Table 2 and [29-38]). Therefore, MBPs absence from some species implies that these have a better ability to metabolize or otherwise excrete MBPs, or that MBPs are not present in their diets. This is in contrast to CB-153, which is present in every species, reflecting the ubiquity and persistence of POPs in the environment [39].

3.3 Stable isotope results

Both carbon and nitrogen stable isotope ratios were measured for every sample and there was excellent agreement between duplicates (Table 4). The $\delta^{15}N$ varied from +8.01‰ for *Calanus sp*. to 10.4±0.9‰ for squid, 12.6±0.9 for fish, and 14.2±1.2‰ for mammals. There was more overlap and a smaller range for the $\delta^{13}C$ values (-20.4‰ to -17.5‰).

3.4 Trophic level assessment by $\delta^{15}N$

In order to provide evidence that MBPs biomagnify, the trophic position of each sampled organism must be accurately assigned. Photosynthetic organisms occupy TL1, obligate herbivores are at TL2, and carnivores inhabit

≥TL3. However, many consumers are omnivores, preying upon more than one trophic level, and trophic positions are rarely delineated easily. Trophic level has traditionally been assessed by observation of foraging behavior, and/or examination of stomach and scat contents of the organisms of interest. These methods are problematic in that they only give a snapshot of possible predator-prey relationships, and are inconvenient for examining a marine food web due to difficulties in sampling [40]. More recently, stable nitrogen isotopic compositions have been used to examine the bioaccumulation of POPs [40, 41] and HNPs [12] within marine food webs. This method takes advantage of nitrogen isotope fractionation during heterotrophy and respiration. For every step up in trophic level, the nitrogen isotopic composition ($\delta^{15}N$) increases. Unlike identifying stomach contents, it integrates over the many different prey consumed, as well as over time [42]. Additionally, this method can account for dimorphism within a species, be it gender or age related.

We used a calculation modified from Hobson et al. (1995) to determine the trophic levels of our samples:

$$TL_{\text{sample}} = 2 + \left(\frac{\delta^{15}N_{\text{sample}} - \delta^{15}N_{Calanus}}{3.6‰} \right)$$

where TL_{sample} is the trophic level of the sample organism, $\delta^{15}N_{Calanus}$ is the nitrogen isotope value of the *Calanus* sample collected (8.01‰), and 3.6‰ is the trophic enrichment factor (Fry, 1988). *C. finmarchicus* species are opportunistic

omnivores, but their trophic level is generally around 2 [43]. Thus, our TL_{sample} is an estimate with uncertainty introduced by both the trophic enrichment factor (3.6‰) and the baseline value for TL2 ($\delta^{15}N$ = 8.01‰). This uncertainty does not affect our determination of biomagnification, which is based on a relative, not absolute, determination of trophic level. The trophic level calculated for fish, squid and mammal samples ranged from 2.4-4.4 (Table 4); thus, we analyzed animals from 3 different trophic levels.

3.5 Evidence for biomagnification of MBPs

In general, MBP concentrations were found to increase with trophic level (Figure 3), which suggests that MBPs may biomagnify in this food web (samples <MDL were not included). At the 95% significance level, the slope of the linear regression was significantly larger than zero for Br_7-MBP-79, MBP-Br_6Cl-*b*, MBP-Br_6-*b*, MBP-Br_5Cl-*d*, and MBP-Br_5Cl-*e* (Table 5). Biomagnification cannot be confirmed until it is clear this concentration increase results from a trophic transfer process. Our measurements show that CB-153 also appears to biomagnify in this food web.

Food web magnification factors (FWMFs) are used to compare the magnitude of biomagnification and are calculated using the slope of the linear regression of the natural log of the concentration versus trophic level [41]:

$$\text{FWMF} = e^{\text{slope}}$$

(Only samples above the detection limit were used in the calculation of the slope.) The FWMFs calculated for the MBPs are quite high (Table 5), ranging from 15 to 82, while CB-153 has the lowest calculated FMWF (9.0). For comparison, FWMFs for DMBPs in the Arctic vary from 5.2 to 14.6 [12], and POPs also tend to have FWMFs in this range [41, 44, 45]. Polybrominated diphenyl ethers (PBDEs) containing 3-7 bromines have FWMFs that are much lower (~1.5) [46]. However, PBDE biomagnification factors (concentration in organism relative to the concentration in diet, equivalent to the FWMF but for only two trophic levels) have been shown to extend up to 76 [47]; these values are consistent with our findings. Numerical modeling investigations of POPs in food webs that include mammals demonstrate biomagnification factors of up to 49 [48]. Additionally, the value calculated for CB-153 agrees well with a previous FWMF calculation of this compound in a marine food web (9.8) [41]. It is important to note that because of the exponential relationship between slope and the FWMF, small variations in the slope result in large changes in the FWMF value [44]. For our compounds, high FWMFs are associated with fewer data points and greater uncertainty (Table 5). Regardless, it is clear that MBPs have FWMFs greater than 1, which supports the hypothesis that MBPs biomagnify in this marine food web.

3.6 Pinniped anomaly

Although MBPs and CB-153 are both present in odontocetes and non-mammals, there are distinct differences in their abundances in pinnipeds. Pinnipeds have much lower body burdens of MBPs than would be expected for their trophic level, yet levels of CB-153 are comparable to those of odontocetes (Tables 3 and 4, and [3]). Previous studies have seen this same discrepancy between POPs and naturally produced compounds in pinnipeds [1, 12, 13]. The cause may be in pinniped diets, and/or in their ability to metabolize HNPs. To examine if foraging preference is responsible for the difference in MBP body burdens, we targeted prey species of the mammals included in this study for inclusion in our analyses.

The marine mammals consume a wide variety of prey [49] and their diets likely contain a similar spectrum of the local prey species. However, studies also indicate that they have distinct prey preferences, as observed from foraging behavior, and stomach and scat contents. The dolphins, *L. acutus* and *D. delphis*, are known to focus their feeding on squid, mackerel, silver hake, and herring [49, 50]. Harbor porpoises, *P. phocoena*, have similar preferences, targeting squid, herring, silver hake, and red/white hake [51, 52]. Squid make up a large portion of pilot whales' diets (~75%, [49]), with the remaining 25% from pelagic fish such as mackerel and, to a lesser extent, cod and hake [53]. In contrast to the odontocetes, both pinniped species in our study consume relatively small

amounts of squid (5%, [49]). Harp seals (*P. groenlandica*) preferentially target polar cod, capelin, amphipods, halibut and herring [54-56]. Gray seals (*H. grypus*) focus on red/white hake, winter flounder, skate, cusk eel, and sand lance [57].

Although we were unable to analyze all prey species of interest, it is noteworthy that mammals with high MBP body burdens preferentially consume large quantities of the prey species that have high MBP concentrations: squid, mackerel and herring. Pinnipeds also consume herring and squid, and therefore do consume MBP-rich prey, yet MBPs are generally far below detection limits in pinniped blubber. Thus, prey preference is not sufficient to explain the dramatic differences between odontocete and pinniped MBP concentrations.

Differences between odontocetes and pinnipeds in the blubber structure may also play a role. Odontocetes have more highly stratified blubber, resulting in outer layers being much colder and less metabolically active. Pinniped blubber, however, is far less stratified, and thus the depth of the blubber is metabolically active. Thus, contaminants in pinniped blubber may be more available for metabolism and removal from the body, while contaminants in odontocete blubber would likely have longer lifetimes.

The presence of CB-153 in blubber of both groups of marine mammals, however, suggests that blubber morphology alone cannot explain the differences between MBPs and CB-153. This is consistent with previous research showing

that pinnipeds with low body burdens of HNPs still contain high concentrations of POPs [1, 3, 12, 13]. As a result, the data presented here strengthens the hypothesis originally put forward by Tittlemier et al. [12], that pinnipeds have an enhanced capability to degrade HNPs relative to odontocetes. If this is the case, then pinniped enzymes used in HNP metabolism cannot adapt, or have not yet adapted, to degrade CB-153 as efficiently as MBPs. Previous investigations into metabolism of PCB congeners have identified that differences in cytochrome P450 enzymes in cetaceans and phocid seals can help explain differences in the metabolism of different types of PCB congeners between these mammalian orders [58]. Phocid seals have a greater ability to metabolize PCB congeners with vicinal H atoms (H atoms adjacent to each other on an aromatic ring system) in the *ortho-* and *meta-*positions, but cetaceans have a greater ability to metabolize PCB congeners with vicinal hydrogens in the *meta-* and *para-* positions [58]. However, since these differences relate to the position of vicinal H atoms, and the MBPs investigated here do not contain vicinal hydrogen atoms, the difference in P450 enzymes between cetaceans and phocid seals cannot explain our results. Thus, investigating differences between the enzymes responsible for MBP metabolism in both groups of marine mammals would provide valuable information on the metabolism of perhalogenated compounds and/or compounds with no vicinal H atoms.

3.7 Differences between MBPs and CB-153

The concentrations of both MBPs and CB-153 both increase with increasing trophic level. On closer examination, however, differences between the PCB and the natural products are apparent. MBPs strongly correlate (ρ>0.9) with each other; the correlation of MBPs with CB-153, while still statistically significant, is weaker (Figure 4, Table 6). (Please note, although the data appear to fall within two groupings, all of the assumptions to statistically test for correlation are met within this data set.) This is consistent with a previous study by Stapleton et al. [59] that showed significant correlations between HNPs, but not between HNPs and POPs. The exception is MBP-Br_5Cl-*e*, which is not as well correlated with either the MBPs or CB-153. This is likely caused by the large error term for in the quantification of this compound (24%), which is much larger than those for the other MBP congeners and CB-153 (Tables 2 and 3, and [3]). For these analyses, both compounds are required to be above the MDL, therefore the pinniped samples were excluded and cannot explain this variation. In fact, the pattern is present in both marine mammals and in fish and squid.

In the non-mammalian samples the difference in correlation strength can be partially explained by examining the stable carbon isotope ratios. A well-established relationship exists between stable carbon isotope ratios and carbon source: the pelagic food web is depleted (-22‰) relative to that of the inshore/benthic food web (-17‰) [60-63]. CB-153 is highest in non-mammalian samples that are the most enriched in ^{13}C and lowest in those that are most ^{13}C

depleted (Figure 5). This pattern is consistent with a terrestrial source of the anthropogenic PCB. In the non-mammalians samples there are two data points for CB-153 that are much higher than those of the other fish, these are for the cod samples. These data points may have disproportionate influence on this trend, but are valid data points in themselves. Thus, we include these points for analysis, but the limitations of this data should be noted. There is no similar relationship between $\delta^{13}C$ and the MBPs, which is not surprising since they have no terrestrial source. The relationship between $\delta^{13}C$ and CB-153 is absent in the mammal samples (Figure 5), which reflects mammals diets of both pelagic and benthic prey [49]. Their mixed diets are also evident in their $\delta^{13}C$ values, which span a more narrow range (relative to non-mammalian samples) from -19.1‰ to -17.5‰ (Table 4).

By measuring an increase in the lipid-normalized MBP concentrations with increasing trophic level, we present strong evidence for the biomagnification of another class of marine natural products. Thus, there is greater confidence in the use of MBPs as natural analogues for POPs in marine ecosystems. MBPs are present in various fish and squid species, though at much lower concentrations than those found in odontocete blubber. The presence of MBPs in pinniped prey and absence in pinniped blubber suggests that these mammals share dietary exposure to MBPs with odontocetes, but have an enhanced ability to metabolize these natural products. Finally, although both

MBPs and CB-153 biomagnify in this food web, MBPs do not display the same coupling to $\delta^{13}C$ as CB-153, which is a further indication of their natural origins.

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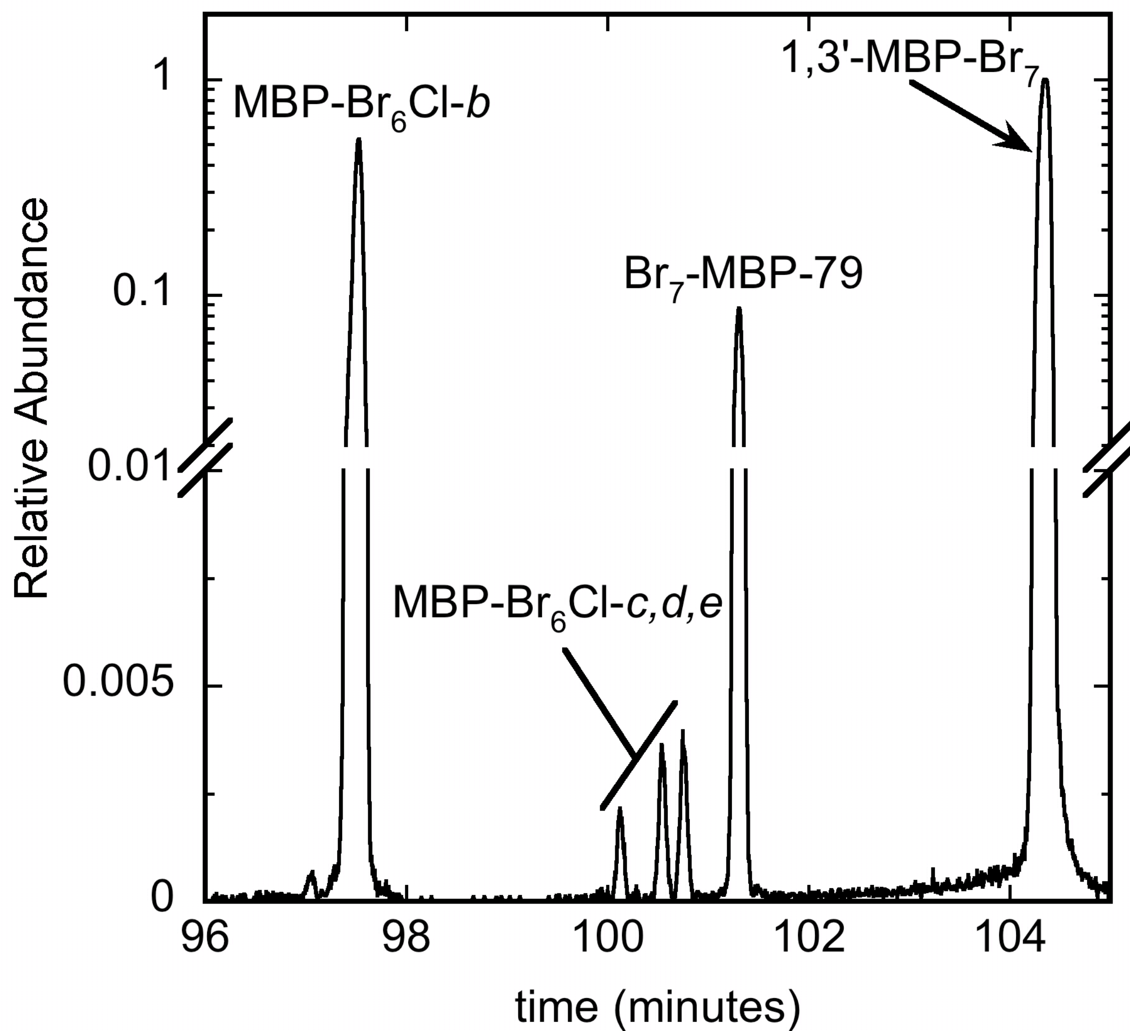

Figure 1. The partial summed ion chromatogram (ions ±0.5: 575.7, 619.7, 653.7, and 697.7) of the Silver Hake 165 extract. The break in the y-axis highlights the newly identified hexabromochloro-MBP congeners, MBP-Br_6Cl-*c*, MBP-Br6Cl-*d*, and MBP-Br6Cl-*e*. Also identified are MBP-Br_6Cl-*b*, which is the most abundant MBP in this sample, MBP-Br_7, and our internal standard, 1,3′-MBP-Br_7.

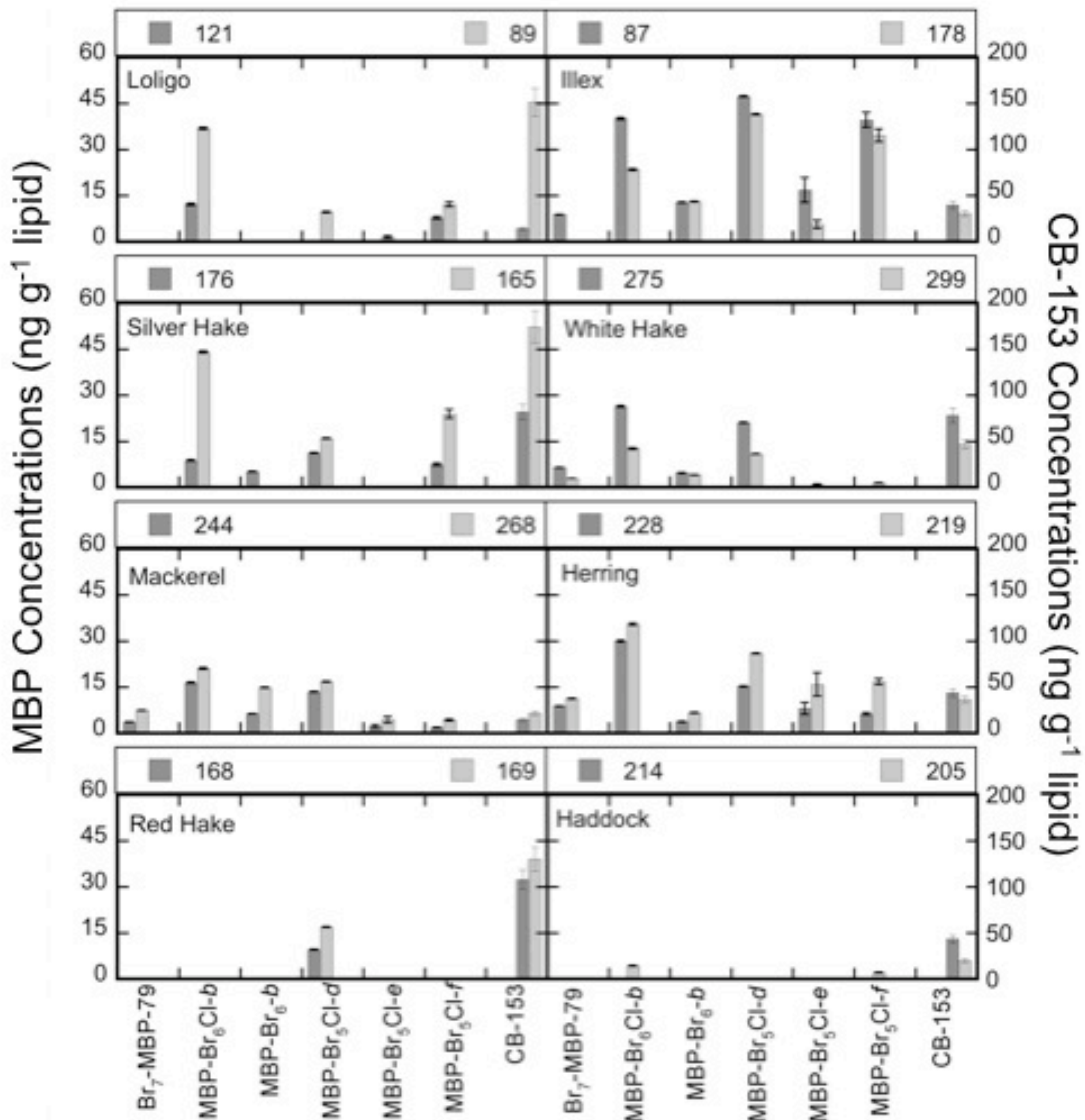

Figure 2. The distribution of MBPs and CB-153 in select non-mammalian samples analyzed in this study (species for which no MBPs were detected – cod and Acadian redfish – are excluded). MBPs are plotted on the left hand axis, and CB-153 concentrations are plotted on the right hand axis. Note the consistent presence of either MBP-Br_6Cl-*b* and MBP-Br_5Cl-*d* in each sample.

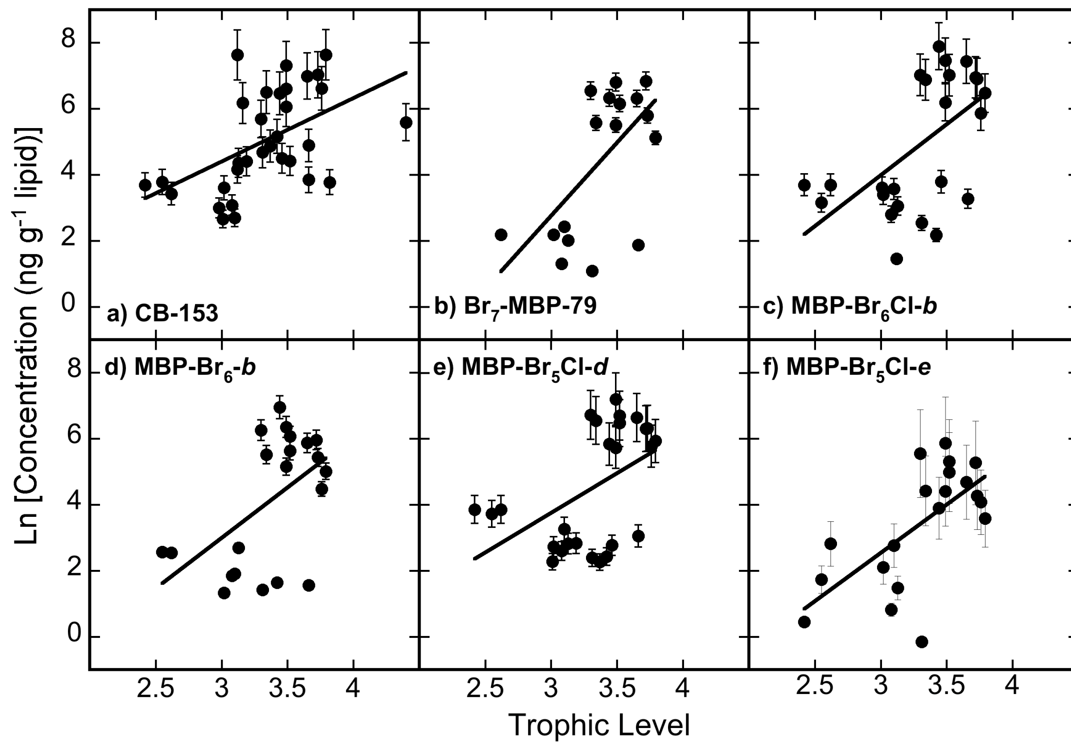

Figure 3. Biomagnification of MBPs and CB-153 is evident by the increase in (lipid-normalized) concentration with increasing trophic level. Here we present the compounds that biomagnify (slopes significantly greater than 0) (a) CB-153, (b) Br_7-MBP-79, (c) MBP-Br_6Cl-*b*, (d) MBP-Br_6-*b*, (e) MBP-Br_5Cl-*d*, and (f) MBP-Br_5Cl-*e*. Regression equations and statistical significance are presented in Table 5.

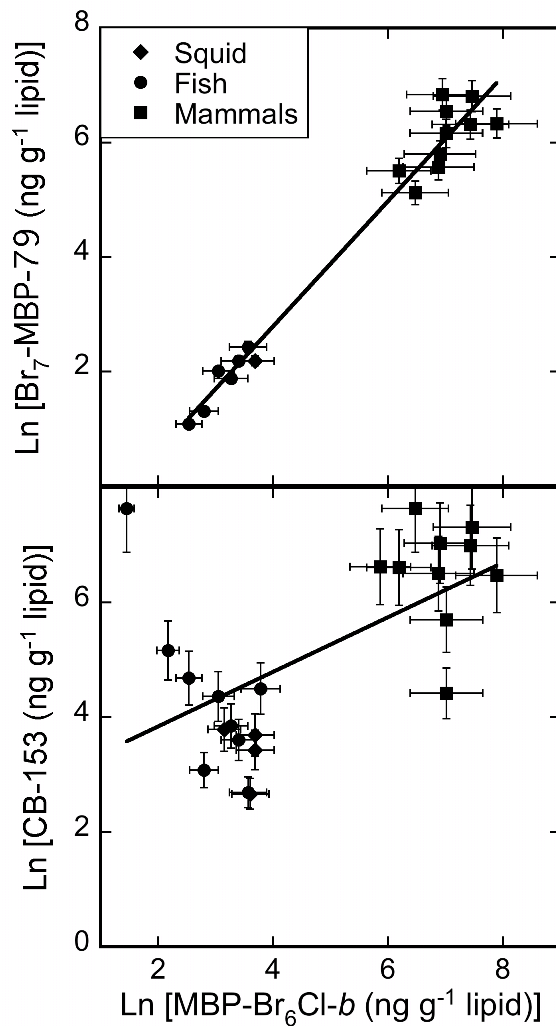

Figure 4. A stronger correlation was found between MBP congeners (top panel) than was found between the MBPs and CB-153 (lower panel).

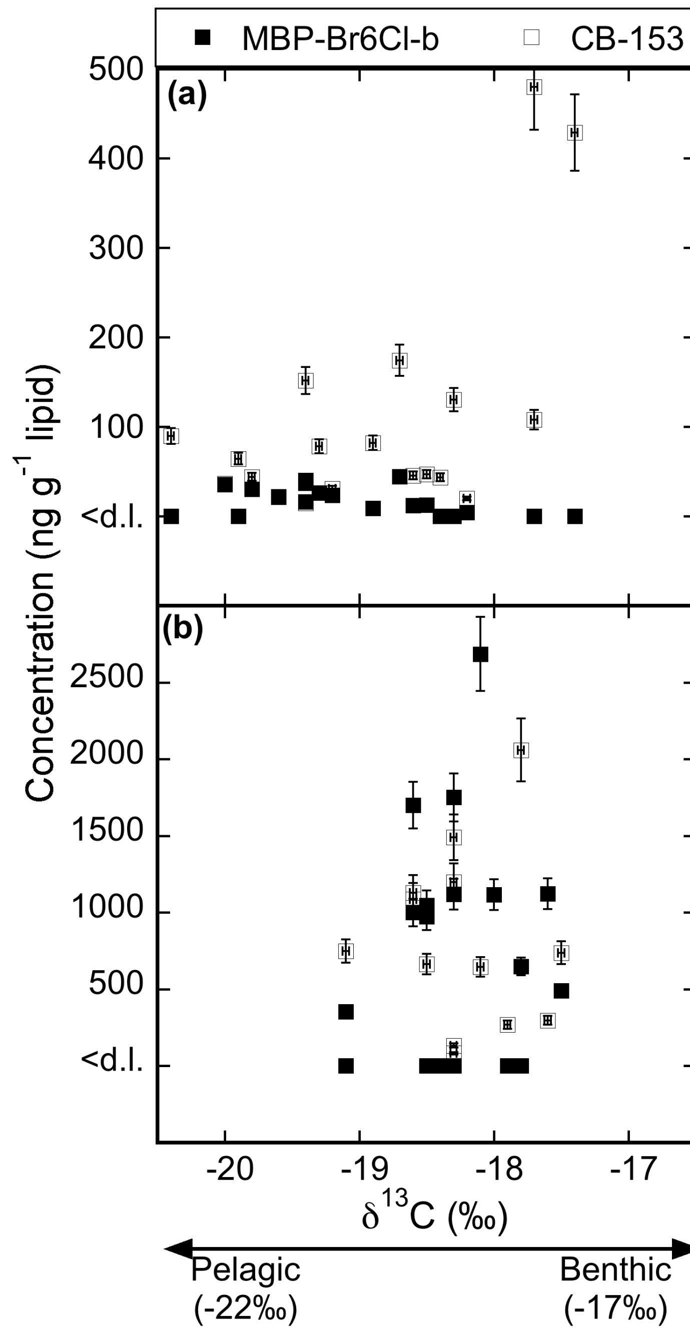

Figure 5. CB-153 and MBP-Br_6Cl-*b* vs. $\delta^{13}C$ in (a) non-mammals and (b) mammals. More enriched (more positive) stable carbon isotope ratios are indicative of a benthic origin (-17‰), and less enriched (more negative) values are indicative of pelagic origin (-22‰) [63]

Table 1. Description of mammals at stranding for the six mammals included in this study and not published previously. For the other mammals in this study, please see [3].

CCSN ID number	gender	age	length (cm)	weight (kg)	condition
CCSN06-019La	male	adult	280	116	robust
CCSN04-218Dd	male	calf	139.2	32	robust
CCSN06-144Dd	male	not recorded	187	not recorded	robust
CCSN07-074Dd	female	adult	201.1	99.8	robust
CCSN07-066Pg	female	juvenile	108	25	thin
CCSN07-076Pg	female	adult	104	63.5	thin

Table 2. Lipid-normalized whole body concentrations (ng g^{-1} lipid) of quantifiable MBPs and CB-153 in non-mammilian samples. The three-digit number in the sample ID is the tow from which the sample was collected; <d.l. indicates that a compound was lower than the detection limit in a sample.

Sample ID	**Br_7-MBP-79 ±4%**	**MBP-Br_6Cl-*b* ±9%**	**MBP-Br_6-*b* ±5%**	**MBP-Br_5Cl-*d* ±6%**	**MBP-Br_5Cl-*e* ±24%**	**MBP-Br_5Cl-*f* ±6%**	**CB-153 ±10%**
MDL (ng µL^{-1})	***2.0***	***2.2***	***.87***	***1.1***	***0.6***	***0.2***	***0.26***
Loligo 121	<d.l.	12	<d.l.	<d.l.	<d.l.	8	46
Loligo 89	<d.l.	37	<d.l.	9.7	2	10	150
Illex 87	8.9	40	13	47	20	40	40
Illex 178	<d.l.	23	13	41	6	30	31
Herring 228	8.9	30	3.8	15	8	6	44
Herring 219	11	36	6.7	26	20	20	37
Mackerel 244	3.7	16	6.3	13	2	2	15
Mackerel 268	7.5	21	15	17	4	4	22
White Hake 275	6.5	26	4.8	21	<d.l.	<d.l.	78
White Hake 299	3.0	13	4.1	11	.9	1	47
Red Hake 168	<d.l.	<d.l.	<d.l.	9.6	<d.l.	<d.l.	110
Red Hake 169	<d.l.	<d.l.	<d.l.	17	<d.l.	<d.l.	130
Silver Hake 176	<d.l.	8.8	5.1	11	<d.l.	8	82
Silver Hake 165	<d.l.	44	<d.l.	16	<d.l.	20	170
Acadian Redfish 258	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	90
Acadian Redfish 257	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	64
Cod 326	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	480
Cod 324	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	430
Haddock 214	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	43
Haddock 205	<d.l.	4.3	<d.l.	<d.l.	<d.l.	2	20

Table 3. MBP and CB-153 concentrations (ng g^{-1} lipid) in the blubber of mammals in this study not previously reported. For the other mammals in this study, please see [3].

ID number	Species	Br_7-MBP-79 ±4%	MBP-Br_6Cl-*a* ±6%	MBP-Br_6Cl-*b* ±9%	MBP-Br_6-*a* ±6%	MBP-Br_6-*b* ±5%	MBP-Br_6-*c* ±29%	MBP-Br_6-*d* ±11%
	MDL (ng g^{-1} lipid)	***130***	***0.7***	***150***	***3***	***59***	***3***	***2***
CCSN06-019La	*L. acutus*	940	13	1000	30	390	40	40
CCSN04-218Dd	*D. delphis*	68	<d.l.	350	<d.l.	88	<d.l.	5
CCSN06-144Dd	*D. delphis*	470	4	1100	30	430	20	4
CCSN07-074Dd	*D. delphis*	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l	<d.l.
CCSN07-066Pg	*P. groenlandica*	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
CCSN07-076Pg	*P. groenlandica*	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.

Table 3. (continued)

CCSN ID number	MBP-Br_5Cl-*a* ±4%	MBP-Br_5Cl-*b* ±7%	MBP-Br_5Cl-*c* ±7%	MBP-Br_5Cl-*d* ±6%	MBP-Br_5Cl-*e* ±24%	MBP-Br_5Cl-*f* ±6%	CB-153 ±10%
MDL (ng g^{-1} lipid)	***8***	***2***	***1***	***73***	***40***	***10***	***62***
CCSN06-019La	51	7	9	550	200	200	not recorded
CCSN04-218Dd	20	3	1	320	60	100	750
CCSN06-144Dd	70	10	10	810	200	300	not recorded
CCSN07-074Dd	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	83
CCSN07-066Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	not recorded
CCSN07-076Pg	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	26	not recorded

Table 4. The stable isotopic ratios (the average of duplicate analyses) and the calculated trophic levels are shown for all samples. *We assume that our *Calanus* sample occupies TL2.

Sample ID	Species (Common name)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	TL
Calanus sp.	Zooplankton	8.01	-19.8	2.0*
Loligo 121	Longfin inshore squid	11.6	-18.6	3.0
Loligo 89	Longfin inshore squid	9.53	-19.4	2.4
Illex 87	Northern shortfin squid	10.3	-19.4	2.6
Illex 178	Northern shortfin squid	10.0	-19.2	2.6
Herring 228	Atlantic herring	11.7	-19.8	3.0
Herring 219	Atlantic herring	12.0	-20.0	3.1
Mackerel 244	Atlantic mackerel	11.9	-19.4	3.1
Mackerel 268	Atlantic mackerel	12.1	-19.6	3.1
White Hake 275	White Hake	14.0	-19.3	3.7
White Hake 299	White Hake	12.7	-18.5	3.3
Red Hake 168	Red Hake	12.9	-17.7	3.4
Red Hake 169	Red Hake	12.3	-18.3	3.2
Silver Hake 176	Silver Hake	13.1	-18.9	3.4
Silver Hake 165	Silver Hake	13.3	-18.7	3.5
Acadian Redfish 258	Acadian Redfish	12.0	-20.4	3.1
Acadian Redfish 257	Acadian Redfish	12.2	-19.9	3.2
Cod 326	Cod	13.4	-17.7	3.5
Cod 324	Cod	14.6	-17.4	3.8
Haddock 214	Haddock	11.5	-18.4	3.0
Haddock 205	Haddock	12.0	-18.2	3.1
CCSN07-023La	Atlantic white-sided dolphin	14.5	-17.8	3.8
CCSN06-019La	Atlantic white-sided dolphin	14.2	-18.5	3.7
CCSN04-218Dd	Common dolphin	14.3	-19.1	3.8
CCSN07-115Dd	Common dolphin	13.4	-18.3	3.5
CCSN07-109Dd	Common dolphin	12.8	-18.5	3.3
CCSN06-119La	Atlantic white-sided dolphin	14.2	-18.6	3.7
CCSN06-144Dd	Common dolphin	13.5	-18.0	3.5
CCSN06-263Dd	Common dolphin	12.7	-17.6	3.3
CCSN06-022La	Atlantic white-sided dolphin	14.0	-18.6	3.7
CCSN07-040Dd	Common dolphin	13.2	-18.1	3.4
CCSN07-074Dd	Common dolphin	13.5	-18.3	3.5
DO7041	Harbor porpoise	13.5	-18.3	3.5
CCSN07-022Gm	Pilot whale	13.4	-17.5	3.5
CCSN07-116Pg	Harp seal	15.6	-18.5	4.1
CCSN07-066Pg	Harp seal	13.6	-18.4	3.6
CCSN07-129Hg	Gray seal	16.6	-17.9	4.4
DO8742	Gray seal	16.7	-17.8	4.4
CCSN07-080Pg	Harp seal	15.1	-19.1	4.0
CCSN07-084Pg	Harp seal	14.0	-18.3	3.7
CCSN07-076Pg	Harp seal	15.4	-18.3	4.1

Table 5. The number of data points (*n*), regression equation, significance level (*p*-value), food web magnification factor (FWMF) and log K_{ow}s are given for each compound in Figure 1.

Compound	*n*	Regression Equation *y* =	*p*-value	FWMF	Log K_{ow} [b]
Br_7-MBP-79	18	4.4*x* – 11	.006	82	8.3
MBP-Br_6Cl-*b*	25	3.2*x* – 5.7	.003	25	8.1
MBP-Br_6-*b*	21	3.1*x* – 6.2	.014	22	7.4
MBP-Br_5Cl-*d*	25	2.7*x* – 4.4	.005	15	7.2
MBP-Br_5Cl-*e*	20	2.9*x* – 6.3	.002	18	7.2
CB-153	33	2.2*x* – 2.1	<.001	9.0	6.9

[a]From [4].

Table 6. Correlation matrix for the compounds in Table 3, calculated with SPSS, PASW® Statistics. The Pearson correlation coefficient (ρ), significance level (p-value) and number of data points (n) are presented.

		MBP-Br_7	MBP-Br_6Cl-*b*	MBP-Br_6-*b*	MBP-Br_5Cl-*d*	MBP-Br_5Cl-*e*	CB-153
MBP-Br_6Cl-*b*	ρ	.987	1				
	p-value (1-tailed)	.000					
	n	18	25				
MBP-Br_6-*b*	ρ	.977	.978	1			
	p-value (1-tailed)	.000	.000				
	n	18	21	21			
MBP-Br_5Cl-*d*	ρ	.977	.963	.963	1		
	p-value (1-tailed)	.000	.000	.000			
	n	18	23	21	25		
MBP-Br_5Cl-*e*	ρ	.945	.910	.895	.954	1	
	p-value (1-tailed)	.000	.000	.000	.000		
	n	17	20	19	20	20	
CB-153	ρ	.918	.908	.869	.843	.788	1
	p-value (1-tailed)	.000	.000	.000	.000	.000	
	n	16	23	19	23	18	33

CHAPTER 4 APPENDIX

Mass of the total lipid extract (TLE), dry mass, and wet mass for all non-mammalian samples.

Sample ID	Mass extracted (g)		
	TLE	dry mass	wet mass
Loligo 121	12.6	139.3	594.3
Loligo 89	12.8	164.9	761.8
Illex 87	14.2	119.4	not recorded
Illex 178	7.98	69.7	263.4
Herring 228	19.7	69.7	283.9
Herring 219	30.5	139.3	453.9
Mackerel 244	23.1	109.5	428.3
Mackerel 268	19.2	129.4	907.6
White Hake 275	13.9	69.7	291.6
White Hake 299	40.7	199.0	not recorded
Red Hake 168	8.16	89.6	376.5
Red Hake 169	2.67	80.6	not recorded
Silver Hake 176	11.2	not recorded	162.4
Silver Hake 165	4.65	not recorded	91.9
Acadian Redfish 258	29.5	179.4	690.5
Acadian Redfish 257	33.5	207.9	794.0
Cod 326	6.16	181.2	not recorded
Cod 324	10.3	184.6	886.1
Haddock 214	11.3	199.0	990.9
Haddock 205	22.2	139.3	679.0

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CHAPTER 5

$\delta^{15}N$ enrichment suggests possible source for halogenated 1′-methyl-1,2′-bipyrroles (MBPs)

Abstract

Highly halogenated 1′-methyl-1,2′-bipyrroles are natural products that bioaccumulate in upper trophic levels of the marine food web. Here we demonstrate that they are dramatically enriched in $\delta^{15}N$ (+20-30‰). This ^{15}N-enrichment is greater than that seen for other biosynthetic organic compounds measured to date. We argue that this enrichment likely stems from enriched precursors and/or fractionation during biosynthesis, and is not the product of MBP degradation. We also consider possible sources of MBPs in light of these results.

1. Introduction

Halogenated 1′-methyl-1,2′-bipyrroles (MBPs) are a class of marine natural products. As their name describes, they are made from two pyrrole rings linked by an *N,C1*-bond, and they have up to seven halogens (bromine or chlorine) on the ring carbons (Figure 1). Originally identified in marine mammal blubber [1-3], they appear to biomagnify through marine food webs to reach the

concentrations observed in blubber samples [4]. Although recent research has elucidated their geographic and trophic distributions [3-12], the origins and physiological role of MBPs remain a mystery.

MBP structure is especially notable in the *N,C1*-linkage of the pyrrole rings; this bipyrrole structure has not been previously seen in naturally produced compounds. The novel pyrrole linkage and the highly halogenated nature of MBPs suggest that the biosynthetic organism(s) may have new genes to add to current knowledge of biosynthetic chemistry. With recent advances in genomic sequencing, the genes involved in biosynthesis can be identified and catalogued in 'libraries' [13]. One route of recent drug development is the use of these biosynthetic pathways to replace or assist in the production of complex chemical structures with specific biological activities [14]. Additionally, many organisms that produce secondary metabolites (compounds not required for primary metabolism, but that benefit the producing organism) synthesize more than one type of compound [15]. For example, the marine bacterium that produces hexabromo-2,2'-bipyrrole also generates other brominated pyrrole structures and biosynthetically-unrelated polysaccharides [16, 17]. Thus, identification of MBPs' producer(s) may result in the discovery of more novel bioactive compounds. To fully realize this potential benefit of MBPs, however, their source must be identified.

One method to infer the origins of a compound is by measuring its isotopic signature, which is imprinted during biosynthesis. This signature is a combination of the isotopic values of the precursors and the fractionation that occurs during the biosynthetic process. Once released into the environment, a compound's isotope signature can be altered if portions of the original inventory undergo reactions with associated isotopic fractionations. However, compounds that biomagnify should not undergo significant metabolism [18], and, theoretically, there is very little opportunity for isotope fractionation to occur. Hence, the isotopic signal of production should remain unchanged within the tissues of the higher trophic level organisms [19]. By isolating compounds from these higher trophic levels, we can determine the isotopic signature resulting from their biosynthesis. Although the process to isolate sufficient quantities of individual compounds is time and labor intensive, it can be performed without altering the isotopic signature of the targeted molecules [20]. Here we employ compound-specific nitrogen isotope analysis to examine the origin of MBPs isolated from the blubber of three common dolphins (*Delphinus delphis*). To the best of our knowledge, these are the first compound-specific ^{15}N isotope analyses of biomagnified compounds.

2. Methods

2.1 Samples

Blubber samples from three *D. delphis* (CCSN04-218Dd, CCSN06-013Dd and CCSN07-074Dd) were acquired through the Cape Cod Stranding Network from dolphins stranded on Cape Cod in 2004-2007. CCSN04-218Dd was a maternally-dependent male calf of normal health who stranded with an adult female; the calf was in poor health due to the stress of stranding and was euthanized. CCSN06-013Dd was a male (195.5 cm length) in robust health, but was euthanized at the site of stranding due to deteriorating condition. CCSN07-074Dd was an adult female in robust health, was lactating at the time of stranding, and died at the stranding site.

2.2 MBP isolation from blubber

The blubber was received and was stored as frozen slabs, and thawed prior to processing. It was homogenized with hexane and filtered to a clear, yellow total lipid extract (TLE). The solvent was removed and the oil was stored at -20 °C.

To isolate large enough quantities of the molecules of interest for compound-specific nitrogen isotope analysis, 250 g of oil was used for each sample. We employed gel permeation chromatography (GPC) to isolate the small organic compounds and remove the lipids from the samples. Briefly, 10 g aliquots of each sample was applied to the top of a 3cm (o.d.) column, which was packed with 100g of SX-8 BioBeads (~45cm, uncompressed). We used a mobile phase of 1:1 dichloromethane:hexane and collected two fractions. The first

fraction (0-150mL) that contained ~70% of the lipids was discarded; the second fraction (150-400mL), containing the remaining lipids and the molecules of interest, was reduced in volume by rotary evaporation. This procedure was repeated with the remaining oil from the initial 250 g sample, then the second fractions were combined and re-applied to the GPC column in 10 g aliquots. This was repeated until < 0.5g lipids remained in the combined second fraction. The extract was further purified by silica/alumina column chromatography, as described in a previous publication [10].

Although we measured $\delta^{15}N$ by gas chromatography isotope-ratio mass spectrometry with a combustion interface (GC-C-IRMS), we chose to first isolate individual MBPs from the concentrated extract by preparative capillary gas chromatography (PCGC) [20]. This choice was determined by MBP structure, where nitrogen is only ~4-5% percent (by mass) of each compound. To produce the required signal strength in the IRMS (see section 2.5.1), the GC column was overloaded. By isolating the individual compounds prior to GC-C-IRMS, the quality of the chromatography did not limit our ability to make accurate measurements. The quantity of compound isolated was estimated by comparison to a synthetic standard of 2,2′,3,4,4′,5,5′-heptabromo-1′-methyl-1,3′-bipyrrole using gas chromatography coupled to a flame ionization detector (GC/FID). The identities of the isolated compounds were confirmed with gas chromatography/electron capture negative ion mass spectrometry (GC/ECNI-

MS). The details of the GC/ECNI-MS method and compound identification have been previously published [10].

2.3 Bulk stable nitrogen isotope analyses

The $\delta^{15}N_{bulk}$ of the dolphins was determined by measuring the stable nitrogen isotope ratios of their muscle tissue. Sampling details are provided in a companion study (Chapter 4, [4]). Analyses were conducted at the Stable Isotope Facility at the University of California at Davis.

2.4 Compound-specific nitrogen isotope analyses

Compound-specific nitrogen isotope analysis of MBPs was performed following a modified procedure of N isotope analysis in organic contaminants using GC-C-IRMS [21]. GC-C-IRMS was chosen for the compounds-specific nitrogen isotope analyses because it requires far smaller quantities of each compound per measurement (~3 nmoles N, equivalent to ~2 μg, see section 2.5.1) than does an elemental analyzer interfaced to an isotope-ratio mass spectrometer (EA/IRMS) (20-150 μg N [22]).

2.5 Quality controls

2.5.1 GC-C-IRMS

Due to the high molecular weight (580-700 amu), large number of halogens (6-7), and low nitrogen content (2 nitrogen atoms per compound) of the analytes, we anticipated difficulties in MBP combustion and the reduction of the subsequent N-containing fragments to N_2 in the GC/IRMS interface. Indeed,

such effects were previously reported for the $\delta^{13}C$, $\delta^{2}H$, and $\delta^{15}N$ analysis of triazine herbicides with a similar experimental setup [23]. Due to MBPs' high molecular weight, the amount required to achieve the recommended signal intensity of 500 mV [24] is too large to reasonably introduce onto a GC column. Therefore, injections were limited to 3-6 nmoles of each MBP, which produced smaller currents. As shown in Figure 2, accurate and precise $\delta^{15}N$ measurements of halogenated bipyrroles are possible at low signal intensities, that is, peak amplitudes between 50 and 500 mV. Excellent agreement between the $\delta^{15}N$ measured by EA/IRMS (-3.9±0.2‰, UC Davis Stable Isotope Facility) and our results (-4.5±1.1‰) was observed during this test.

The $\delta^{15}N$ values of the synthetic standard DMBP-Cl_6 were monitored after every 3-6 MBP measurements to ensure accuracy. The low abundances used for these measurements resulted in an average of -2.9±1.4‰, which is 2‰ removed from the EA/IRMS measurement. To be conservative, we use an error of ±2‰ to account for this variation. To clarify, this error accounts for the entire method; the instrumental error for low signal strengths is ±1.1‰, as described above.

2.5.2 Extraction and purification procedure

The purification process to isolate MBPs was lengthy and involved multiple steps. Although no isotopic fractionation would be expected during column chromatography, previous work has shown that PCGC is capable of substantially altering the isotopic signature (up to 9.4‰ for ^{13}C) of isolated

compounds if a peak is not captured in its entirety due to cross-peak inhomogeneities [20]. To be cautious, the entire extraction method was assessed to determine if fractionation occurred during the isolation of MBPs from blubber. A 10 g sample of vegetable oil was spiked with DMBP-Cl_6 and subjected to the isolation procedure (GPC – silica/alumina chromatography – PCGC). The spiked DMBP-Cl_6 recovered from the vegetable oil was analyzed alongside a stock solution of DMBP-Cl_6. The samples had the same $\delta^{15}N$ within the error of the measurement (±2‰), which indicates that our procedure can be used to determine the compound-specific isotope ratios of halogenated bipyrroles.

2.6 Nomenclature

Details on our nomenclature system are available in our prior publications [10, 12], and consistency between the publications allows results to be easily compared. The two compounds discussed here are 2,3,3′,4,4′,5,5′-heptabromo-1′-methyl-1,2′-bipyrrole (Br_7-MBP-79) and the most abundant hexabromochloro-MBP congener (MBP-Br_6Cl-*b*). The acronym for the perbrominated congener is consistent with the nomenclature system developed by Vetter et al. [25] for MBPs of known structure.

3. Results and Discussion

3.1 MBPs enriched in ^{15}N

The stable nitrogen isotopic ratios of the MBPs measured ranged from +19-28‰ (Table 1). Four individual compounds were analyzed (in triplicate):

Br_7-MBP-79 in all three blubber samples, and MBP-Br_6Cl-*b* in CCSN07-074Dd. The Br_7-MBP-79 isolated from the female dolphin was enriched relative to MBP-Br_6Cl-*b* also isolated from the same blubber (+28‰ and +22‰, respectively). The MBPs are enriched relative to $\delta^{15}N_{bulk}$ of the dolphins from which they were isolated (Table 1). To our knowledge, the extent of this enrichment, even with our conservative consideration of error (±2‰), is unusual in comparison to other naturally produced compounds.

A thorough literature review of stable nitrogen isotope measurements of organic components in environmental or biological samples (commercially available standards were excluded) shows that most naturally occurring compounds have $\delta^{15}N$ values that typically fall from -10‰ to +10‰ (Figure 3). The amino acids are the most analyzed compound class, and the measurements come from a large range of sources: bacteria [26], plankton [27-30], soil [31], plants [32, 33], collagen [34, 35], and human blood plasma [36]. Perhaps not surprisingly, amino acids also span the largest range of isotope ratios. DNA shows the most limited range, but is from only one study measuring soil extracts [37]. The pigment measurements are for chlorophyll and other porphyrins, and they have been measured in multiple studies, including plants [38], phytoplankton [39, 40], lake and marine sediments [41-43], and a saline meromictic lake [44]. Many are tetrapyrroles and thus may share biosynthetic precursors with MBPs. Secondary metabolites show a similar distribution to the

pigments, which both tend to be depleted in ^{15}N relative to the cellular inventory of nitrogen [45].

MBPs have much higher $\delta^{15}N$ values compared with these other biosynthetic organic compounds (Figure 3). When making this comparison it is important to note that compound-specific $\delta^{15}N$ measurements are far less common than those made for $\delta^{13}C$, which limits our ability to make comparisons. Nevertheless, the $\delta^{15}N$ enrichment seen for MBPs is a distinct isotopic signal. MBPs are putative secondary metabolites made of two pyrrole rings. Thus, their enrichment is especially notable compared with the much lower $\delta^{15}N$ values measured for secondary metabolites (Figure 3: heroin, morphine, cocaine, nicotine, caffeine and methyl-*N*-methyl-anthranilate, all isolated from various terrestrial plants [45]) and the tetrapyrrolic pigments.

3.2 Origin of enrichment

There are at least three possible explanations for the enrichment we observe in the MBP congeners: enriched precursors, biosynthetic fractionation, and degradative pathways that introduce isotopic fractionation. These three explanations are not mutually exclusive; in fact, all may contribute. MBPs are relatively newly discovered, and details about their synthesis and degradation are still uncertain. Thus, we will examine potential biosynthetic routes of MBPs, including precursors, but first we will discuss the possibility of nitrogen isotope fractionation during degradation of MBPs.

3.2.1 Fractionation during degradation

Although MBPs biomagnify, which indicates that they are resistant to degradation, they are not necessarily immune to degradation. In fact, enzymes involved in the degradation of halogenated organic compounds are ubiquitous, and likely evolved to process HNPs [46, 47]. Such capabilities exist not only in microbes [48], but also in larger organisms, including fish [49] and mammals [50, 51]. In these larger organisms, biotransformation of halogenated organic compounds can proceed via both oxidative pathways and reductive dehalogenation [49, 52]. Thus, it is reasonable to consider that some fraction of any biomagnifying compound may be metabolized during trophic transfer.

Any metabolism that occurs can have an associated isotopic fractionation, deriving from a kinetic isotope effect. Bond strength is slightly mass dependent, and heavy isotopes form stronger bonds than do light isotopes. Thus, bonds containing the light isotope break more quickly, leading to a faster reaction rate for these compounds. This difference in reaction rates results in enrichment of the heavy isotope in the remaining parent compound. Therefore, if degradation occurs during trophic transfer, we expect to see this enrichment in the remaining compounds, i.e., those that accumulate in the next trophic level. Every link in the food web allows an additional opportunity for fractionation, thus, compounds in the highest trophic levels should show the largest isotopic enrichment. This effect has been observed for 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT);

DDT isolated from the blubber of a grey seal was enriched in ^{37}Cl relative to commercially produced DDT [53].

If MBPs are subject to dehalogenation during trophic transfer, the nitrogen isotopic enrichment would likely be very small. As there are no halogen-nitrogen bonds in MBPs, dehalogenation is a secondary isotope effect with respect to nitrogen. (I.e., the bond involved in the reaction center is adjacent to nitrogen atom, thus, the nitrogen atom is not directly involved in the reaction.) Since they are not directly involved in the reaction, secondary isotope effects are generally quite small relative to primary isotope effects [54]. This is apparent in the minute secondary isotope effects documented for chlorine in dehalogenation (0.3‰), relative to the primary isotope effects in carbon (7.2‰ and 8.5‰ for vinyl chloride and *cis*-1,2-dichloroethane, respectively) [55]; and nitrogen in enzymatic nitrobenzene oxidation (0.75‰), again relative to carbon (3.9‰) [56]. Thus, any fractionation in nitrogen isotopes during MBP dehalogenation is likely to be minor.

The potential for oxidative degradation is more difficult to assess as the degradative pathways of MBPs are as of yet unknown. However, the degradation of halogenated bicyclic ring compounds and nitrogen-containing aromatic compounds have been scrutinized. Oxidative metabolism of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) produces hydroxylated metabolites [50, 52, 57], and PBDE metabolites also

include brominated phenols [52]. Like dehalogenation, hydroxylation would result in a secondary isotope effect with respect to nitrogen. The production of brominated phenols, however, requires separation of the two aromatic rings in each PBDE at the ether linkage. MBPs have no ether linkage, but are joined through a C-N bond. It is not clear whether the enzymes that produced brominated phenols from PBDEs would similarly act upon the C-N bond in MBPs. Two oxidative paths are suggested by the metabolism of chlorotoluenes: hydroxylation on the aromatic ring (as described for the PBDEs) and at the methyl carbon [58]. The latter results in the formation of a benzyl alcohol, which would produce a secondary isotope effect for the nitrogen atom in position 1'.

Overall, the most probable metabolic pathways for MBPs, dehalogenation and hydroxylation, would result in secondary isotope effects with respect to nitrogen. Keeping in mind the extent of $\delta^{15}N$ enrichment in MBPs, it seems unlikely that secondary isotope effects could contribute significantly to their isotopic signature. If we assume that MBPs are initially introduced into the food chain with a $\delta^{15}N$ of nitrate (~5‰) [59] and that secondary isotope effects are responsible for degradation ($\varepsilon \sim 1‰$, a conservative estimate), we can calculate the fraction of MBPs remaining (f = fraction remaining) by Rayleigh distillation of kinetically fractionated isotopes (Eq. 1).

$$\delta_{\text{MBP blubber}} = \delta_{\text{MBP initial}} - \varepsilon \ln(f) \qquad \text{(Eq. 1)}$$

Here, $\delta_{MBP\ blubber}$ is the $\delta^{15}N$ of MBPs isolated from blubber (here we use 20‰), and $\delta_{MBP\ initial}$ is the $\delta^{15}N$ of MBPs when they enter the food web (5‰, as described above). Under these assumptions, this calculation yields f = 0.0000003, or less than 0.000001% of the original MBP remaining. Using a more conservative estimation of $\delta_{MBP\ initial}$ = 10‰ results in an estimate of f = 0.00005. This seems unreasonable, as a similar calculation for DDT results in ~10% of the original DDT remaining [53], and DDT is transformed in the environment relatively quickly to dechlorinated metabolites. Thus, the $\delta^{15}N$ enrichment of MBPs isolated from whale blubber is unlikely to be due to degradation.

3.2.2 *Enriched precursors and biosynthesis*

During biosynthesis the isotopic composition of a compound is set and reflects the source of nutrients, the uptake fractionation, and the internal fractionation between the target compound and the cellular biomass [60]. Most important to our investigation are the two pyrrole rings in each MBP, as they contain the nitrogen atoms of interest. Pyrrole biosynthesis is an active area of study, as many secondary metabolites contain pyrroles or pyrrole-derived moieties [61], and a number of pyrrole biosynthetic pathways have been determined (Figure 4). Although there are a few routes to pyrrole synthesis, all derive nitrogen from amino acids [61-65]. Amino acids are synthesized during proteinogenesis, a fraction of which can be drawn off to natural product

pathways [61]. Amino acids shown to be pyrrole precursors are glycine, proline, alanine, ornithine, serine, and methionine [61-65].

Compound-specific nitrogen isotope analyses have been performed on over 17 amino acids from a variety of sources, for a total of over 360 measurements (Figure 3). Amino acids $\delta^{15}N$ values tend to vary from -10‰ to +10‰, but individual amino acids show distinct patterns. Of those shown to contribute to pyrrole biosynthesis (see above), alanine, ornithine and proline are more enriched, and glycine and methionine are more depleted, relative to the amino acid mean $\delta^{15}N$ (Figure 5). For amino acids that are polynitrogeneous, intramolecular isotopic evidence shows that the side-chain nitrogen is consistently enriched relative to the peptide nitrogen [66]. Similarly, nitrogen atoms involved in heteroaromatic binding (such as the nitrogen in pyrroles) are enriched relative to amino acids' peptide nitrogen [45]. Thus, it is possible that part of the enrichment observed in MBPs is due to the incorporation of relatively enriched amino acids (such as alanine, ornithine, and proline) or nitrogen from the side chains of polynitrogeneous amino acids.

Reactions during biosynthesis may also impart associated frationations. The observed enrichment could occur through pyrrole halogenation or during the linkage of the two pyrrole rings. Halogenation of the pyrrole ring would occur by electrophilic aromatic substitution, which would disrupt the aromaticity of the pyrrole. This change in the molecular electronic structure could thus result

in fractionation for nitrogen atoms. Reaction at the nitrogen atom is a primary isotope effect, and is thus most likely to impart a strong isotopic signature. It seems reasonable that the mechanism linking the two pyrrole rings may be responsible for the enriched nitrogen isotope values we observe.

An alternative possibility for precursor enrichment is $\delta^{15}N$ enrichment in the available nitrogen pools. One source of enriched nitrogen is from higher trophic levels. Predators are enriched relative to their prey by ~3‰ [67]. Thus, top predators are enriched in ^{15}N, such as the dolphins in this study ($\delta^{15}N$~14‰). By acquiring nitrogen from top predators by parasitic activity, recycling of carcasses, etc., a very enriched source of nitrogen could be tapped.

Another source of enriched N could be nitrate. Nitrate is the most abundant source of nitrogen in the oceans, which typically has $\delta^{15}N$ values ~5‰ [59]. However, this value can vary widely, especially in regions where denitrification has depleted the pool of nitrate. Denitrification is strongly fractionating, leaving the remaining pool of bioavailable nitrogen enriched in $\delta^{15}N$ (e.g., $\delta^{15}N_{nitrate}$=12.5‰ North Pacific Subtropical Gyre) [68]. Thus, MBP ^{15}N enrichment may derive from biosynthesis in areas of denitrification, or other areas of ^{15}N-enriched nitrate.

3.3 A hypothesis for MBPs origins

Actinomycetes and *Pseudoalteromonas* species are prolific producers of secondary metabolites [69, 70], and are already known to produce halogenated

bipyrroles [63, 69, 71, 72]. Interestingly, some actinomycete species and *Pseudoalteromonas denitrificans* are capable of denitrification [67-69] and thus can access nitrogen pools enriched in δ^{15}N. Thus, actinomycetes and/or *Pseudoalteromonas* seem a likely source of MBPs. Although these bacterial orders have been extensively examined for natural product production, MBPs have never been identified [69, 70]. This is unsurprising, as only a small fraction of existing species have been cultured and examined for secondary metabolite production, and most of these are soil-dwelling species [73]. Future research on the origins of MBPs may want to focus on new strains of marine actinomycetes and *Pseudoalteromonas*, particularly those isolated from regions where nitrate has high δ^{15}N values.

MBPs are a remarkable class of environmental contaminants. They are highly halogenated, bioaccumulative, natural products, and the research presented here demonstrates that they are dramatically enriched in δ^{15}N (+20-30‰). This ^{15}N-enrichment is greater than that seen for other terrestrially produced organic compounds that have been measured. The enrichment likely stems from enriched precursors and/or fractionation during biosynthesis. One source of enriched nitrogen in the oceans is the residual nitrate in areas of denitrification and two bacterial species known to produce halogenated bipyrroles are from genera with denitrifying capabilities. Thus, we hypothesize that marine actinomycetes and/or *Pseudoalteromonas* produce MBPs.

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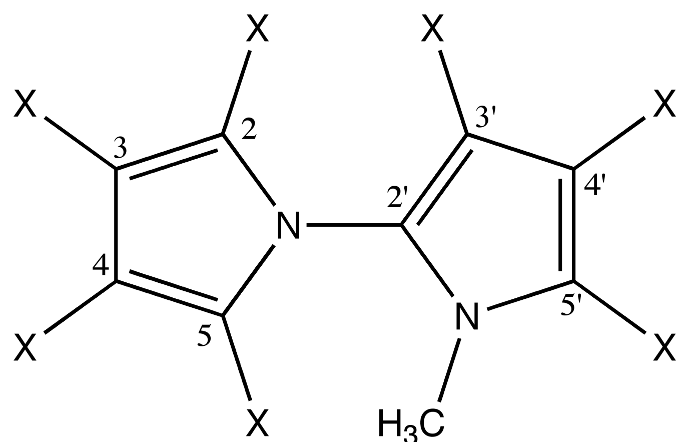

Figure 1. The general structure of MBPs, where X represents Br, Cl or H.

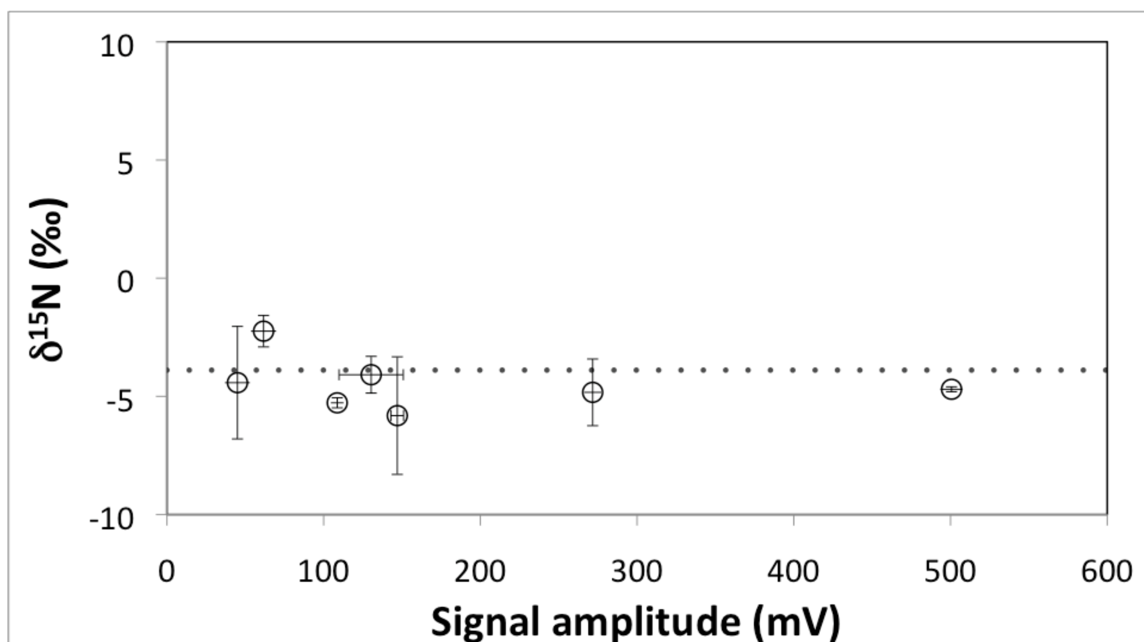

Figure 2. Linearity test of the GC-C-IRMS system for measuring low abundances of halogenated bipyrroles. Each circle represents one measurement on the GC-C-IRMS system (average of -4.5±1.1‰) and the dotted line represents the EA/IRMS measured $\delta^{15}N$ value of the standard DMBP-Cl_6 (-3,9±0.2‰).

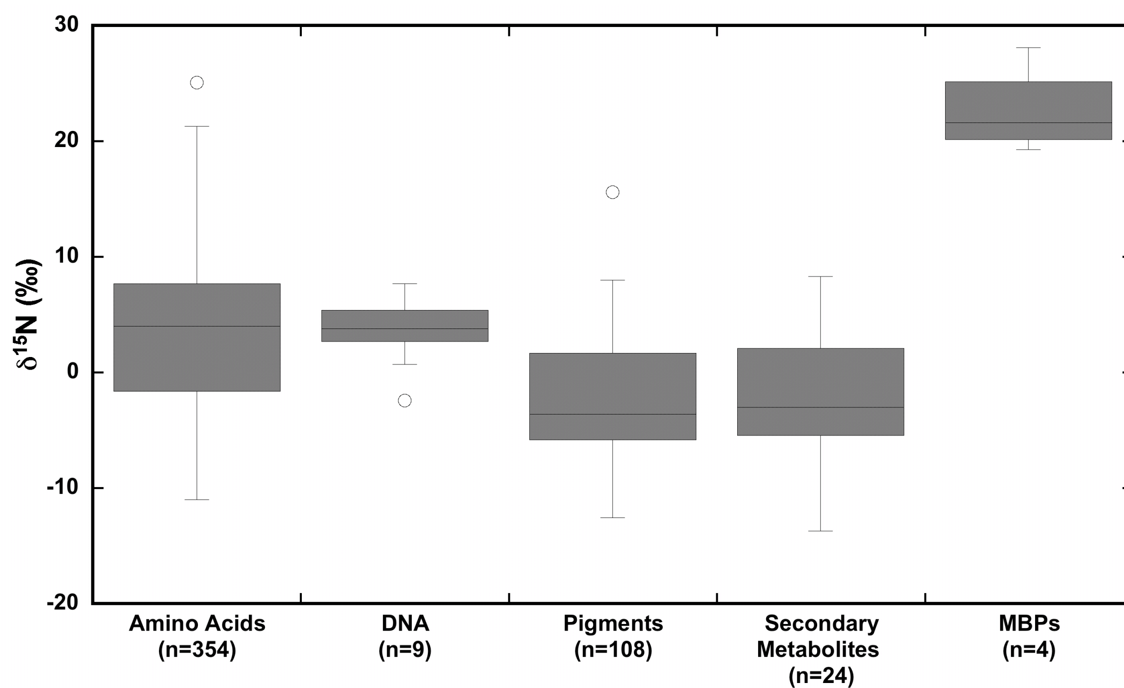

Figure 3. Box plots of selected $\delta^{15}N$ ranges from a literature review of compound-specific nitrogen isotope analyses compared with the MBPs presented in this study. Data from: amino acids [26-31, 33-36, 66, 74], DNA [37], pigments [38-44], secondary metabolites [45].

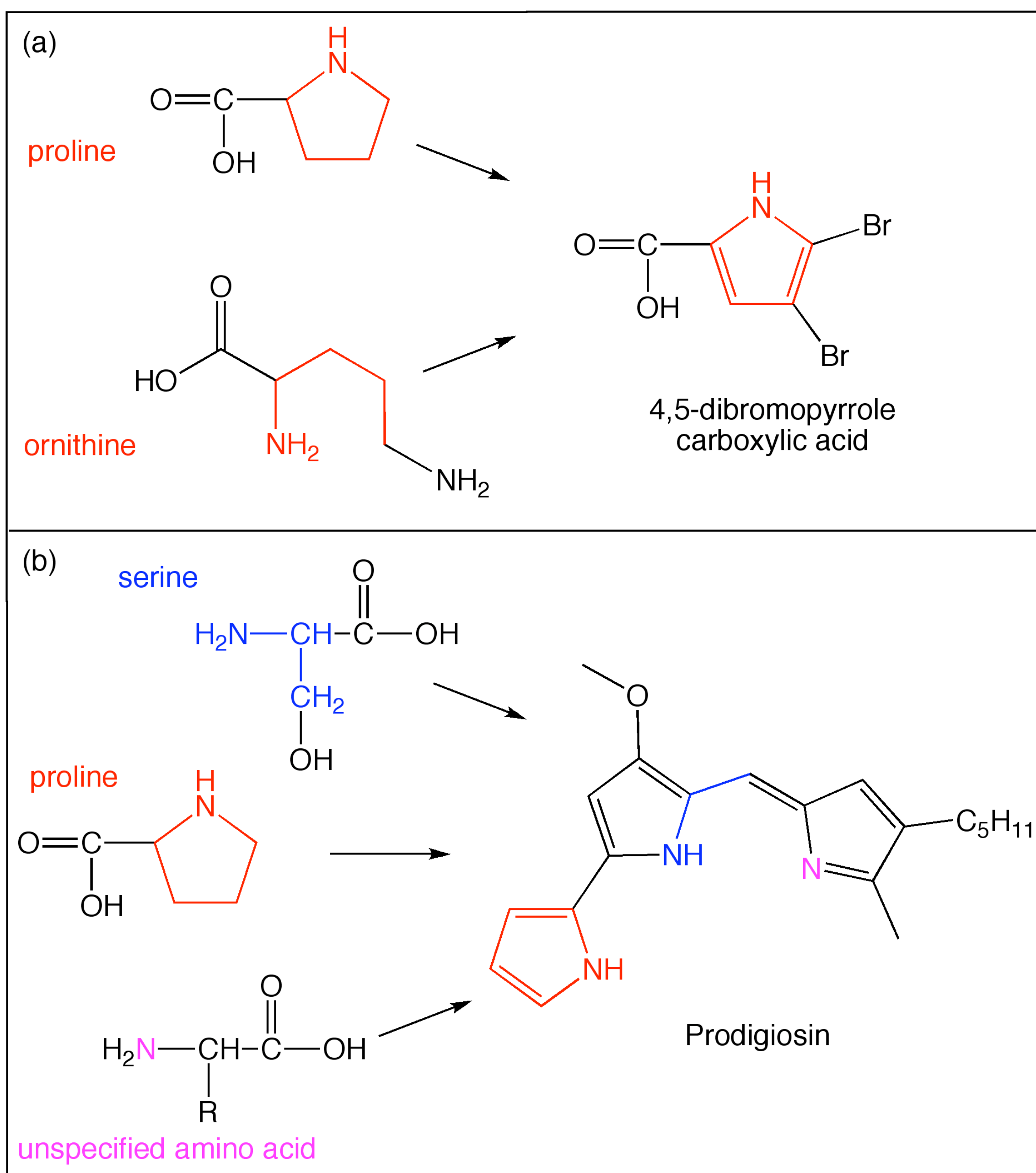

Figure 4. Selected pathways of pyrrole biosynthesis. The pyrrole moiety in the oroidin family of bromopyrrole alkaloids (produced in a number of marine sponge genera) is generated from proline and ornithine [75]. Proline is also the basis for one of the three pyrrole rings (in red) in Prodigiosin biosynthesis, the other nitrogens derive from serine (in blue) and an additional (unidentified) amino acid (in purple) [63].

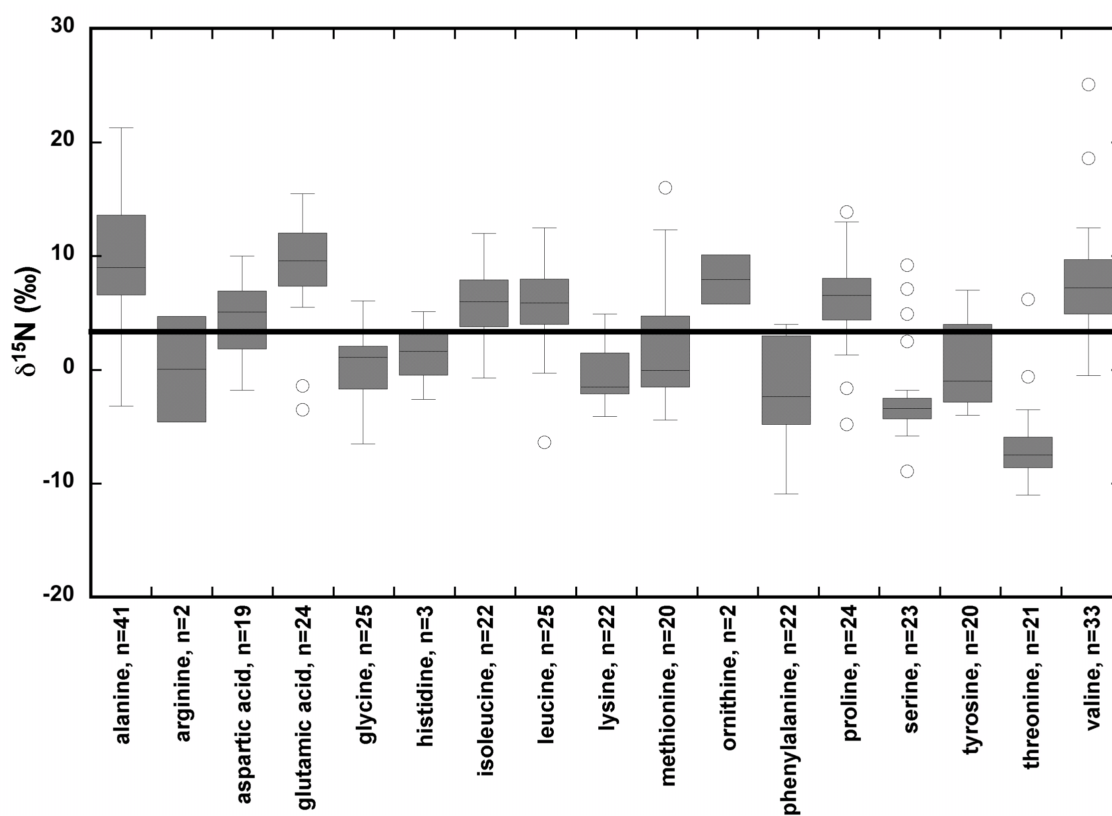

Figure 5. Box plots of the $\delta^{15}N$ ranges of selected amino acids (amino acids with only one reported value were not included). The horizontal bar represents the average value of all amino acids from this literature review (+3.4‰). Data from [26-31, 33-36, 66, 74].

Table 1. The $\delta^{15}N$ of four MBPs isolated from the blubber of three common dolphins, and the gender, age, and $\delta^{15}N_{bulk}$ of the dolphins. Measurements were made in triplicate, all three measurements are presented in italics and the average in bold.

Sample ID	gender	age	$\delta^{15}N_{bulk}$	$\delta^{15}N$ (‰) (±2‰)	
				Br_7-MBP-79	MBP-Br_6Cl-*b*
CCSN04-218Dd	**male**	**calf**	**14.3**	**+19.3**	
			replicate 1	*+21.6*	
			replicate 2	*+20.0*	
			replicate 3	*+16.2*	
CCSN06-013Dd	**male**	**adult**	**13.2***	**+21.0**	
			replicate 1	*+21.6*	
			replicate 2	*+20.3*	
			replicate 3	*+21.0*	
CCSN07-074Dd	**female**	**adult**	**13.5**	**+28.1**	**+22.2**
			replicate 1	*+28.3*	*+25.5*
			replicate 2	*+30.4*	*+19.7*
			replicate 3	*+25.6*	*+21.3*

*Muscle was not sampled, and no bulk $\delta^{15}N$ was measured. The value was estimated based on the values of other adult male common dolphins in a companion study (Chapter 4 [4]).

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CHAPTER 6

Conclusions and Future Directions

Halogenated 1′-methyl-1,2′-bipyrroles (MBPs) are a distinctive class of marine organic compounds. They are naturally produced, have a unique carbon structure, are highly halogenated (with bromine and/or chlorine), appear to biomagnify, and are considerably enriched in ^{15}N relative to other terrestrial and marine naturally produced organic molecules.

When these compounds were initially detected in blubber samples, only a few of the most abundant MBP congeners were identified [1-3]. Specifically, only one MBP-Br_6Cl congener was detected (out of a possible five), prompting researchers to speculate that the halogenation process during biosynthesis was regioselective and that congeners with fewer than seven halogens were the dehalogenation products of biosynthetic perhalogenated congeners [3]. However, over twenty perhalogenated MBPs have now been detected [4-6], which diminishes the theory that MBP halogenation is regioselective. The origins of MBPs with fewer than seven halogens are not yet clear. They may be synthesized concomitantly with their perhalogenated counterparts, they may be products of dehalogenation, or both processes may contribute to their presence in environmental samples.

One line of evidence for MBPs as marine natural products is their detection only in marine samples. Even the carbon structure (*N.C1*-linked bipyrrole) had never been reported prior to the identification of MBP-79 [7]. However, it should be noted that a thorough search for MBPs in terrestrial environments has not yet been performed. Since pyrroles are relatively common, as are opportunities for halogenation, a more thorough screening of terrestrial samples would be beneficial supporting evidence. Further, the abiotic condensation of pyrroles in the presence of halogenating agents or enzymes should also be investigated. Unintentional abiotic generation of MBPs may be unlikely (lab-based chemical synthesis of these molecules suffers from very low yields [2, 8]), but still requires investigation to rule out.

The evidence for MBPs biomagnification is extremely strong. As demonstrated in this dissertation, lipid-normalized MBPs concentrations increase with increasing trophic level. Additionally, MBPs display the biomagnification patterns seen in the environment for persistent organic pollutants (POPs) and that have been demonstrated mathematically by numerical models. In adult males, MBP concentrations increase with age; in juveniles there is a growth dilution pattern; and adult females have much lower body burdens than males, likely due to MBP transfers during lactation and gestation. All of our data is consistent with biomagnification as the process responsible for the environmental distribution of MBPs. Since biomagnification is a critical feature

of the environmental chemistry of POPs, the data presented here affords greater confidence in the use of MBPs as natural analogues for these anthropogenic contaminants.

It remains to be determined why MBPs and other similar naturally produced compounds are persistent in the environment. Although halogenated natural products are commonly hypothesized to be defensive compounds [9], further hypotheses have also been proposed, such as forms of chemical communication, settling deterrents/anti-fouling agents, and protection from UV radiation [9, 10]. Persistence is not an obviously desirable attribute for chemicals involved in communication or that might be toxic. However, as proposed by Prof. William Fenical (personal communication) the lipophilicity of many of these persistent chemicals may be the mechanism that prevents them from over-accumulating in the ecosystem in which they are produced.

However, the similarities to POPs are inevitably limited, as demonstrated in this dissertation by the comparison of MBPs and a POP, 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153). POPs and marine halogenated natural products (HNPs), such as MBPs, have very different sources, and this can lead to differences in their distributions. POPs are now ubiquitous [11], but they are produced terrestrially, and their inputs to the ocean are thus tied to their terrestrial sources. MBPs, however, have only a marine source, and have no association with terrestrial inputs. Further, it is likely that just one species or an

assemblage of species produces them, thus their source is far more limited than POP sources. The differences in the geography and magnitude of sources between MBPs and POPs may lead to variations in the ways that they enter and transit through the food webs. Thus, it takes longer for concentrations to accumulate in blubber because MBPs have a more limited magnitude and distribution relative to PCBs and exposure to MBPs is reduced. This theory is strengthened by the consistent presence of CB-153, and its relative abundance, compared to the more variable, but consistently less abundant, distribution of MBPs in marine species.

The differences between MBPs and POPs are not limited to their sources and geographic distributions. This dissertation presents evidence suggesting that marine mammals process these compounds differently. MBPs appear to be more easily mobilized from blubber during times of nutritional stress, and this may include periods of gestation and lactation in adult females. Further, the pinniped species analyzed in this dissertation (*P. groenlandica* and *H. grypus*) have far lower MBP blubber concentrations than would be expected for their trophic positions, yet they still have the expected high levels of CB-153. Such differences between HNPs and POPs were noted in previous studies of pinnipeds [12-14], which suggested that these pinnipeds had enhanced metabolic capabilities to process HNPs. By determining that pinnipeds are exposed to MBPs through their diets, the data presented in Chapter 4 considerably

strengthens this argument. Investigating the enzymatic mechanisms of HNP and POP metabolism may elucidate which structural features facilitate MBP metabolism.

Finally, MBPs have high $\delta^{15}N$ values, enriched relative to other naturally produced organic compounds and to the bulk tissue of the dolphins from which they were isolated. This enrichment appears to be a signature of biosynthesis, and it is unlikely to have been introduced by metabolism or abiotic degradation. Thus, their enrichment is tied to biosynthetic fractionation and/or enriched precursors. Two potential sources of biosynthetic enrichment can be tested rather easily. The isotope effects of halogenation of pyrrole rings can be examined through the abiotic process to determine if changes in aromaticity result in an associated fractionation. The fractionation associated with linking the pyrrole rings could be examined by comparing the $\delta^{15}N$ of MBPs and marinopyrroles with that of DMBPs. MBPs and marinopyrroles are both linked through a nitrogen atom from one of the pyrrole rings, while DMBPs are only linked through carbon atoms. Thus, if the enrichment we observe in MBPs is due to formation of the *C1,N* bond, than similar enrichment should be observed in the marinopyrroles and no such enrichment would be present in the DMBPs.

The biosynthetic mechanism of MBPs is, of course, unknown, but structurally similar compounds are produced by other marine bacteria, specifically the marinopyrroles from an actinomycete strain (CNQ-418) [15] and

3,3′,4,4′,5,5′-hexabromo-2,2′-bipyrrole from *Pseudoalteromonas luteoviolacea* [16, 17]. A source of enriched nitrogen is common to both groups. Species from both groups are involved in denitrification, a process that strongly fractionates nitrogen isotopes, leaving the residual nitrate enriched in ^{15}N. Consequently, MBP's $\delta^{15}N$ signatures suggest that species from the *Actinomycetes* or *Pseudoalteromonas* may be responsible for MBP biosynthesis. Future searches for MBP producers may find it most productive to focus on these species.

Much remains to be learned about the controls on MBP cycling and fate, as well as about MBP biosynthesis and chemical ecology. Ultimately, the questions that drive my interest in MBPs have not yet been answered: who make these compounds, how are they made, what function do they serve, and why are they required to be environmentally persistent? The dissertation provides the foundation necessary to find these answers by expanding our understanding of environmental distributions and providing initial constraints on MBP biosynthesis.

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Appendix

The lipid content of mammalian samples. Blubber percentages are given on a wet weight basis, liver percentages are given on a dry weight basis. The abbreviations are: 'n.r.' – not recorded; 'n/a' – not applicable because no liver samples were available.

CCSN number	% lipid	
	blubber	liver
CCSN07-023La	83.3	13.4
CCSN06-119La	77.6	12.5
CCSN06-022La	75.5	19.8
CCSN06-264Dd	69.3	10.5
CCSN07-109Dd	57.3	10.0
CCSN07-115Dd	56.7	11.0
CCSN05-316Dd	n.r.	12.6
CCSN06-013Dd	n.r.	13.6
CCSN06-029Dd	80.5	8.73
CCSN06-263Dd	85.2	7.23
CCSN07-040Dd	71.5	6.00
CCSN06-024Dd	79.0	23.1
CCSN06-096Dd	83.8	19.6
CCSN06-137Dd	63.6	12.1
CCSN07-036Dd	n.r.	7.35
CCSN07-041Dd	53.8	11.1
D08742	n.r.	9.94
CCSN07-129Hg	78.0	11.0
D07041	63.1	15.7
CCSN07-063Pg	81.4	22.8
CCSN07-080Pg	84.3	5.66
CCSN07-084Pg	79.5	16.3
CCSN07-116Pg	85.5	5.34
CCSN06-133Pg	67.3	15.2
CCSN07-022Gm	n.r.	9.48
CCSN06-019La	80.0	n/a
CCSN04-218Dd	59.9	n/a
CCSN06-144Dd	72.9	n/a
CCSN07-074Dd	n.r.	n/a
CCSN07-066Pg	77.5	n/a
CCSN07-076Pg	76.3	n/a