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**IDENTIFICATION AND CHARACTERIZATION OF THE
ENVIRONMENTAL CHEMISTRY, BIOACCUMULATION, AND
TOXICOLOGY OF HALOGENATED DIMETHYL BIPYRROLES**

Sheryl A. Tittlemier

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

Carleton University

Ottawa, Ontario

November 26, 2001

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The undersigned recommend to the Faculty of Graduate Studies
and Research acceptance of the thesis

**“Identification and Characterization of the Environmental Chemistry,
Bioaccumulation, and Toxicology of Halogenated Dimethyl Bipyrrroles”**

submitted by Sheryl Angela Tittlemier, B.Sc.(Hons.)
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

Chair
Thesis Supervisor
Ross J. Norstrom
External Examiner
Gordon W. Gribble

Date:

Abstract

A group of unknown halogenated compounds, initially observed in biota in 1988, was identified, characterized, and examined within the framework of environmental chemistry. Various mass spectrometry experiments, including isotope exchange positive chemical ionization mass spectrometry, were used to identify the novel compounds as halogenated dimethyl bipyrrroles (HDBPs). The fundamental physical properties – aqueous solubilities, liquid sub-cooled vapour pressures, Henry's Law constants, and octanol/water partition coefficients – were characterized using established methods. Results of a simple Level I equilibrium distribution model predicted HDBPs to be associated mainly with soil and sediments, and to have low mobilities in the environment.

HDBPs were found to be globally distributed in marine mammal blubber and present in seabird eggs from both the Atlantic and Pacific coasts of Canada. Highest concentrations of HDBPs were observed in biota from Pacific as opposed to Atlantic-influenced environments. In some instances, concentrations of total HDBPs were at levels comparable to ubiquitous anthropogenic organohalogens such as chlordanes. The geographical distribution of HDBPs did not resemble the distribution of the recalcitrant polychlorinated biphenyl (PCB) congener CB-153. CB-153 was at its highest concentrations in samples obtained near industrialized areas such as the St. Lawrence River, Mediterranean, Baltic, and Wadden Seas. HDBP concentrations in samples from these areas were among the lowest observed.

Examination of an Arctic marine food web determined that HDBPs biomagnify to a similar extent as recalcitrant anthropogenic compounds such as CB-153. However, ringed seals appeared to have the ability to metabolize HDBPs.

HDBPs displayed some dioxin-like activity in a chicken embryo hepatocyte *in vitro* bioassay. Their ability to induce the production of metabolizing enzyme cytochrome P450 1A was comparable to PCB congeners containing one chlorine atom occupying an ortho position. However, a mixture of HDBPs did not elicit any negative effects on reproductive or morphological endpoints in American kestrels exposed via diet.

Circumstantial evidence indicates that HDBPs may have a natural origin. A structural resemblance to a known marine natural product and geographical distribution unlike anthropogenic compounds lend some support to the natural production hypothesis. If proven to be biogenic in origin, HDBPs will be the first identified class of bioaccumulative naturally-produced organohalogens.

List of Publications Associated with the Dissertation

Refereed Journals

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S.A. Tittlemier, A. Borrell, J. Duffe, P.J. Duignan, P. Fair, A. Hall, P. Hoekstra, K.M. Kovacs, M.M. Krahn, M. Lebeuf, C. Lydersen, D. Muir, T. O'Hara, M. Olsson, J. Pranschke, P. Ross, U. Siebert, G. Stern, S. Tanabe, R. Norstrom (2001) "Global Distribution of Halogenated Dimethyl Bipyrrroles in Marine Mammal Blubber". *Archives of Environmental Contamination and Toxicology*. Submitted July 30, 2001.

S.A. Tittlemier, A.T. Fisk, K.A. Hobson, and R.J. Norstrom (2001) "Examination of the bioaccumulation of halogenated dimethyl bipyrrroles in an Arctic marine food web using stable nitrogen isotope analysis". *Environmental Pollution*. 116: 85-93.

S.A. Tittlemier, M. Simon, W.M. Jarman, J.E. Elliott, and R.J. Norstrom (1999) "Identification of a novel $C_{10}H_6N_2Br_4Cl_2$ heterocyclic compound in seabird eggs. A bioaccumulating marine natural product?" *Environmental Science & Technology*. 33: 26-33.

Conference Extended Abstracts

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S.A. Tittlemier, A.T. Fisk, K.A. Hobson, and R.J. Norstrom (2000) "Bioaccumulation of HDBPs in an Arctic marine food web". *Organohalogen Compounds*. 49: 410-413.

S.A. Tittlemier, D.H. Blank, G.W. Gribble, and R.J. Norstrom (2000) "Structure elucidation of a novel, bioaccumulating organohalogen using isotope exchange positive chemical ionization mass spectrometry and retention time comparison". *Proceedings of the Third Biennial International Conference on Monitoring and Measurement of the Environment*. pp.61-66.

S.A. Tittlemier, R.J. Norstrom, and J.E. Elliott (1998) "Levels of a possibly naturally-produced bromo/chloro compound in bird eggs from the Great Lakes, Atlantic, and Pacific coastal areas". *Organohalogen Compounds*. 39: 117-120.

S.A. Tittlemier, M. Simon, and R.J. Norstrom (1998) "Mass spectrometry of a novel brominated chlorinated nitrogen-containing heterocycle". Proceedings of the Second Biennial International Conference on Chemical Measurement and Monitoring of the Environment. pp.111-116.

Conference and Workshop Presentations

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List of Symbols and Acronyms

A	Clausius-Clapeyron integer
AhR	aryl hydrocarbon receptor
ANCOVA	analysis of covariance
ANOVA	analysis of variance
B	Clausius-Clapeyron integer
CB-	chlorobiphenyl congener
CEH	chicken embryo hepatocyte
CHL	chlordan compound
CIBZ	chlorobenzene compound
CYP1A	cytochrome P4501A enzyme
Da	Dalton
DBP-	dimethyl bipyrrole congener
<i>p,p'</i> -DDD	<i>bis</i> -2,2'-(4-chlorophenyl)-1,1-dichloroethane
<i>p,p'</i> -DDE	<i>bis</i> -2,2'-(4-chlorophenyl)-1,1-dichloroethene
<i>p,p'</i> -DDT	<i>bis</i> -2,2'-(4-chlorophenyl)-1,1,1-trichloroethane
ΣDDT	sum of <i>p,p'</i> -DDD, <i>p,p'</i> -DDE, <i>p,p'</i> -DDT
DMSO	dimethylsulfoxide
ECNI	electron capture negative ionization
ED ₅₀	effective dose required to produce half maximal response
EI	electron impact
EROD	ethoxyresorufin- <i>o</i> -deethylase
eV	electron volt

FID	flame ionization detection
GC-ECD	gas chromatography – electron capture detection
GC-MS	gas chromatography – mass spectrometry
GC-RT	gas chromatography – retention time
GPC	gel permeation chromatography
H	Henry's Law constant
H₂₅	Henry's Law constant at 25 ⁰ C
ΔH_{vap}	enthalpy of vaporization
HCH	hexachlorocyclohexane
HDBP	halogenated dimethyl bipyrrole
ΣHDBP	sum of HDBP congeners
HPLC	high pressure liquid chromatography
HPO	haloperoxidase
i.d.	inner diameter
IEF	induction equivalency factor
IE-PCI-MS	isotope exchange positive chemical ionization mass spectrometry
K_{ow}	octanol/water partition coefficient
δ¹⁵N	difference of ¹⁵ N/ ¹⁴ N ratios between sample and standard
NMR	nuclear magnetic resonance
NOW	Northwater polynya
P	vapour pressure
P_{GC}	uncorrected vapour pressure determined by GC-RT
P⁰_L	liquid sub-cooled vapour pressure

$P^0_{L,lit}$	literature value liquid sub-cooled vapour pressure
PA	proton affinity
Pa	Pascal
PAH	polynuclear aromatic hydrocarbon
PBDE	polybrominated diphenyl ether
PCA	principal components analysis
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo- <i>p</i> -dioxin
PCDE	polychlorinated diphenyl ether
PCDF	polychlorinated dibenzofuran
PCI	positive chemical ionization
POP	persistent organic pollutant
ppb	parts per billion
ppm	parts per million
PTFE	polytetrafluoroethylene
S_L	liquid sub-cooled aqueous solubility
$S_{L,25}$	liquid sub-cooled aqueous solubility at 25°C
SPE	solid phase extraction
S_w	aqueous solubility
$S_{w,25}$	aqueous solubility at 25°C
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TCDD-EQ	TCDD equivalents
TL	trophic level

T_m	melting point
TMF	trophic magnification factor
UHC	unknown halogenated compound

CHAPTER 1.

GENERAL INTRODUCTION

1.1 Naturally-produced organohalogens

1.1.1 Introduction

It is often thought that all halogenated organic compounds are “man-made”, or anthropogenic, in origin. Even though nearly 3600 naturally-produced organohalogens have already been identified (Gribble 1999), natural sources are often ignored (Gribble 1994). A 1992 survey on naturally-produced organohalogens (Gribble 1992), drew on two examples to illustrate this observation. The first involves a report from the Science Advisory Board to the International Joint Commission on the Great Lakes which stated: “There is something non-biological about halogenated organics (excluding iodinated compounds)...” (Science Advisory Board of the International Joint Commission on the Great Lakes 1989). The other example is taken from an article in Science which contained the quote “...some types of synthetic compounds, including halogenated hydrocarbons such as PCB, are not found in nature.” (Marx 1990)

This incorrect conception of organohalogens as “unnatural” is somewhat understandable considering that incidents involving industrially produced organohalogens, or their byproducts, are well publicized. Events including the leaching of contaminants from Love Canal in 1980 in Niagara Falls, New York resulting in the relocation of a number of families, and the discovery of dioxin-contaminated animal feed

in Belgium in 1999 are just two examples of occurrences involving industrially produced organochlorines which received much attention by the popular media.

Knowledge of naturally-produced organohalogens is essential when regulations concerning anthropogenic organohalogens are being drafted since many organohalogens have both natural and anthropogenic sources. For example, methyl bromide has a large number of natural sources including giant kelp (Manley and Dastoor 1987), Antarctic ice algae (Sturges et al. 1993), and marine phytoplankton (Tokarczyk and Moore 1994). It is also industrially produced for use as a soil fumigant.

1.1.2 Definition of “naturally-produced organohalogens”

There is some ambiguity surrounding the term “naturally-produced organohalogen” since the mechanisms of formation of most compounds deemed as such are not well characterized. Other factors also contribute to the vagueness of the expression “naturally-produced organohalogen”. For example, there are circumstances in which anthropogenic organohalogens undergo biotransformation to form metabolites which may be considered to be “naturally-produced organohalogens” under a liberal use of the expression. However, this wide application of the expression fails to capture the concept of the process of halogenation as performed by a living entity. Further complicating this situation is the possibility that organisms may be producing organohalogens as a response to stress placed upon them by human activities. Finally, organohalogens may be produced by non-biogenic natural processes, such as combustion and volcanic eruptions (Stoiber et al. 1971). For the purpose of this work, “naturally-produced organohalogen” will be taken to mean an organic compound possessing at least

one carbon-halogen bond that has been associated with, and likely halogenated by, a living organism in its natural habitat.

1.1.3 Sources of naturally-produced organohalogens

By far, the majority of naturally-produced organohalogens are formed by marine organisms since they have ready access to the large pool of halide ions in seawater. Concentrations of halides in seawater are approximately 0.5 M chloride, 0.8 mM bromide, and 1 mM iodide (Gillespie et al. 1989). The principal producers of natural organohalogens are certain types of red alga (Fenical 1975); but sponges, tunicates, fungi, polychaetes, bryozoans, and some bacteria have also been shown to manufacture mixed brominated and chlorinated compounds (Gribble 1996).

1.1.4 Structures of naturally-produced organohalogens

Structures of naturally-produced organohalogens range from simple haloalkanes such as bromomethane (Harper 1994) to polycyclic compounds, such as the complex indole prosurugatoxin (Kosuge et al. 1985) (Figure 1.1). The extensive review written by Gribble (1996) illustrates the wide variety of structures of naturally-produced organohalogens. Many organohalogens produced by marine organisms are mixed halogenated, that is, they contain more than one type of halogen. More than half of the 3600 identified naturally-produced organohalogens contain bromine (Gribble 1999), even though bromide ions are present at a concentration in seawater approximately 600 times less than that of chloride. The

Figure 1.1 Structures of a simple and a complex marine halogenated marine natural product.

proliferation of organobromine compounds is most likely due to the low ionization potential of the bromine atom (11.8 eV) as compared to chlorine (13.0 eV) (CRC Handbook of Chemistry and Physics, 1996).

1.1.4 Formation mechanisms of naturally-produced organohalogens

The main mechanism in the formation of naturally-produced organohalogens is halogenation involving special enzymes called haloperoxidases. Haloperoxidases (HPOs) catalyze the oxidation of a halide ion by hydrogen peroxide, thereby enabling the oxidized halide to undergo electrophilic addition to an organic substrate. Nearly all

classes of marine organisms, and many terrestrial organisms, including humans, contain HPOs (Butler and Walker 1993). There are two types of marine HPOs, differentiated by their prosthetic groups: FeHeme and vanadium HPOs.

FeHeme HPO is the most common of the two HPOs encountered in marine organisms. The general halogenation mechanism involving FeHeme HPO is illustrated in Figure 1.2. The native heme-Fe³⁺ state of the HPO is oxidized to heme-Fe⁴⁺ by hydrogen peroxide. Heme-Fe⁴⁺ can then remove two electrons from a halide ion, which exists in its oxidized state as hypohalous acid, dihalogen, or trihalide ion. These species, which then halogenate an organic compound by electrophilic addition, are not enzyme bound.

Vanadium HPO catalyzes peroxidative halogenation in a similar manner as FeHeme HPO. The general mechanism is shown in Figure 1.3. There are two differences in this mechanism from that of FeHeme HPO, the first involving the binding of H₂O₂, most likely by coordination, to the vanadium (Tromp et al. 1990) atom in the HPO. The second difference is that it is not known whether or not the oxidized halide ion (ie. the hypohalous acid, dihalogen, trihalide ion, or other species containing a "halonium" moiety) is enzyme bound or free in solution.

It should be noted that halogenation does not appear to be the final step in the synthesis of many naturally-produced organohalogens. Laboratory studies in which marine organisms were deprived of certain halide ions did not result in the accumulation of the dehalo organic product (Fowden 1968).

Figure 1.2 FeHeme haloperoxidase mechanism of peroxidative halogenation.

Adapted from Butler and Walker (1993).

Figure 1.3 Vanadium haloperoxidase mechanism of peroxidative halogenation.

Adapted from Butler and Walker (1993).

1.1.5 Function of naturally-produced organohalogens

It appears that naturally-produced marine organohalogens are formed primarily for use as a chemical defense (Gribble 2000). The prolific producers of natural organohalogens tend to be soft-bodied, sessile organisms which lack physical defenses such as spines or shells. One study by Tuomi et al. (1989) supports the chemical defense hypothesis by demonstrating that within-plant concentrations of naturally-produced phenols were higher in the softer sections of a species of brown alga.

Many isolated naturally-produced organohalogens act as feeding deterrents in laboratory studies (Paul et al. 1993; Meyer and Paul 1995; Kinnel et al. 1977). Others have been implicated as anti-fouling agents (Paul 1987). It is almost certain that there are other functions of naturally-produced organohalogens than those listed above, since the structures of these compounds vary widely.

1.2 Environmental chemistry of naturally-produced organohalogens

Naturally-produced organohalogens are generally not examined within the framework of environmental chemistry as are anthropogenic organohalogens. The focus of natural products research is based on a pharmaceutical point of view concerned with the bioactivity of these compounds (Faulkner 1995). Many naturally-produced organohalogens display antiviral, antitumour, or antibiotic behaviour (Scheuer 1989), which is not surprising considering that many compounds are likely produced as chemical defense agents and thus likely display bioactive behaviour.

A number of naturally-produced organohalogenes have similar structures, or structural moieties, as many anthropogenic persistent organic pollutants (POPs). Similarities include the presence of aromatic systems and relatively high halogen contents (greater than three halogen atoms). Both of these properties contribute to a compound's resistance to biodegradation and abiotic transformations.

Molecular structure also affects the physical properties and environmental behaviour and fate of chemicals. Since there are structural similarities between some naturally-produced organohalogenes and anthropogenic POPs, one would therefore expect some naturally-produced organohalogenes to behave as anthropogenic POPs in the environment. Structural similarities will specifically affect the properties which ultimately govern the effects of chemicals on the ecosphere, such as persistence, bioaccumulation, biomagnification, and toxicological activity. The persistence, bioaccumulation, and biomagnification of a compound all affect the degree of exposure that an organism experiences whereas the toxicological activity reflects the hazard, or potential of the compound to cause negative effects. When combined together, exposure and hazard define the risk a compound poses to an organism.

1.2.1 Persistence

As described above, persistence is a resistance to abiotic and biotic degradation reactions occurring in the environment. Abiotic degradation occurs via hydrolysis, reduction, photolysis, and reaction with ozone or hydroxyl radicals in the atmosphere. Biotic degradation involves metabolic transformations such as dehalogenation, or the insertion of polar functional groups on relatively non-polar molecules which usually

renders compounds susceptible to elimination from organisms. General structure activity relationships have been constructed which define structures of persistent organohalogenes (Braune and Norstrom 1989). However, these relationships can be organism-specific, since metabolic capabilities vary widely between species. For example, seals generally have a higher capability than zooplankton, fish, and cetaceans, but lower than birds and terrestrial mammals, for metabolism of organohalogen pesticides and *m,p*-substituted polychlorinated biphenyls (PCBs) (Boon et al. 1989).

1.2.2 Bioaccumulation and biomagnification

Bioaccumulation is a general term referring to the accumulation of a compound in biota from its food or environment (including respired air or water and cutaneous absorption). Bioaccumulation occurs when compounds are ingested but not excreted or otherwise removed from an organism efficiently. Organic compounds which bioaccumulate are mostly lipophilic, but may also be metabolized and covalently bound to fatty acids (Larsen et al. 1992) or proteins (Sandau et al. 2000). Thus, resistance to biotransformation affects bioaccumulation. A compound which is easily transformed into a polar, water soluble metabolite will be easily excreted, and will not have the chance to accumulate in the organism. This is the case for the overwhelming majority of biogenic compounds, including those which contain halogens. Without protection from bioaccumulation, living organisms would soon be overwhelmed by exposure to lipophilic compounds.

Biomagnification is a more specific term describing the process in which a compound bioaccumulates in an organism to higher concentrations than what is found in

its food. Biomagnification factors can be calculated to quantify the extent to which the concentration of a compound increases over one, or multiple levels, of a food web. As with bioaccumulation, biotransformation affects biomagnification. Biomagnification of organochlorine pesticides and PCBs in aquatic food webs has been related to their octanol/water partition coefficients (K_{ow}) (Kidd et al. 1998). K_{ow} is a measure of a compound's hydrophobicity. Biomagnification of PCBs in a bird, the American kestrel, has also been shown to significantly increase with K_{ow} (Drouillard et al. 2001). However, a curvilinear relationship was observed between biomagnification and K_{ow} in juvenile rainbow trout (Fisk et al. 1998). Compounds with $\log K_{ow} > 7$ exhibited a decrease in biomagnification. This decrease in biomagnification was caused by an increase in the elimination rates of the highly hydrophobic compounds, possibly due to disequilibrium in the fish, reduced membrane passage, or elimination into feces. It is possible that the relationships between biomagnification and K_{ow} discussed above are not relevant to mammals or other species which have not been examined in this context.

1.2.3 Toxicological activity

The complexity of living organisms provides many opportunities for exogenous compounds to affect normal functions. There are many *in vivo* systems for POPs to disturb. Effects elicited can include reproductive, teratogenic, neuro- and cognitive developmental, immunotoxic, and genotoxic effects. The area of POPs toxicology is quite large and encompasses many specific biochemical and physiological effects. Furthermore, the examination of toxicological activity becomes more complex since the

susceptibility of organisms to these negative effects can vary widely between sexes, ages, and species (Stegeman et al. 2001; Bosveld et al. 1997; Okey et al. 1994).

One example of the range of effects that can be caused by exposure to anthropogenic POPs are "dioxin-like effects". These effects refer to those observed after exposure to polychlorinated dibenzo-*p*-dioxins and structurally similar compounds, such as polychlorinated dibenzofurans and non-ortho PCBs. Whole organism effects include chloracne observed in humans (Nishizumi 1996) and the fatal chick edema disease observed in some birds (Ludwig et al. 1993) and fish blue sac syndrome (Walker and Peterson 1994). Biochemical effects include the prolonged induction of metabolizing enzymes, such as cytochrome P4501A.

Most dioxin-like effects appear to be mediated by binding of a compound to a protein called the aryl hydrocarbon receptor (AhR). Ligands which bind with the strongest affinities for the AhR are more structurally similar to the most potent polychlorinated dibenzo-*p*-dioxin congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). These ligands are usually planar, aromatic, and have electron acceptor properties at lateral positions of the molecule (Mekenyan et al. 1996). There are other compounds which act as ligands of the AhR which do not contain these structural characteristics, however they generally do not bind with the same affinity as TCDD (Ciolino et al. 1999; Washburn et al. 1997; Phelan et al. 1998; Schaldach et al. 1999).

Effects related to disruption of the endocrine system have also been observed after exposure to some POPs. In these cases, exposure results in a disturbance of hormone systems which function as chemical messengers regulating various processes in growth and development. Effects of endocrine disruption by halogenated POPs have been

associated with responses such as the feminization of males (Guillette et al. 1995), birth defects (Colborn et al. 1993), and eggshell thinning (Peakall 1993). Specific biochemical effects include interference of endogenous ligands binding to receptors (Lutz and Kloas 1999) or transport proteins (Sandau et al. 2001).

There are no well-defined structure activity relationships for endocrine disruption, possibly because there is such a wide variety of effects mediated by many pathways (Bradbury et al. 1998; McKinney and Waller 1998). However, many halogenated aromatic POPs have been labeled as endocrine disrupters in various systems, including some tetra- to hexachlorinated PCB congeners and their hydroxylated metabolites (Andersson et al. 1999), *o,p'*-dichlorodiphenyltrichloroethane (Celius et al. 1999), γ -hexachlorocyclohexane (Celius et al. 1999), and certain polybrominated biphenyl congeners (Johnston et al. 1980).

1.3 Discovery of an unknown halogenated compound

During a routine monitoring program in 1988, relatively high levels of an unknown halogenated compound (UHC) were detected in Leach's storm-petrel eggs from both the Pacific and Atlantic coasts of Canada (Elliott et al. 1992). Mass spectra of the UHC indicated that it may be pentabrominated, and that the compound did not resemble any known anthropogenic contaminant. The presence of the UHC in eggs alongside commonly analyzed POPs such as PCBs and chlordane compounds suggested that it was another persistent hydrophobic compound that bioaccumulates in upper trophic level organisms.

The discovery of new organohalogens is not too uncommon, especially considering that measures of total organic chlorine or total organic bromine in biota often do not correspond to the concentrations of quantitated anthropogenic organohalogens (Kawano et al. 1995). New halogenated compounds have been identified as metabolites of anthropogenic organohalogens in plasma (Sandau et al. 2001), impurities in technical mixtures of industrially-produced POPs (Haglund et al. 1995), and truly unknown compounds (Vetter et al. 2000). Research into novel persistent and bioaccumulative halogenated compounds will likely increase as concentrations and research interest in routine analytes such as PCBs decrease.

1.4 Thesis purpose and outline

The purpose of this thesis is to describe the identification and subsequent examination of the environmental chemistry, bioaccumulation, and toxicology of the unknown halogenated compound (UHC) described in section 1.3. The research described in this thesis was performed in an attempt to evaluate the hypothesis:

“the unknown halogenated compound, and related compounds, are naturally-produced, globally distributed, persistent, bioaccumulative organohalogens that exhibit toxicological activity.”

The following three thesis chapters are concerned with the identification and characterization of the UHC and related compounds. Chapter 2 reports the tentative identification of UHC and three similar compounds as halogenated dimethyl bipyrrroles (HDBPs) and examination of the occurrence of the predominant UHC in seabird eggs from Canada. Chapter 3 describes the elucidation of the main UHC's structure using

isotope exchange positive chemical ionization mass spectrometry as 1,1'-dimethyl-tetrabromo-dichloro-2,2'-bipyrrrole. The determination of the positioning of the halogen atoms by comparison of mass spectral and retention time data between UHCs extracted from biota and synthesized HDBPs is also included. Chapter 4 contains results from the characterization of HDBP fundamental physical properties and a Level I equilibrium model describing their distribution.

The next two chapters focus on the distribution of HDBPs in biota. Chapter 5 reports on their global distribution in marine mammal blubber. Chapter 6 examines their distribution and potential to biomagnify in an Arctic marine food web.

The final chapters deal with the evaluation of the toxicological activity of the HDBPs. Chapter 7 describes the *in vitro* dioxin-like activity of HDBPs in chicken embryo hepatocytes. Since *in vitro* assays do not adequately represent effects to a whole organism. Chapter 8 evaluates the *in vivo* reproductive and morphological affects on American kestrels (*Falco sparverius*) exposed to HDBPs in their diet.

In total, this thesis represents the first evaluation of a class of possible naturally-produced compounds within the broad framework of environmental chemistry. This process provides results which describe the distribution, bioaccumulation, and possible effects of these compounds within the ecosphere.

CHAPTER 2.

IDENTIFICATION OF A NOVEL MIXED HALOGENATED HETEROCYCLIC COMPOUND IN SEABIRD EGGS*

2.1 Introduction

The known occurrences of mixed brominated and chlorinated compounds fall into two general areas: incineration by-products and marine natural products. Mixed halogenated dibenzodioxins/furans (Heeb et al. 1995) and phenols (Müller and Buser 1986) have already been detected and identified as incineration by-products. These compounds are formed in high temperature environments, such as combustion engines and municipal waste incinerators, that contain traces of aromatic and halogenated species (Heeb et al. 1995). Incineration by-products are presumed to be formed via free radical halogenation of the aromatic compounds.

Marine natural products are the second source of mixed brominated and chlorinated organic species. There is a wide variety of such compounds, including polyhalogenated monoterpenes (Faulkner et al. 1973; Fuller et al. 1992), phenols (Chen and Gerwick 1994), diphenyl ethers (Gribble 1996), pyrroles, and indoles (Gribble 1992). Sources of these compounds appear to be limited to sessile or slow-moving organisms with limited physical defenses, such as sponges and algae (Gribble 1996).

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adapted from Tittlemier et al. (1999a).

In 1988, relatively high levels of an unknown halogenated compound (UHC) were detected in Leach's storm-petrel eggs from both the Pacific and Atlantic coasts (Elliott et al. 1992). It appeared to be pentabrominated, but the exact halogen content was not determined. In this study, the novel UHC was characterized using various GC-MS techniques, including low and high resolution electron impact ionization (EI), electron capture negative ionization (ECNI), and positive chemical ionization (PCI). The more energetic ionization technique of EI provides information on the fragmentation of the compound, while the less energetic ionization techniques of ECNI and PCI provide information on the identity of the molecular ion. The high resolution mass spectrometry determines the specific elemental composition of the ions. The distribution of the novel compound was determined in bird eggs from the Great Lakes, Atlantic, and Pacific areas, and compared to that of a major bioaccumulating PCB, CB-153.

2.2 Experimental Section

2.2.1 Isolation of UHC

Bald eagle (*Haliaeetus leucocephalus*) liver samples from Port Hardy, BC were used as a source because of their relatively high levels of UHC. Two 12 g samples of homogenized liver were each combined with 145 g of activated Na₂SO₄ and mixed until free-flowing mixtures were obtained. The mixtures were extracted with dichloromethane/hexane (1:1) for 17 hours on a Soxhlet extraction apparatus. The extracts were reduced to approximately 5 mL and filtered using pre-cleaned 0.2 µm PTFE disposable filters and plastic syringes.

Lipids were removed from both samples according to the method described by Letcher et al. (1995). After lipid removal, the samples were reduced to approximately 1 mL and separated on a Florisil column into three fractions. The procedure outlined by Norstrom et al. (1988) was used, with the following changes: F1 36 mL hexane, F2 36 mL dichloromethane/hexane (85%/15%), F3 56 mL dichloromethane/hexane (1:1). The F2 fractions from both samples were combined and reduced to a final volume of 390 μ L.

2.2.2 Identification of UHC

The molecular formula of UHC was determined using both low and high resolution mass spectrometry. The initial mass spectrometry experiments were performed at unit resolution on a Hewlett Packard 5987B mass spectrometer in the electron impact ionization mode (electron energy 70 eV). The mass spectrometer was linked via a 5988 GC/MS direct interface to a 5890 Series II GC equipped with a 30m x 0.25mm i.d. DB-5MS column with a film thickness of 0.25 μ m (J&W Scientific). The initial temperature and hold time was 85°C for 3.00 min, first ramp 10°C/min to 180°C, second ramp 5°C/min to 300°C, injector temperature 250°C, interface temperature 280°C, and source temperature 190°C. Splitless injection mode was used, with volume of 2.0 μ L injected by a Hewlett Packard 7673 automatic injector.

Low resolution ECNI and PCI mass spectra were also obtained on the Hewlett Packard 5987B mass spectrometer to help in the identification of the unknown compound. These spectra were run under the same GC and interface temperature conditions used above. The ECNI spectrum was obtained using CH_4 (99.97%) at a pressure of 1.0 Torr measured at the source inlet, and a source temperature of 175°C.

The PCI spectrum was obtained at a source temperature of 150°C and electron energy of 100 eV using NH₃ (99.999%, 0.8 Torr) as a reagent gas. The ECNI and PCI spectra were full scans, from 75 to 650 and 200 to 750 Da, respectively.

The high resolution mass spectrometry was performed at resolutions of 10000 and 20000 using a VG AutoSpec double-focusing mass spectrometer in full scan electron impact ionization mode (electron energy 70 eV). The mass spectrometer was linked to a Hewlett Packard 5890 Series II GC fitted with 30 m x 0.25 mm i.d. DB5-MS column (J & W Scientific). The GC conditions used were as follows: initial temperature 100°C held for 3 min, initial ramp 20°C/min to 180°C, and final ramp 5°C/min to 300°C. The injector, interface, and source were all kept at a temperature of 280°C. Volumes of 1.0 µl were injected in the splitless mode by a CTC Analytics A200S automatic injector.

2.2.3 Quantification of UHC in eagle liver sample

The concentration of UHC in the combined F2 and F3 extracts from the bald eagle liver was determined using gas chromatography with flame ionization detection (FID). Determination of the molecular formula by mass spectrometry indicated that the compound contained 10 carbon atoms. Yieru et al. (1990) demonstrated that carbon weight response factors for a variety of compounds are constant even though FID responses are affected by the presence of heteroatoms (Tong and Karasek 1984). As a precaution, a variety of compounds were used to ensure adequate representation of all of the structural elements that could be present in the unknown compound. The following chemicals were used as standards in the quantification: nicotine, hexachlorobenzene, heptachlor epoxide, pyrene, 2,2',4,4',5,5'-hexachlorobiphenyl, 2,3,3,4',5,5',6-

heptachlorobiphenyl, mirex, t-chlordane, 2-(p-tolyl)-pyridine, α,α,α -tribromoquinaldine, 1,3-dibromoadamantane, and 2,3,3',4,4',5,6-heptabromodiphenyl ether.

The compounds were analyzed with a Hewlett Packard 5890 GC with FID detection under the following conditions: detector temperature 270°C, initial oven temperature 85°C held for 3.00 min, initial ramp of 20°C/min to 200°C, final ramp of 5°C/min to 300°C, and held for 3.00 min. A volume of 0.5 μ L of each solution was injected onto a 30m x 0.25mm i.d. DB-5MS column (0.25 μ m film thickness, J&W Scientific) using automated cool on-column injection to avoid discrimination against the higher molecular weight compounds. All solutions were injected in triplicate. Average carbon weight response factors (Yieru et al. 1990) were calculated for each of the 12 compounds. After determination of its carbon content by the mass spectrometry experiments, the carbon concentration of the UHC in the eagle liver extract was determined using the mean of the average carbon weight response factor for each of the 12 compounds. The UHC concentration was then determined from a knowledge of its molecular formula.

2.2.4 Sample analysis

Seabird and gull egg samples were obtained from the National Wildlife Research Centre Specimen Bank. The samples (sampling locations shown in Figure 2.1) represent offshore surface feeders [Leach's storm petrel (*Oceanodroma leucorhoa*) and Black-footed albatross (*Diomedea nigripes*)], offshore subsurface feeders [Rhinceros auklet (*Cerorhinca monocerata*) from the Pacific and Atlantic puffin (*Fratercula arctica*) from the Atlantic], and inshore omnivores [Glaucous-winged gulls (*Larus glaucescens*) from

the Pacific and herring gulls (*Larus argentatus*) from the Atlantic and Great Lakes]. The Leach's storm petrel was classified as a surface feeder even though it feeds on vertically migrant fish and crustaceans, because it also consumes fish, crustaceans, and soft-bodied animals that inhabit the surface layer (Huntington et al. 1996). Accurately weighed amounts of homogenized egg contents (around 5 g) were mixed with a ten-fold amount of activated Na₂SO₄. The mixtures were then spiked with an internal standard (¹³C-labelled heptachlor epoxide, 20 µL, 100 ng/µL). After 45 minutes the mixtures were wet-packed with dichloromethane/hexane (1:1) into a glass column (2 cm i.d.) and extracted with 220 mL of dichloromethane/hexane (1:1). After extraction, the lipid content of the samples was determined, and the lipid was then removed by GPC (Letcher et al. 1995). The GPC fractions were reduced to approximately 1 mL and the organohalogenes were fractionated on a Florisil column following the same procedure as for the bald eagle liver. Fractions F2 and F3 were combined and reduced to exactly 2.00 mL. Fraction F1 was also reduced to a final volume of 2.00 mL. Four method blanks containing no sample were used. One blank was carried through all of the steps of the sample preparation and analysis with each set of samples run.

The F1 fractions were analyzed for CB-153 using a method similar to the one outlined by Norstrom et al. (1988). The combined F2 and F3 fractions were analyzed for UHC using gas chromatography electron capture negative ionization mass spectrometry (GC-ECNI-MS) in the single ion monitoring mode. The analyses were performed on a Hewlett Packard 5987B mass spectrometer connected to a 5890 Series II GC. The GC was equipped with a 30m x 0.25mm i.d. DB-5MS column with a film thickness of 0.25

Figure 2.1 Seabird and gull egg sampling sites.

A, Storm Islands, BC; B, Cleland Island, BC; C, Five Finger Rocks, BC; D, Silver Islet, Lake Superior; E, Big Sister Island, Lake Michigan; F, Chantry Island, Lake Huron; G, Middle Island, Lake Erie; H, Snake Island, Lake Ontario; I, Machias Seal Island, NB; J, Kent Island, NB; K, Sable Island, NS; L, Gull Island, NF. The Sand Island, Pacific Ocean site is part of the Midway Island group and is not shown on this map.

μm (J&W Scientific). The temperature program was as follows: injector temperature 250°C, initial temperature and hold time 85°C for 3.00 min, first ramp 10°C/min to 180°C, second ramp 5°C/min to 300°C. Splitless injection mode was used, with an injection volume of 2.0 μL. The mass spectrometer was operated in the negative chemical ionization mode with CH₄ (99.97%, 1.0 Torr) as the moderating gas, an interface temperature of 280°C, and a source temperature of 190°C. Previous to injection, 50.0 μL of each sample and standard were spiked with 1.0 μL of a performance standard of decachlorodiphenyl ether (50 ng/μL). Quantitation was performed using the bald eagle liver extract UHC solution.

2.3 Results

2.3.1 Identification of UHC

High resolution mass spectra of the UHC were obtained to determine its molecular formula. Software provided as part of the OPUS operating system of the VG AutoSpec was used to determine the possible elemental compositions of the ions studied by comparing the experimentally determined mass to theoretical masses of species provided as possibilities by the software. Limits were set on the possible elements, and their amounts, present in the suggested fragments. The possibilities given by the software were restricted to those that contained C (0-15), H (0-15), N (0-4), O (0-4), S (0-4), P (0-4), Br (0-4), and Cl (0-4). Seven different ions from the most abundant clusters in the EI mass spectrum of the major congener were analyzed under the high resolution

conditions. The most probable elemental composition of each ion was the composition which gave a theoretical mass with the smallest difference from the experimentally determined mass (Table 2.1) and contained the correct proportion of bromine and chlorine to produce the isotopic pattern observed for UHC. The experimental masses listed in Table 2.1 were obtained at a resolution of 10000.

Table 2.1. Elemental composition of ions from the major UHC congener found in bald eagle liver extract, analyzed at a resolution of 10000.

Experimental Mass	Theoretical Mass	Difference	Most Probable Elemental Composition
139.031067	139.029623	0.001444	C ₉ H ₃ N ₂
190.913635	190.913738	-0.000103	C ₅ H ₃ NBrCl
366.802704	366.804001	-0.001297	C ₉ H ₃ N ₂ Br ₂ Cl ₂
381.828430	381.827476	0.000954	C ₁₀ H ₆ N ₂ Br ₂ Cl ₂
419.719086	419.719263	-0.000177	C ₈ H ₃ NBr ₃ Cl ₂
460.748535	460.745812	0.002723	C ₁₀ H ₆ N ₂ Br ₃ Cl ₂
539.664063	539.664148	-0.000085	C ₁₀ H ₆ N ₂ Br ₄ Cl ₂

2.3.2 EI mass spectrum of $C_{10}H_6N_2Br_4Cl_2$

The low resolution EI mass spectrum of $C_{10}H_6N_2Br_4Cl_2$ is shown in Figure 2.2. The molecular ion was assigned to the ion cluster starting at 540 Da. The identities of some of the fragment ions are indicated on the mass spectrum. The only fragmentations observed are successive losses of Br[•] and Cl[•], loss of CH₃[•], and loss of C₂H₃NBr. The fragmentation pathways are similar for all of the congeners in the series.

2.3.3 ECNI mass spectrum of $C_{10}H_6N_2Br_4Cl_2$

Four main clusters can be seen in the ECNI mass spectrum (Figure 2.3). The apparent molecular ion from the EI spectrum is also the base peak at 544 Da in the ECNI spectrum. A fragment corresponding to loss of Br[•] is seen at 461 Da. Another fragment ion cluster occurs around 372 Da, and is likely a combination of two or more overlapping patterns.

2.3.4 NH₃ PCI mass spectrum of $C_{10}H_6N_2Br_4Cl_2$

The NH₃ PCI mass spectrum is shown in Figure 2.4. There are no ions of mass greater than 541 Da observed. Only two other ion clusters are observed: one at 384 Da, and the other at 463 Da. The identity of the first cluster is not known, while the second cluster is a composite of two overlapping clusters - [M+H-Br]⁺ and [M+2H-Br]⁺. The pathways that form these ions must be of very low energy if they are accessible by a molecular ion produced by NH₃ PCI-MS.

2.3.5 Quantification of $C_{10}H_6N_2Br_4Cl_2$ in eagle liver extract

The 12 standards used in the FID quantification of $C_{10}H_6N_2Br_4Cl_2$ had similar carbon weight response factors (mean: 1888 ± 165 area/g of carbon). Comparable results were found using a wider variety of compounds by Yieru et al. (1990). Since the carbon weight response factors are similar for a variety of organic compounds regardless of their type or structure, the $C_{10}H_6N_2Br_4Cl_2$ could be quantitated using standards that are structurally dissimilar. Thus, the structure of $C_{10}H_6N_2Br_4Cl_2$ does not have to be known for adequate standards to be found. In the eagle liver extract the concentration of $C_{10}H_6N_2Br_4Cl_2$ was calculated to be 160 ± 20 ng/ μ L.

The ECNI total ion chromatogram of the eagle liver extract is shown in Figure 2.5. The four congeners of the $C_{10}H_6N_2Br_mCl_n$ series are present. However, only the major congener, $C_{10}H_6N_2Br_4Cl_2$, was present at a large enough amount to be quantitated using FID.

Figure 2.2 EI mass spectrum of C₁₀H₆N₂Br₄Cl₂ from bald eagle liver extract.

Figure 2.3 ECNI mass spectrum of C₁₀H₆N₂Br₄Cl₂ from bald eagle liver extract.

Figure 2.4 NH₃ PCI mass spectrum of C₁₀H₆N₂Br₄Cl₂ from bald eagle liver extract

2.3.6 Distribution of $C_{10}H_6N_2Br_4Cl_2$ in egg samples

Absolute concentrations of $C_{10}H_6N_2Br_4Cl_2$, and concentrations relative to CB-153 are shown in Table 2.2. No corrections were made to the $C_{10}H_6N_2Br_4Cl_2$ concentrations because recoveries of the internal standard were all above 70%. The concentration of the mixed halogenated compound is given as a ratio to that of CB-153 to normalize its concentration to the levels of other halogenated compounds in the eggs. This ratio allows the bioaccumulation of $C_{10}H_6N_2Br_4Cl_2$ to be compared among species relative to a ubiquitous environmental contaminant.

2.4 Discussion

2.4.1 Identification of UHC

The UHC was first detected in 1988 during a screening program to identify contaminants in Atlantic and Pacific coast seabirds using low resolution EI-MS. At the time, the compound was believed to be a pentabrominated species (Elliott et al. 1992). Further low resolution EI-MS work on a highly contaminated bald eagle liver sample indicated that the unknown compound contained not only bromine, but chlorine as well. The observed isotopic patterns of the unknown compound most closely matched four bromine and two chlorine atoms. A pentabromo isotopic cluster looks very similar to a tetrabromodichloro cluster, except the latter contains seven ions.

Table 2.2. Concentrations of $C_{10}H_6N_2Br_4Cl_2$ in various seabird egg samples from Canada.

Sample collection location ^a	Fig. 1 Map Reference	Ocean / Great Lakes	Number of eggs in pool	Collection date	$C_{10}H_6N_2Br_4Cl_2$ (ng/g sample, wet weight)	Ratio of $C_{10}H_6N_2Br_4Cl_2$ to CB-153
<i>Offshore Surface Feeders</i>						
LSP, Storm Islands, BC	A	Pacific	6	02/07/94	140	2.1
LSP, Cleland Island, BC	B	Pacific	3	30/06/94	120	0.96
ALB, Sand Island, Pacific Ocean	-	Pacific	10	03/12/93	32	0.34
LSP, Gull Island, NF	L	Atlantic	10	22/06/96	4.6	0.097
LSP, Kent Island, NB	J	Atlantic	10	16/06/96	4.8	0.029
<i>Offshore Subsurface Feeders</i>						
RA, Cleland Island, BC	B	Pacific	5	29/04/90	3.1	0.050
RA, Storm Islands, BC	A	Pacific	5	04/05/90	1.8	0.043
AP, Gull Island, NF	L	Atlantic	10	15/06/96	1.7	0.048
AP, Machias Seal Island, NB	I	Atlantic	10	19/05/96	2.5	0.017
<i>Inshore Omnivores</i>						
GWG, Five Finger Rocks, BC	C	Pacific	10	28/06/90	5.6	0.10
HG, Silver Islet, Lake Superior	D	G. Lakes	7	18/05/96	nd ^b	-
HG, Middle Island, Lake Erie	G	G. Lakes	13	28/04/96	nd	-
HG, Snake Island, L. Ontario	H	G. Lakes	13	26/04/96	nd	-
HG, Chantry Island, L. Huron	F	G. Lakes	13	23/04/96	nd	-
HG, Big Sister Island, L. Michigan	E	G. Lakes	13	03/05/96	nd	-
HG, Sable Island, NS	K	Atlantic	10	13/05/96	1.6	0.0096
HG, Gull Island, NF	L	Atlantic	4	20/05/96	0.61	0.0088

^aLSP = Leach's storm-petrel, RA = rhinoceros auklet, GWG = glaucous-winged gull, ALB = black-footed albatross,

HG = herring gull, AP = Atlantic puffin.

^bnd = not detected

Figure 2.5 ECNI total ion chromatogram of bald eagle liver extract

According to the results of the high resolution mass spectrometry given in Table 2.1, the parent ion at 540 Da has a molecular formula of $C_{10}H_6N_2Br_4Cl_2$. There were other possible elemental compositions provided by the program, but they did not contain both bromine and chlorine; or if they did, it was not in the right proportion that would produce isotopic patterns that resembled those of the major congener. There were some suggested compositions which did contain the right proportion of bromine and chlorine, however their theoretical masses ranged from 1 to 27 mDa higher than the experimental masses listed in Table 2.1.

The ion of largest mass in the EI spectrum (540 Da) was assumed to be the molecular ion. This assignment was confirmed in the PCI experiments. The even mass is consistent with a compound containing two nitrogen atoms according to the nitrogen rule (McLafferty 1966). Subsequent examination of the bald eagle liver sample revealed that a series of four mixed hexahalogenated congeners existed (Figure 2.5), with the halogen content ranging from Br_6 to Br_3Cl_3 . Therefore, the series of unknown compounds in the eagle liver have the general formula $C_{10}H_6N_2Br_mCl_n$, where $m=3-6$, $n=0-3$, and $m+n=6$. The major congener, $C_{10}H_6N_2Br_4Cl_2$, was present at an amount roughly 10 times greater than the other three congeners.

One of the most likely structures for $C_{10}H_6N_2Br_4Cl_2$ is 1,1'-dimethyl-tetrabromodichloro-2,2'-bipyrrole (Figure 2.6). A related compound, hexabromo-2,2'-bipyrrole, is a known marine bacterial product (Andersen et al. 1974). Loss of $CHNBr$ from M^+ is important in the EI mass spectrum of hexabromo-2,2'-bipyrrole.

Figure 2.6 Proposed structure of $C_{10}H_6N_2Br_4Cl_2$: 1,1'-dimethyl-tetrabromodichloro-2,2'-bipyrrrole.

The letter X represents a bromine or chlorine atom such that total Br = 4 and total Cl = 2.

Also present in the EI mass spectrum is what appears to be a doubly charged ion at 191 Da. corresponding to $[M-2Br]^{++}$. The spacing of the peaks in the isotopic pattern is 1 as opposed to 2 Da. which is indicative of a doubly charged ion. The isotopic pattern of this cluster should be identical to that of the cluster for $[M-2Br]^+$ at 382 Da. however it is not. The presence of the doubly charged ion was confirmed by lowering the ionizing electron energy to 30 eV. Doubly charged ions have high appearance potentials. thus electrons of low kinetic energy (30 eV) will not transfer an adequate amount of energy to the substrate to form the ions (Bieman 1962). When the electron energy was lowered. the isotopic cluster for $[M-2Br]^{++}$ disappeared. leaving only the cluster for $[M-3Br-2Cl-C_2H_3N]^+$ at 190 Da present. The overlapping isotopic pattern from $[M-3Br-2Cl-C_2H_3N]^+$ caused the isotopic patterns at 191 and 382 Da to differ.

Multiply charged ions can supply structural information on the analyte molecule because they are formed only under certain circumstances. They are only formed from highly stabilized molecules with high π -electron density such as fused-ring aromatics

(McLafferty 1966), or from compounds that contain a nitrogen atom (Hamming and Foster 1972). In this case, high resolution mass spectrometry confirmed that the doubly charged ion $[C_{10}H_6N_2Br_2Cl_2]^{++}$ contained two nitrogen atoms. It is also probable that the doubly charged ion has an aromatic structure; the unsaturation number (Lambert et al. 1987) of the parent compound is 6, indicating that an aromatic moiety is most likely present.

The abundance of the multiply charged ion can also provide some structural information. The doubly charged ion in the mass spectrum is about 45% of the base peak. Usually doubly charged ions are less than 10% of the base peak (Benyon and Williams 1959). An exception to this general rule occurs in the case of alkyl indoles. This suggests that an alkyl indole-type structure is another possibility.

As expected, fewer fragment ions were observed in the ECNI mass spectrum (Figure 2.3) than the EI spectrum. In ECNI, a moderating gas acts as a source of secondary electrons, and creates an environment for thermalization of primary electrons and stabilization of negative molecular ions (Ong and Hites 1994). Less energy is transferred during the capture of a thermalized electron by an analyte molecule. Less energy transferred generally results in less fragmentation of the analyte.

There is also evidence of bromide ion attachment in this mass spectrum. The fourth cluster at 619 Da corresponds to $[M+Br]^-$, and the isotopic pattern is indicative of an ion containing five bromine and two chlorine atoms. This seems to be the first noted instance of bromide ion attachment. The formation of the bromide ion/molecule adduct is analogous to the chloride ion attachment that has been observed for polychlorinated

aromatic compounds (Dougherty et al. 1975) and hexachlorocyclopentadiene derivatives (Stemmler and Hites 1985).

PCI, like ECNI, is a softer ionization technique than EI, therefore the PCI spectrum shows fewer fragment ions than the EI spectrum. However, PCI is unlike ECNI in the mechanism of ionization. In ECNI, thermalized electrons ionize the analyte molecule. In PCI, the analyte molecule is ionized via addition of H^+ or [reagent gas molecule] $^+$. The difference in proton affinities of the analyte and reagent gas determine which ionized products are formed and the extent to which they will fragment.

The degree of fragmentation can be controlled by using various reagent gases. A reagent gas with a proton affinity very close to, but less than, the analyte will transfer H^+ to the analyte, and keep the internal energy of MH^+ lower than the analogous parent ion in EI or ECNI. A low amount of internal energy of MH^+ will result in less fragmentation. Therefore, using a reagent gas with a relatively high proton affinity, such as NH_3 , will reduce fragmentation, and provide supporting evidence for the identity of the molecular ion. Thus, the lack of ions of mass greater than 541 Da is strong evidence that the cluster at 541 Da is the molecular ion cluster, $[M+H]^+$.

2.4.2 Distribution of $C_{10}H_6N_2Br_4Cl_2$ in seabird eggs

From the data in Table 2.2, it is apparent that $C_{10}H_6N_2Br_4Cl_2$ is widespread in marine seabirds. By far the highest concentrations were found in Pacific Ocean surface feeders, the mid-ocean feeding albatross and Leach's storm-petrels, which feed approximately 100-200 km from the coast (Huntington et al. 1996). Concentrations in Pacific Ocean storm-petrels were 28 times higher than in the same species in the Atlantic

Ocean. Concentrations in the offshore, subsurface piscivores, rhinoceros auklet and Atlantic puffin, were similar in the Atlantic and Pacific Oceans. The bald eagle liver sample contained a higher level of $C_{10}H_6N_2Br_4Cl_2$ than each of the seabird samples because it feeds at a higher trophic level than the seabirds.

The compound was not detected in any of the herring gull samples from the Great Lakes. This absence from Great Lakes samples suggests that long-range atmospheric transport does not play a role in the distribution of these compounds. It also tends to rule out combustion sources, such as municipal incinerators. The herring gull samples are geographically representative of the Great Lakes since the year-round movements of adult herring gulls are within the Great Lakes drainage basin (Gilman et al. 1977).

Ratios of $C_{10}H_6N_2Br_4Cl_2$ to CB-153 and absolute concentrations of $C_{10}H_6N_2Br_4Cl_2$ from the Pacific and Atlantic samples were compared using the Mann-Whitney U test. This test was used because the unequal variances of the Pacific and Atlantic data sets affected the t-test. The concentrations of $C_{10}H_6N_2Br_4Cl_2$ across the different species were much more uniform in the Atlantic samples than in the Pacific samples. The absolute concentrations and ratios were found to be significantly ($p=0.037$) higher in the Pacific samples. The ratio of $C_{10}H_6N_2Br_4Cl_2$ to CB-153 was 24, 11 and 1.4 times greater in Pacific than Atlantic petrels, gulls and alcid, respectively.

The ratio of $C_{10}H_6N_2Br_4Cl_2$ to CB-153 in Pacific Ocean surface feeders was 0.34 to 2.1, indicating that $C_{10}H_6N_2Br_4Cl_2$ ranks as a major contaminant in these species along with well-known globally-distributed organohalogen pollutants. In the Pacific Ocean, Leach's storm-petrels had ratios 15 and 33 times greater than the glaucous-winged gull

and rhinoceros auklets. For the Atlantic samples, the ratios were 6.8 and 1.9 times greater for Leach's storm-petrels than the herring gull and Atlantic puffins.

2.5 Conclusions

The widespread occurrence of $C_{10}H_6N_2Br_4Cl_2$ in Pacific Ocean and Atlantic Ocean seabirds coupled with its absence in the Great Lakes indicates that atmospheric transport is not involved in its distribution, nor is combustion likely to be a source. The high nitrogen and mixed chlorine/bromine content suggests a marine natural product, since synthetic organohalogenes which are produced in large volume (e.g., flame retardants) are either chlorinated or brominated, but usually not both. Sources of naturally-produced organohalogenes include many types of marine bacteria and algae (Gribble 1996). An extensive literature search revealed no previous mention of a compound with this molecular formula. The concentration of $C_{10}H_6N_2Br_4Cl_2$ was much higher in Pacific Ocean surface-feeding birds than in subsurface-feeding piscivorous birds from the same area and in the same species in the Atlantic Ocean. This finding suggests that the compound is produced in highest quantities in the Pacific Ocean surface layer. Another possibility is that the compound is produced and accumulated at lower depths in the Pacific Ocean, and transported to the surface by organisms such as jellyfish and crustaceans. The structure suggested by the EI spectrum, 1,1'-dimethyl-tetrabromodichloro-2,2'-bipyrrrole, is a good candidate. A closely related hexabrominated compound has been identified in Pacific Ocean marine chromobacteria (Andersen et al. 1974). Furthermore, a bipyrrrole with perhalogenated ring carbons and N,N'-dimethyl substitution is likely to be more resistant towards biotransformation than most possible

structures, and therefore has bioaccumulation potential in birds. Structures that contain primary or secondary amine groups can probably be ruled out as they are readily biotransformed in most species.

It is extremely difficult to speculate on a possible natural source of HDBPs since there are numerous marine organisms which produce organohalogens (Gribble 1996). At this point, the likeliest source is the bacterium, or a related species, which produces the structurally similar hexabromo-2,2'-bipyrrrole. This bacterium, referred to as *Chromobacter* I-L-33 by Andersen et al. (Andersen et al. 1974), was physiologically, culturally, and morphologically similar to *Chromobacterium marinum* (Hamilton and Austin 1967). *C. marinum* was isolated from a surface seawater sample, and its behaviour in culture suggested that it is confined to the surface layers. If the source of $C_{10}H_6N_2Br_4Cl_2$ can be confirmed to be *C. marinum* or another marine organism, this would be the first instance of a naturally-produced organohalogen bioaccumulating in higher trophic level organisms.

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CHAPTER 3.

STRUCTURE ELUCIDATION OF FOUR POSSIBLE BIOGENIC ORGANOHALOGENS USING ISOTOPE EXCHANGE MASS SPECTROMETRY*

3.1 Introduction

There exists a large number of biogenic organohalogen compounds in the environment in addition to the ubiquitous anthropogenic organohalogenes routinely observed. Naturally-produced organohalogenes are of interest since they share a chemical characteristic with most persistent bioaccumulative contaminants, namely they contain halogen substituents. The strengths of the halogen-carbon bonds contribute to the resistance to degradation and bioaccumulation of persistent organic pollutants. Structures of naturally-produced organohalogenes range from simple halomethanes to halogenated polycyclic glycopeptides (Gribble 1996). The variety of molecular structures of biogenic organohalogenes reflects the importance of these compounds.

Our recent study measured levels in bird eggs from across Canada of a mixed brominated chlorinated compound that was proposed to be naturally-produced (Tittlemier et al. 1999a). The molecular formula of the unknown halogenated compound was determined to be $C_{10}H_6N_2Br_4Cl_2$ by high resolution mass spectrometry. This particular compound was also found to be the most abundant congener in a series of four

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* adapted from Tittlemier et al. (2001a).

hexahalogenated compounds ($C_{10}H_6N_2Br_3Cl_3$, $C_{10}H_6N_2Br_4Cl_2$, $C_{10}H_6N_2Br_5Cl$, and $C_{10}H_6N_2Br_6$) that apparently shared a common skeletal structure. Concentrations of the major congener ranged from 32 to 140 ppb in eggs from offshore surface feeding species from the Pacific, such as the Black-footed albatross and Leach's storm-petrel. These levels were roughly 0.3 to 2 times the concentration of CB-153, the most abundant PCB congener in the samples.

The accumulation to relatively high levels in seabird eggs indicated an apparent resistance of the $C_{10}H_6N_2Br_4Cl_2$ to biotransformation. This resistance suggested that the compound contains tertiary amine groups since primary or secondary amine functional groups are less sterically hindered and thus more accessible to oxidative enzymes. In addition, $C_{10}H_6N_2Br_4Cl_2$ and the other congeners were proposed to contain a 2,2'-bipyrrole moiety based on EI mass spectral similarities to hexabromo-2,2'-bipyrrole (Andersen et al. 1974). The skeletal structure of the hexahalogenated compounds was proposed to be 1,1'-dimethyl-2,2'-bipyrrole (Figure 2.6) based on the above evidence (Tittlemier et al. 1999a).

Isotope exchange positive chemical ionization mass spectrometry (IE-PCI-MS) was initially used in this study to help determine the structure of $C_{10}H_6N_2Br_4Cl_2$. It has been shown using isotopically labeled reagent gases that hydrogen atoms in certain chemical environments can be exchanged for deuterium atoms. Aromatic hydrogens in alkylbenzenes, oxygenated benzenes, thiophenes, metallocenes, and some polycyclic aromatic hydrocarbons (PAHs) have been exchanged using D_2O , EtOD, and ND_3 (Hunt and Sethi 1980). In addition, hydrogen atoms bonded to heteroatoms in functional

groups such as alcohols, phenols, carboxylic acids, amines, amides, and mercaptans were exchanged using D₂O (Hunt et al. 1972).

This exchange phenomenon has been used to differentiate between isomeric primary, secondary, and tertiary amines (Hunt et al. 1971), and to characterize amine-substituted PAHs and azaarenes using ND₃ (Buchanan 1982). The degree of substitution is determined by separately obtaining PCI mass spectra using native and deuterated reagent gases. A shift in mass of the quasimolecular ion occurs since the labile hydrogen atoms are exchanged with heavier deuterium atoms. From the shift in mass, the number of exchangeable hydrogen atoms, and therefore the degree of substitution of the amine, can be determined. In this experiment NH₃ and ND₃ were used as reagent gases in IE-PCI-MS to characterize the two nitrogen atoms in C₁₀H₆N₂Br₄Cl₂ as mono-, di-, or tri-substituted.

Once the nitrogen atoms were characterized, molecular structures of the four hexahalogenated compounds were proposed, and the corresponding compounds were synthesized. Retention times and full scan electron capture negative ionization (ECNI) mass spectra of the synthesized compounds were then compared to C₁₀H₆N₂Br₃Cl₃, C₁₀H₆N₂Br₄Cl₂, C₁₀H₆N₂Br₅Cl, and C₁₀H₆N₂Br₆ found in biota extracts to confirm the proposed structures.

3.2 Experimental Section

3.2.1 Chemicals

Nineteen chemicals (Table 3.1) were used as reference compounds in the IE-PCI-MS experiment. Reference compounds were chosen to represent primary, secondary, and tertiary amines in both aromatic and aliphatic chemical environments. Five reference compounds contained two nitrogen atoms. Six compounds containing no nitrogen atoms were included to act as control compounds. Solutions of each compound were made in the 38-60 ng/ μ L range. Bald eagle (*Haliaeetus leucocephalus*) liver and Northern elephant seal (*Mirounga angustirostris*) blubber were used as sources of $C_{10}H_6N_2Br_3Cl_3$, $C_{10}H_6N_2Br_4Cl_2$, $C_{10}H_6N_2Br_5Cl$ and $C_{10}H_6N_2Br_6$. The extraction and isolation from bald eagle liver has been described in detail elsewhere (Tittlemier et al. 1999a). A similar method was used for the extraction and isolation of the four hexahalogenated compounds from Northern elephant seal blubber.

3.2.2 IE-PCI-MS

All mass spectrometry was performed on a Hewlett Packard 5987B mass spectrometer coupled via a 5988 GC/MS direct interface to a 5890 Series II GC. The interface was maintained at a temperature of 280°C. The GC was equipped with a 30 m x 0.25 mm i.d. DB5-MS column with a film thickness of 0.25 μ m (J & W Scientific). Sample volumes of 2.0 μ L were injected using a Hewlett Packard 7673 automatic injector. The mass spectra were obtained in the positive chemical ionization mode at a source temperature of 190°C and ionizing electron energy of 85 eV. Both NH_3

(99.999%, Praxair Products Inc.) and ND₃ (D₃, 99%; Cambridge Isotope Laboratories, Inc.) were used as reagent gases in IE-PCI-MS. They were maintained at a pressure of 1.3 Torr, measured at the source inlet. Full scans were obtained from 65-650 Da for NH₃, and 90-650 Da for ND₃.

3.2.3 Synthesis of halogenated dimethyl bipyrrroles (HDBPs)

The 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrrole and 1,1'-dimethyl-3,3',4,4',5,5'-hexabromo-2,2'-bipyrrrole were synthesized and characterized using X-ray crystallography, ¹H and ¹³C NMR as described in Gribble et al. (1999). The 1,1'-dimethyl-3,3',4,4'-tribromo-5,5'-dichloro-2,2'-bipyrrrole was the tribrominated dimethyl bipyrrrole (DBP) thought to be formed as a byproduct in the synthesis of 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrrole. The final DBP – 1,1'-dimethyl-3,3',4,4',5-pentabromo-5'-chloro-2,2'-bipyrrrole – was synthesized using a slightly different procedure than the other HDBPs. The 1,1'-dimethyl-5-chloro-2,2'-bipyrrrole was first synthesized from 1,1'-dimethyl-2,2'-bipyrrrole and one equivalent of N-chlorosuccinimide. The monochlorinated compound was then brominated using five equivalents of N-bromosuccinimide. The pentabrominated monochlorinated compound was characterized using ¹H and ¹³C NMR (Gribble et al. 1999).

3.2.4 Retention time and ECNI full scan comparison

Five GC columns with stationary phases of varying polarity were used in the retention time and ECNI full scan comparisons to reduce the chances of coincidental co-

elution. The GC columns used were: DB5-MS (5% phenyl- 95% methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 μ m, J&W Scientific), DB-1701 (14% cyanopropylphenyl- 86% methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 μ m, J&W Scientific), DB-210 (50% trifluoropropyl- 50% methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 μ m, J&W Scientific), DB-WAX (polyethylene glycol, 30 m x 0.25 mm i.d., film thickness 0.25 μ m, J&W Scientific), and Rtx-2330 (10% cyanopropylphenyl- 90% biscyanopropylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.2 μ m, Restek). Identical GC temperature programs were used to obtain full scan spectra of the synthesized compounds and the compounds extracted from biota. Mass spectrometric analysis was performed in the ECNI mode with CH_4 (99.97%, 1.0 Torr) as the moderating gas. Spectra were obtained from 75 to 650 Da.

3.3 Results

3.3.1 Isotope exchange mass spectrometry

Results for NH_3 and ND_3 PCI experiments are listed in Table 3.1. As expected from a low energy ionization technique, all PCI spectra showed little to no fragmentation. For all of the amines, including $\text{C}_{10}\text{H}_6\text{N}_2\text{Br}_4\text{Cl}_2$, the base peak of the mass spectra corresponded to addition of a proton or deuteron (plus H/D exchange in the cases of primary and secondary amines) to the analyte molecule. Some amines also underwent addition of NH_4^+ or ND_4^+ , but the intensities of these ions were always less than that of the base peak. The predicted mass shifts of the quasimolecular ions between the NH_3 and ND_3 obtained mass spectra are also listed in Table 3.1.

Table 3.1. Results from isotope exchange PCI-MS analysis of C₁₀H₆N₂Br₄Cl₂.

Compound	Amine Type	Predicted Mass Shift	Molec. Weight	NH₃ PCI spectra Quasimolecular ion^{a,b}	ND₃ PCI spectra Quasimolecular ion
Control Compounds					
pyrene		1	202	M ⁺ m/z 202, (15%) [M+H] ⁺ m/z 203, (100%) NS	M ⁺ m/z 202, (40%) [M+D] ⁺ m/z 204, (100%) [M-H+2D] ⁺ m/z 205, (23%) NS
3,3',4,4',5-pentachlorobiphenyl		1	324	NS	NS
2,3,3',4,5,5',6-heptachlorobiphenyl		1	392	NS	NS
naphthalene		1	128	M ⁺ m/z 128, (100%) [M+H] ⁺ m/z 129, (20%)	M ⁺ m/z 128, (100%)
fluoranthene		1	202	M ⁺ m/z 202, (100%) [M+H] ⁺ m/z 203, (30%)	M ⁺ m/z 202, (100%)
indene		1	116	NS	NS
Amines					
1-adamantanamine	1 ^o	3	151	[M+H] ⁺ m/z 152, (100%)	[M-2H+3D] ⁺ m/z 155, (100%)
2-amino-3,5-dibromopyridine	1 ^o , 3 ^o	3	250	[M+H] ⁺ m/z 251, (100%)	[M-2H+3D] ⁺ m/z 254, (100%)
2,4-dichlorobenzylamine	1 ^o	3	175	[M+H] ⁺ m/z 176, (100%)	[M-2H+3D] ⁺ m/z 179, (100%)
1-aminoanthracene	1 ^o	3	193	[M+H] ⁺ m/z 194, (100%)	[M-2H+3D] ⁺ m/z 197, (100%)
indole	2 ^o	2	117	[M+H] ⁺ m/z 118, (100%)	[M-H+2D] ⁺ m/z 120, (44%) [M-2H+3D] ⁺ m/z 121,

Compound	Amine Type	Predicted Mass Shift	Molec. Weight	NH ₃ PCI spectra Quasimolecular ion ^{a,b}	ND ₃ PCI spectra Quasimolecular ion
2-phenyl-2-imidazoline	2°, 3°	2	146	[M+H] ⁺ m/z 147, (100%)	(100%) [M-H+2D] ⁺ m/z 149, (100%)
iminodibenzyl	2°	2	195	[M+H] ⁺ m/z 196, (100%)	[M-H+2D] ⁺ m/z 198, (100%)
indazole	2°, 3°	2	118	[M+H] ⁺ m/z 119, (100%)	[M-H+2D] ⁺ m/z 121, (100%)
nicotine	3°, 3°	1	162	[M+H] ⁺ m/z 163, (100%)	[M+D] ⁺ m/z 164, (100%)
2-(p-tolyl)-pyridine	3°	1	169	[M+H] ⁺ m/z 170, (100%)	[M+D] ⁺ m/z 171, (100%)
5-bromo-2,4-dichloropyrimidine	3°, 3°	1	226	[M+H] ⁺ m/z 227, (100%)	[M+D] ⁺ m/z 228, (100%)
N-phenylpiperidine	3°	1	161	[M+H] ⁺ m/z 162, (100%)	[M+D] ⁺ m/z 163, (100%)
α,α,α-tribromoquinoline	3°	1	377	[M+H] ⁺ m/z 378, (100%)	[M+D] ⁺ m/z 379, (100%)
Unknown Compound					
C ₁₀ H ₆ N ₂ Br ₄ Cl ₂	3°, 3°	1	540	[M+H] ⁺ m/z 541, (100%)	[M+D] ⁺ m/z 542, (100%)

^a values in brackets are relative abundances of ions

^b NS = not seen in mass spectrum

3.3.2 Retention time and ECNI full scan comparison

Absolute retention times of $C_{10}H_6N_2Br_3Cl_3$, $C_{10}H_6N_2Br_4Cl_2$, $C_{10}H_6N_2Br_5Cl$, and $C_{10}H_6N_2Br_6$ found in the bald eagle liver, $C_{10}H_6N_2Br_3Cl_3$, $C_{10}H_6N_2Br_4Cl_2$, and $C_{10}H_6N_2Br_5Cl$ in the Northern elephant seal extracts, and the analogous synthesized HDBPs are listed in Table 3.2. Since the full scan ECNI mass spectra contained very few ion clusters, comparison of mass spectra of the synthesized and extracted compounds was based on the presence of the molecular ion $[M]^+$ and the $[M-Br]^+$ ion clusters.

3.4 Discussion

3.4.1 Isotope exchange mass spectrometry

The predicted mass shifts of the quasimolecular ions between the NH_3 and ND_3 obtained mass spectra were observed for 17 of the 19 reference compounds. The two unexpected results were obtained for indole and pyrene, which resulted from replacement of an aromatic hydrogen atom. In the case of indole, one aromatic hydrogen was exchanged in addition to the hydrogen bonded to the nitrogen atom. The presence of a $[M-H+2D]^+$ ion at m/z 120 indicated that the exchange of the aromatic hydrogen was only partial. If the exchange was complete, the ND_3 PCI spectrum would show only $[M-2H+3D]^+$ at m/z 121. Aromatic hydrogen exchange occurs if the site of protonation is on the ring, rather than on a substituent. The replaced aromatic hydrogen must be at a site on the ring where the proton affinity (PA) would be greater than that of ND_3 (857.7 kJ mol⁻¹) (Hunter and Lias 1998), otherwise the exchange reaction would be endothermic and energetically unfavourable.

Table 3.2. Comparison of retention times (min) between synthesized halogenated dimethyl bipyrroles and extracted compounds.

Stationary phase	Retention times of synthesized compounds					Retention times of compounds extracted from bald eagle liver, (Northern elephant seal)				
	Br ₃ Cl ₃	Br ₄ Cl ₂	Br ₅ Cl	Br ₆		Br ₃ Cl ₃	Br ₄ Cl ₂	Br ₅ Cl	Br ₆	
DB-5MS	22.51	24.06	25.69	27.30		22.52, (22.51)	24.08, (24.08)	25.69, (25.69)	27.31	
DB-1701	25.82	27.47	29.45	31.85		25.83, (25.82)	27.50, (27.50)	29.45, (29.45)	31.85	
DB-210	18.67	19.84	21.27	22.94		18.67, (18.67)	19.89, (19.87)	21.26, (21.26)	22.94	
DB-WAX	33.99	41.77	53.80	71.50		33.98, (33.99)	41.82, (41.84)	53.73, (53.78)	71.41	
Rtx-2330	25.78	28.83	33.07	38.88		25.84, (25.80)	28.96, (28.89)	33.14, (33.08)	38.98	

Similar to indole, the exchange involving a non-amino hydrogen atom on pyrene was only partial. There was still some formation of M^{+} indicating that exchange of the aromatic hydrogen was not complete. Only partial exchange occurred, since the rate of proton transfer was decreased due to the small difference in PAs of analyte and reagent gas (Hunt and Sethi 1980). The difference in PAs of pyrene and NH_3 is only 11.3 kJ mol^{-1} (Hunter and Lias 1998). Characterization of the reference and control compounds was deemed successful, even though the anomalous results discussed above occurred for one reference compound and one control compound.

As shown in Figure 3.1, the base peaks in both the NH_3 and ND_3 obtained mass spectra for $C_{10}H_6N_2Br_4Cl_2$ show only proton and deuterium attachment, respectively. The mass shift of the quasimolecular ion was 1 Da, indicating that no proton/deuteron exchange occurred for $C_{10}H_6N_2Br_4Cl_2$. Since there were no hydrogen atoms bonded to either nitrogen atom it was concluded that both N atoms are tri-substituted.

3.4.2 Retention time and ECNI full scan comparison

Very similar retention times and ECNI full scan mass spectra were found between samples and standards on all five of the GC columns used. In general, retention time differences between samples and standards were all less than four seconds. There were some instances where the deviation was larger than four seconds. These all occurred on the Rtx-2330 column, suggesting that the stationary phase was not particularly stable between the standard and sample runs.

From the above results, the molecular structures of the four hexahalogenated compounds found in biota samples were confirmed to be those of the synthesized

Figure 3.1 IE-PCI mass spectra of $C_{10}H_6N_2Br_4Cl_2$ extracted from bald eagle liver.

Top spectrum obtained using NH_3 , bottom spectrum obtained using ND_3 as a reagent gas.

compounds. The structures of $C_{10}H_6N_2Br_4Cl_2$, $C_{10}H_6N_2Br_5Cl$, and $C_{10}H_6N_2Br_6$ were unequivocally demonstrated to be those given in Figure 3.2. However, since the synthesized $C_{10}H_6N_2Br_3Cl_3$ was not fully characterized by NMR or X-ray crystallography, the structure shown in Figure 3.2 remains to be confirmed, although it is the same compound as the unknown.

Figure 3.2 Molecular structures of the four hexahalogenated dimethyl bipyrroles.

There is evidence that 1,1'-dimethyl-3,3',4,4'-tribromo-5,5'-trichloro-2,2'-bipyrrole is the correct structure of $C_{10}H_6N_2Br_3Cl_3$. The chlorination step in the synthesis proceeds via an electrophilic addition mechanism. The 5/5' positions have the highest electron density, thus addition of an electrophilic chlorine species will most likely occur at the 5/5' positions. Steric effects will direct subsequent chlorination to the 4/4' positions. Furthermore, the EI mass spectrum of the synthesized $C_{10}H_6N_2Br_3Cl_3$ displays a prominent $[M-C_2H_3NBr]^+$ ion. This is a major ion found in all other HDBPs, and appears to be indicative of bromine substituents at the 3/3' positions.

3.5 Conclusions

The fully halogenated, tertiary diamine structure is consistent with the bioaccumulation of $C_{10}H_6N_2Br_4Cl_2$ and apparent lack of metabolism observed in seabirds in a previous study (Tittlemier et al. 1999a). Bioaccumulation and metabolism are governed by the physicochemical properties of the target compounds and the structural requirements of the binding sites of the metabolizing enzymes. For example, oxidative enzymes, which act by inserting an electrophilic oxygen onto a nitrogen atom, will be affected by the nucleophilicity of the nitrogen atom. Factors such as the degree of substitution of an amine, which affect the electronic and steric aspects of the lone pair of electrons on the nitrogen atom, will affect metabolism. Therefore, in general, primary and secondary amines would be metabolized more quickly and to a greater extent because of the presence of labile hydrogen atoms attached to the nitrogen atoms and lack of steric hindrance from substituent groups (Cho et al. 1984).

The absence of unsubstituted positions on the bipyrrrole rings is also consistent with the apparent lack of metabolism of HDBPs. In the analogous case of PCBs, it has been shown that congeners containing vicinal unsubstituted sites are metabolized whereas congeners containing no adjacent unsubstituted sites are metabolized very slowly, or not at all (Braune and Norstrom 1989).

The presence of only one structure for each of the tri-, tetra-, and pentabrominated homologue groups is indicative of a selective process occurring, either during synthesis or degradation. Since HDBPs appear to be persistent compounds, a selective synthesis seems likelier. The uniform chlorination of the 5/5' positions indicates that synthesis involves an electrophilic reaction, since these positions are the most electrophilic on the pyrrole rings. Electrophilic addition of chlorine is consistent with reactions involving haloperoxidases, enzymes involved in the formation of naturally-produced organohalogens.

The structural similarity of HDBPs to the known marine natural product hexabromo-2,2'-bipyrrrole lends more support to the hypothesis that HDBPs are naturally-produced. There are a number of naturally-produced halogenated pyrroles since rates of electrophilic bromination of pyrroles are approximately 3×10^8 times faster than benzene (Linda and Marino 1968). Further studies are required to determine whether or not their source is biogenic. If proven to be biogenic in origin, this would be the first instance of a naturally-produced organohalogen bioaccumulating in higher trophic level organisms.

3.6 Acknowledgements

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CHAPTER 4.

VAPOUR PRESSURES, AQUEOUS SOLUBILITIES, HENRY'S LAW CONSTANTS, AND OCTANOL/WATER PARTITION COEFFICIENTS OF A SERIES OF MIXED HALOGENATED DIMETHYL BIPYRROLES*

4.1 Introduction

Halogenated organic compounds are present throughout the environment and found in virtually every location and sample matrix studied. These compounds have been detected in many environmental media, including sediment (Müller et al. 1999; Sellström et al. 1998), water (Iwata et al. 1993; Tanabe and Tatsukawa 1980), air (Halsall et al. 1995; Rappe et al. 1988), and biota (Muir et al. 1992; Watanabe et al. 1987).

Organohalogens have been found in samples obtained from locations worldwide, including areas considered to be pristine environments, such as the Arctic (Muir et al. 1992) and Antarctica (Bacci et al. 1986). The wide-ranging distribution of these compounds indicates that they undergo transport away from their sources. The transport and fate of organohalogens are governed by physical properties such as vapour pressures, aqueous solubilities, Henry's Law constants, and octanol/water partition coefficients. These fundamental physical properties have been well characterized for most classes of commonly analyzed organohalogens.

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* adapted from Tittlemier et al. (2001d)

Aqueous solubility (S_w) describes the ability of a solute to dissolve in water (Opperhuizen et al. 1990; Shiu et al. 1990). The environmental mobility of a compound is related to its S_w . Compounds with high S_w tend to become quickly dispersed in the environment via the hydrological cycle, are associated with hydrophobic sediment and biological phases to a lesser extent, and often have higher degradation rates relative to less soluble compounds (Lyman 1982).

Vapour pressure (P) characterizes the equilibrium partitioning of a chemical between its pure condensed and gaseous states at a specified temperature (Schwarzenbach et al. 1993). In environmental fate modeling, P 's are used in the estimation of evaporation rates. In addition, the magnitude of P can be used to evaluate the tendency of a compound to adsorb to particulate matter from the vapour phase (Eisenreich et al. 1981).

Henry's Law constant (H) represents the partitioning between air and water. This variable is dependent upon both P and S_w , as opposed to simply P , and describes the volatilization of compounds from water and wet soils. Henry's Law constants can be expressed as the ratio P/S_w for compounds that are sparingly soluble in water.

The octanol/water partition coefficient (K_{ow}) is used as a surrogate to describe the equilibrium partitioning of compounds between environmental and physiological organic matter and an aqueous phase. The log K_{ow} has been related to biomagnification of hydrophobic persistent organochlorines in a freshwater food web (Kidd et al. 1998).

These fundamental physical properties affecting the movement of halogenated dimethyl bipyrroles (HDBPs) have not yet been characterized. The presence of HDBPs in seabird eggs from both the Atlantic and Pacific coasts of Canada (Tittlemier et al.

1999a) indicate that they may undergo transport. In this study, the vapour pressures, aqueous solubilities, Henry's Law constants, and K_{ow} s of five HDBP congeners were determined using the established methods of the gas chromatography-retention time technique, generator column technique, and slow-stirring method. The effects of substitution of a bromine for a chlorine atom on these physical properties were also examined. A simple four-compartment Level I model was constructed using the experimentally determined physical properties, and was used to illustrate the equilibrium partitioning tendencies of two HDBP congeners among various environmental media.

4.2 Experimental section

4.2.1 Chemicals

HDBP congeners were synthesized and characterized according to the methods outlined in Gribble et al. (1999). Three congeners used in this study were of known structure – 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrole (DBP-Br₄Cl₂); 1,1'-dimethyl-3,3',4,4',5-pentabromo-5'-chloro-2,2'-bipyrrole (DBP-Br₅Cl); and 1,1'-dimethyl-3,3',4,4',5,5'-hexabromo-2,2'-bipyrrole (DBP-Br₆). The two remaining congeners were hypothesized to be 1,1'-dimethyl-3,3',4-tribromo-4,5,5'-trichloro-2,2'-bipyrrole (DBP-Br₃Cl_{3a}) and 1,1'-dimethyl-3,4,4'-tribromo-3',5,5'-trichloro-2,2'-bipyrrole (DBP-Br₃Cl_{3b}) based on their presence as by-products in the electrophilic substitution formation of DBP-Br₄Cl₂. Four of these congeners (DBP-Br₃Cl_{3a}, DBP-Br₄Cl₂, DBP-Br₅Cl, and DBP-Br₆) are environmentally-relevant compounds found in biota (Tittlemier et al. 2001a).

4.2.2 Vapour pressures

The gas chromatographic-retention time (GC-RT) technique described in Hinckley et al. (1990) was used to determine liquid sub-cooled vapour pressures (P_L^0) of the HDBPs. The GC-RT method estimates the liquid sub-cooled vapour pressure as opposed to vapour pressure (Bidleman 1984). The liquid sub-cooled vapour pressure is a property which takes into account the energy required to overcome attractive forces in the solid state and is greater than the vapour pressure of the solid.

The GC-RT method relates P_L^0 of a compound to its retention time (relative to a reference compound) obtained during an isothermal GC run according to the following equations:

$$\log\left(\frac{t}{t_{\text{ref}}}\right) = \left[1 - \left(\frac{\Delta H_{\text{vap}}}{\Delta H_{\text{vap.ref}}}\right)\right] \log P_{L,\text{ref}}^0 - C \quad (1)$$

$$\log P_L^0 = \left(\frac{\Delta H_{\text{vap}}}{\Delta H_{\text{vap.ref}}}\right) \log P_{L,\text{ref}}^0 + C \quad (2)$$

where ΔH_{vap} and $\Delta H_{\text{vap.ref}}$ are the enthalpies of vaporization of the test and reference compounds, respectively. Relative retention times are obtained over a range of temperatures and plotted versus P_L^0 of the reference compound using equation (1) to determine ΔH_{vap} and the constant C . P_L^0 of the test compound is then calculated using ΔH_{vap} and C in equation (2).

Mirex, *t*-chlordane, and the polychlorinated biphenyl congeners CB-4, CB-15, CB-118, CB-153, CB-180, CB-187, CB-194, CB-202, CB-206, and CB-209 were used as standards for the GC-RT method. These compounds, whose P_L^0 s are well defined, were

selected to represent a range of vapour pressures. Solutions of all standards and HDBPs were made in isooctane in the 50-100 pg/ μ L range and spiked with the reference compound *p,p'*-DDT.

The instrumentation used was the same as that described in Tittlemier et al. (2001b). Retention times for all compounds on a DB-1 column (2 m x 0.250 mm i.d., film thickness 0.25 μ m, J&W Scientific, Folsom, CA, USA) were determined during six isothermal runs performed at oven temperatures ranging from 140°C to 165°C at 5°C intervals.

4.2.3 Aqueous solubility

Aqueous solubilities at 25°C ($S_{w,25}$) of DBP-Br₅Cl and DBP-Br₆ were measured using the generator column technique (May et al. 1978). Due to impurities in the three other synthesized congeners, only the water solubilities of DBP-Br₅Cl and DBP-Br₆ could be determined. The experimental apparatus used is described in Tittlemier et al. (2001b). Briefly, approximately 5 mg of crystals of the HDBP congener (>99% purity) were dissolved in acetone and sorbed onto pre-cleaned 60-80 mesh glass beads. Two pre-cleaned stainless steel HPLC columns (250 x 3.9 mm; 100 x 3.9 mm) were then packed with the HDBP-coated glass beads. The larger column was connected to an HPLC pump using only stainless steel tubing and flushed with HPLC grade water at a flow rate of 1 mL/min to create an aqueous solution saturated with HDBP. An external column heater was used to maintain the column at a temperature of 25 ± 1 °C. To ensure that all active sites within the column, fittings, and tubing had been coated with

compound, water was pumped through the packed column for 1 hour prior to collecting the first samples.

Three replicates of HDBP-saturated water (10.0 mL each) were collected in volumetric flasks after 1 hour of equilibration. A second smaller HPLC column similarly packed with HDBP-coated beads was connected in series after the first column to ensure that the aqueous eluate was saturated. An additional three eluate samples were collected after water was pumped through the two columns to coat all active sites. Collected samples were spiked with recovery standard (CB-153) and extracted with 1:1 dichloromethane/hexane (3 x 8 mL). Pure HPLC grade water was used as a method blank with every six eluate samples collected. Organic fractions were combined, dried using activated Na₂SO₄, and reduced on a rotary evaporator. Performance standard (mirex) was added to all samples and external standards prior to analysis by GC-ECD. The eluate was deemed saturated if the concentrations of HDBP in water samples collected from one and two columns did not differ significantly from each other.

4.2.4 Henry's Law constants

Henry's Law constants (H) for DBP-Br₅Cl and DBP-Br₆ were estimated at 25°C using the following equation:

$$H = \frac{P_{L,25}^0}{S_{L,25}} \quad (3)$$

where $P_{L,25}^0$ and $S_{L,25}$ are the respective liquid sub-cooled vapour pressures and aqueous solubilities at 25°C. $S_{L,25}$ was calculated from $S_{w,25}$ using equation (4) since experimental values obtained from the generator column method are for the solid, rather than sub-

cooled liquid, phase. Equation (4) uses melting point (T_m) to calculate and correct for the energy of fusion required to move from the solid to the sub-cooled liquid phase (Kan and Tomson 1996).

$$S_{L.25} = \frac{S_{w.25}}{e^{-0.023(T_m - 25)}} \quad (4)$$

T_m values used in equation (4) were taken as the midpoint of the melting range.

4.2.5 Octanol/Water Partition Coefficients

Octanol/water partition coefficients of the five HDBPs were evaluated using the slow-stirring method (de Bruijn et al. 1989). Water-saturated octanol and octanol-saturated water were prepared by gently stirring HPLC grade water and octanol continuously for 72 hours. Approximately 50 mg each of the five HDBPs and a series of PCB congeners used as standards (CB-153, CB-180, CB-202, CB-206, CB-209) were dissolved in 200 mL of water-saturated octanol. Octanol-saturated water (3.5 L) and organohalogen octanol solution (20 mL) were added to four pre-cleaned and dried Erlenmeyer flasks. The mixture was gently stirred with a Teflon stir bar so that a vortex no greater than 1 cm in height was formed. On days 2, 4, 7, 10, and 14 of the slow-stirring experiment, 1 mL of the octanol and 500 mL of the water phases were collected using a volumetric pipette and flask. The initial preparation of water-saturated octanol and octanol-saturated water, plus the slow-stirring experiment, were performed in a controlled environment room at a constant temperature of $25 \pm 1^\circ\text{C}$.

The water samples were spiked with recovery standard (octachloronaphthalene) and extracted with 1:1 dichloromethane/hexane (3 x 50 mL). Anhydrous NaCl (10g) was

added to the water phase to facilitate extraction of the organohalogens. The dichloromethane/hexane fractions were combined, dried over anhydrous Na₂SO₄, and reduced to 1 mL. Octanol phase samples were diluted by a factor of 1000 with hexane. Prior to analysis, the water extracts and 1 mL aliquots of the diluted octanol samples were spiked with performance standard (¹³C₁₂-CB-138). Organohalogens were quantitated by gas chromatography-electron capture negative ionization-mass spectrometry in the selected ion mode using a method similar to the one outlined in Tittlemier et al. (2001c).

4.3 Results

4.3.1 Vapour pressures

Table 4.1 lists values of P_{L25}^0 , ΔH_{vap} , and the coefficients of the integrated Clausius-Clapeyron equation for HDBPs. Values of ΔH_{vap} were determined from $\Delta H_{\text{vap,DDT}} = 89.9 \text{ kJ/mol}$ (Lei et al. 1999) and the slope of the line described by equation (1) shown in Figure 4.1. The coefficients B and A were calculated from the integrated form of the Clausius-Clapeyron equation (5), which describes the change in vapour pressure of a compound with temperature.

$$\log P_L^0 = -\frac{B}{T} + A \quad (5)$$

where

$$B = -\frac{\Delta H_{\text{vap}}}{2.303R} \quad (6)$$

The P_{L25}^0 values in Table 4.1 are those derived by using a calibration curve to correct vapour pressure values obtained directly from the GC-RT method (designated

P_{GC}). The calibration curve was constructed from the correlation between P_{GC} and literature values of sub-cooled liquid vapour pressures ($P_{L,lit}^0$) of the standard compounds. In the past, P_{GC} values have been corrected by using a calibration curve constructed from P_L^0 values derived from other methods (Bidleman 1984; Hinckley et al. 1990; Lei et al. 1999). In this study, the $P_{L,lit}^0$'s used were values obtained from other GC-RT studies and thus were themselves previously corrected by comparison to P_L^0 derived from other methods (Figure 4.2). Vapour pressure values given in Table 4.1 were corrected using the regression equation obtained from the $P_{GC} - P_{L,lit}^0$ correlation in Figure 4.2:

$$\log P_{GC} = 1.065(\log P_{L,lit}^0) + 0.2395 \quad (7)$$

Table 4.1. Halogenated dimethyl bipyrrrole (HDBP) enthalpies of vaporization (ΔH_{vap}), integrated Clausius-Clapeyron integers (B, A from equation 5), and liquid sub-cooled vapour pressures at 25°C ($P_{L,25}^0$).

HDBP	ΔH_{vap} (kJ/mol)	B	A	$P_{L,25}^0$ (Pa)
DBP-Br ₃ Cl ₃ a	96 ± 11	-5000 ± 600	13 ± 4	0.00019 ± 0.00003
DBP-Br ₃ Cl ₃ b	100 ± 7	-5200 ± 400	13 ± 2	0.000094 ± 0.000009
DBP-Br ₄ Cl ₂	104 ± 6	-5400 ± 300	14 ± 2	0.000041 ± 0.000003
DBP-Br ₅ Cl	109 ± 6	-5700 ± 300	14 ± 2	0.000017 ± 0.000001
DBP-Br ₆	113 ± 3	-5900 ± 200	15 ± 1	0.0000076 ± 0.0000003

Figure 4.1 Plot of retention times of HDBP congeners relative to *p,p'*-DDT versus *p,p'*-DDT liquid sub-cooled vapour pressures.

Figure 4.2 Correlation between experimentally determined (P_{GC}) and literature ($P^{\circ}_{L,lit}$) liquid sub-cooled vapour pressures at 25°C for standard compounds.

The solid regression line denotes the relationship between experimental and literature values for the standards. The dotted line represents the ideal 1:1 correlation.

4.3.2 Aqueous solubility and Henry's Law constant

The $S_{w,25}$ and H_{25} for DBP-Br₅Cl and DBP-Br₆ are listed in Table 4.2. These properties were not examined for the remaining HDBP congeners due to impurities in the synthesized crystals which appeared to affect aqueous solubility determinations.

4.3.3 Octanol/water partition coefficients

The HDBP K_{ow} s determined by the slow-stirring experiment are listed in Table 4.2. The means and standard deviations were calculated from the Day 14 aliquots for the four replicate flasks. Log K_{ow} s of the five PCB congeners used as standards were well correlated with literature values [literature log K_{ow} = 1.17 (experimental log K_{ow}) - 0.761; $R^2=0.788$]. No significant trend of log K_{ow} with bromine content was observed (Figure 4.4).

Table 4.2. Halogenated dimethyl bipyrrrole (HDBP) aqueous solubilities at 25°C ($S_{w,25}$), liquid sub-cooled aqueous solubilities at 25°C ($S_{L,25}$), melting points (T_m), Henry's Law constants at 25°C (H_{25}), and octanol/water partition coefficients at 25°C (K_{ow}).

Mean $S_{w,25} \pm$ standard deviation of three replicates, mean log $K_{ow} \pm$ standard deviation calculated from four replicates.

HDBP	$S_{w,25}$ (g/L)	$S_{L,25}$ (g/L)	T_m (°C)	H_{25} (Pa m ³ /mol)	log K_{ow}
DBP-Br ₃ Cl ₃ a	na ^d	na	na	na	6.5 ± 0.3
DBP-Br ₃ Cl ₃ b	na	na	na	na	6.4 ± 0.2
DBP-Br ₄ Cl ₂	na	na	na	na	6.5 ± 0.3
DBP-Br ₅ Cl	(2.7 ± 0.2) × 10 ⁻⁵	(1.5 ± 0.7) × 10 ⁻³	197-200	0.0068 ± 0.0009	6.6 ± 0.3
DBP-Br ₆	(1.4 ± 0.3) × 10 ⁻⁵	(2.3 ± 0.6) × 10 ⁻³	247-248	0.0020 ± 0.0006	6.7 ± 0.3

^dna = not analyzed

4.4 Discussion

4.4.1 Vapour pressures

The values obtained for $P^{\circ}_{L,25}$ of the five HDBP congeners studied were comparable to those for hepta- to decachlorinated PCBs (Mackay et al. 1999) and tetra- to hexabrominated diphenyl ethers (Tittlemier et al. 2001b; Wong et al. 2001).

Figure 4.3 Effect of bromine/chlorine substitution on the liquid sub-cooled vapour pressure at 25°C ($P^{\circ}_{L,25}$) of HDBPs.

The relative amounts of chlorine and bromine on the HDBP molecule significantly affected $P^{\circ}_{L,25}$. Replacement of a chlorine with a bromine atom resulted in a

2.6-fold decrease in $P^0_{L,25}$ (Figure 4.3). The magnitude of this decrease is less than for hydrogen/chlorine (approximately 4-fold decrease) (Ballschmiter and Zell 1980; Drouillard et al. 1998; Falconer and Bidleman 1994) or hydrogen/bromine substitution (approximately 7-fold decrease) (Tittlemier and Tomy 2001; Wong et al. 2001; Tittlemier et al. 2001b).

The smaller effect of chlorine/bromine substitution is consistent with the effects of size on vapour pressure. In general, compounds occupying larger molar volumes have higher vapour pressures than those which are smaller due to stronger intermolecular attractions in the liquid phase. Substitution of a chlorine for a bromine atom results in a smaller volume change than substitution of a hydrogen for either a bromine or chlorine atom.

4.4.2 Aqueous solubility

The $S_{w,25}$'s determined for DBP-Br₅Cl and DBP-Br₆ are comparable to the range reported for pentachlorinated PCBs (1.23×10^{-5} to 6.13×10^{-5} g/L) (Mackay et al. 1999). Due to the low number of congeners analyzed, it is not possible to clearly determine the relationship between chlorine/bromine substitution and $S_{w,25}$. However the $S_{w,25}$ of DBP-Br₆ was half that of DBP-Br₅Cl (Student's t-test, $p < 0.04$). A greater decrease (three-fold decrease) in $S_{w,25}$ has been observed with an increase in bromine content of polybrominated diphenyl ether (PBDE) congeners (Tittlemier et al. 2001b). A decrease in the $S_{w,25}$ of the larger HDBP congener is expected since an increase in total surface area has been associated with lower aqueous solubilities (Schwarzenbach et al. 1993).

More energy is required to disrupt the stronger molecular interactions of the larger congeners and to create a larger cavity in the water, resulting in a lower value of $S_{w,25}$.

4.4.3 Henry's Law constants

The estimated Henry's Law constants at 25°C (H_{25}) are given in Table 4.2. The values for DBP-Br₅Cl and DBP-Br₆ are on the same order as those for penta- to heptabrominated PBDEs (Tittlemier et al. 2001b). As with the aqueous solubility data, trends of H_{25} with bromine content could not be determined from the data set containing only two HDBP congeners. There does appear to be an approximately 3-fold decrease in H_{25} for the HDBP congeners, due to a greater change in $S_{L,25}$ than $P^0_{L,25}$ with bromine content, but with only two values of H_{25} it is difficult to determine whether this is a real trend of H_{25} with bromine content.

4.4.4 Octanol/Water Partition Coefficients

The log K_{ow} values determined for HBDPs are approximately 0.5 units lower than those predicted by the fragment constant method (6.9 for DBP-Br₃Cl₃ to 7.3 for DBP-Br₆) (Lyman 1982). That is, measured K_{ow} values are about three times lower than calculated. Measured log K_{ows} are similar to log K_{ows} obtained for penta- and hexachlorinated PCBs and PCDEs using generator column and retention indices techniques (Hawker and Connell 1988; Kurz and Ballschmiter 1999). The values of HBDP log K_{ows} obtained are also comparable to the range determined for tetra- and hexabrominated diphenyl ethers using the slow stirring method (Braekevelt et al. 2001).

Figure 4.4 Effect of bromine/chlorine substitution on the octanol/water partition coefficient at 25°C (K_{ow}) of HDBPs.

The HBDP log K_{ow} s are consistent with their biomagnification observed in an Arctic marine food web (Tittlemier et al. 2001c). HDBPs increased in concentration over trophic levels to a similar extent as CB-153, a compound with a similar log K_{ow} (Hawker and Connell 1988). The degree of biomagnification of hydrophobic organochlorines in invertebrates and fish has been shown to be related to K_{ow} in a freshwater food web (Kidd et al. 1998).

As illustrated in Figure 4.4, there was a slight increase in HDBP log K_{ow} with an increase in bromine content of the congener. However, the increase was not statistically significant ($p=0.997$). The similarity of log K_{ow} among HDBP congeners is somewhat unexpected since S_w and S_L were significantly different between DBP-Br₅Cl and DBP-Br₆ and log K_{ow} has been shown to be negatively correlated to S_L for numerous classes of compounds (Schwarzenbach et al. 1993).

4.4.5 Environmental Significance of Physical Properties

It is well accepted that physical properties govern the behaviour of contaminants in the environment. Values of basic physical properties such as vapour pressure, water solubility, and the octanol/water partition coefficient (K_{ow}) can thus be used to model and predict the movement, distribution, and fate of contaminants. The distribution of DBP-Br₅Cl and DBP-Br₆ in a simple four compartment environment at equilibrium was estimated (Figure 4.5) using physical property data determined in this work. The equilibrium distributions of a group of anthropogenic organochlorines are also shown in Figure 4.5 for comparison. The model environment consisted of air (1000 m x 1000 m x 6000 m), soil (1000 m x 300 m x 0.15 m, 2% organic content by volume), water (1000 m x 700 m x 10 m), and sediment (1000 m x 700 m x 0.03 m, 5% organic content by volume) (Mackay et al. 1999).

The equilibrium distributions of the two HDBP congeners most closely resembled the distribution of CB-202 and CB-209, the two highly chlorinated PCB congeners. Greater than 99% of CB-202 and CB-209 and each HDBP was distributed in the soil and sediment compartments. Approximately 0.2% of each HDBP congener was present in

the water compartment as compared to less than 0.05% of the two PCB congeners. Less than 0.0005% of the total amount of HDBPs in the simplified environment was present in the air compartment.

Figure 4.5 Predictions of equilibrium partitioning of HDBPs and some organochlorine compounds in a simple four compartment environment using a Level I model.

The variations in physical properties observed with changes in chlorine and bromine content of HDBPs indicate that there may be some differences in their distribution and fate. However, since the substitution of a bromine for a chlorine atom affected P and K_{ow} less than hydrogen/halogen substitution, the differences in HDBP

congener distributions are not expected to be large. As anticipated, only slight differences in distribution between DBP-Br₅Cl and DBP-Br₆ were predicted.

4.5 Conclusions

The characterization of fundamental physical properties and the results of the simple modeling exercise suggest that the majority of DBP-Br₅Cl and DBP-Br₆ would be present mainly in relatively immobile environmental media such as suspended particulate matter and sediment. Distribution of organohalogens in the environment is thought to occur mainly by atmospheric transport, although ocean currents may also play a role. Based on similarity of physical properties, HDBPs appear to behave similar to octa- and higher chlorinated PCB congeners in their potential for global distribution. It is expected that the other HDBP congeners whose physical properties were not fully characterized would behave similarly since the hexa-, octa-, and decachlorinated PCB congeners examined did not display significantly different behaviour amongst themselves.

4.6 Acknowledgment

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CHAPTER 5.

GLOBAL DISTRIBUTION OF HALOGENATED DIMETHYL BIPYRROLES IN MARINE MAMMAL BLUBBER*

5.1 Introduction

Halogenated dimethyl bipyrroles (HDBPs) are a family of mixed halogenated nitrogen-containing heterocycles. The molecular structures of the four environmentally-relevant HDBPs have recently been determined (Tittlemier et al. 2001a) and are shown in Figure 3.2. The distribution of HDBPs in the environment is not well known since only two studies concerning their occurrence have been completed (Tittlemier et al. 1999a; Tittlemier et al. 2001c). The initial study described the levels of the most abundant congener, 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrole (DBP-Br₄Cl₂), in seabird eggs obtained from the Great Lakes, Pacific, and Atlantic coastal regions of Canada. DBP-Br₄Cl₂ was found at the highest concentrations in samples from the Pacific coast, followed by those from the Atlantic coast, and was not observed in Great Lakes samples (Tittlemier et al. 1999a). All four HDBP congeners given in Figure 3.2 were found in a marine food web from the Canadian high Arctic consisting of three species of zooplankton (*Calanus hyperboreus*, *Mysis oculata*, and *Sagitta* sp.), Arctic cod (*Boreogadus saida*), four species of seabirds [dovekie (*Alle alle*), black guillemot (*Cephus grylle*), black-legged kittiwake (*Rissa tridactyla*), and glaucous

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*adapted from Tittlemier et al. (2001e).

gull (*Larus hyperboreus*)], and ringed seal (*Phoca hispida*) (Tittlemier et al. 2001c). The results of these two studies indicated that HDBPs are persistent, bioaccumulative, and widely distributed throughout the marine environment of North America.

Marine mammals can be good indicators of the distribution of persistent organohalogen compounds in marine ecosystems because they tend to biomagnify hydrophobic organohalogenes. Many pinnipeds and odontocetes occupy the upper levels of marine food webs and thus accumulate relatively high levels of organohalogenes (Muir et al. 1988; Nakata et al. 1997; Tanabe et al. 1997). It has also been shown that pinnipeds and small cetaceans generally have lower capabilities than birds and terrestrial mammals to metabolize organohalogenes such as PCBs unsubstituted by chlorine in adjacent *meta-para* position, which is consistent with low or absent inducible CYP2B enzyme activity in marine mammals (Boon et al. 1989; Tanabe et al. 1988). In general, organohalogenes are less likely to undergo metabolism and subsequent excretion in marine mammals than in terrestrial mammals and birds. Consequently, relatively high levels of persistent organohalogenes are maintained in marine mammal blubber.

In addition, marine mammal blubber is a good sample matrix for the examination of the widespread distribution of persistent organohalogenes such as HDBPs since marine mammals inhabit virtually every ocean and sea, as well as some land-locked lakes such as Lake Baikal. In this work, the global distribution of the four identified HDBP congeners in marine mammals was studied. Levels of the four HDBP congeners were also normalized to concentrations of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153), one of the most ubiquitous anthropogenic organohalogen compounds. Comparison of this ratio

facilitated comparison among species and geographical locations, and provided additional evidence about the nature of the source.

5.2 Experimental Section

5.2.1 Organohalogen analyses

Blubber samples from a variety of cetacean and pinniped species were obtained from the locations shown in Figure 5.1. Detailed information concerning sampling locations, sample size, age class, and sex is summarized in Table 5.1.

Since the samples were supplied by various research groups, the method of organohalogen extraction varied among the samples. The specific method utilized for each sample is indicated in Table 5.1. The majority of samples underwent column extraction (Method A). In this method, accurately weighed amounts of blubber (about 2 g) were cut into small pieces and mixed with a ten-fold amount of activated Na_2SO_4 . The mixtures were then spiked with an internal standard solution of ^{13}C -labelled organochlorines ($^{13}\text{C}_6$ -pentachlorobenzene, $^{13}\text{C}_6$ -hexachlorobenzene, $^{13}\text{C}_{12}$ -CB118, $^{13}\text{C}_{12}$ -CB153, $^{13}\text{C}_{12}$ -CB180, $^{13}\text{C}_{12}$ -CB194). The mixtures were wet-packed with dichloromethane/hexane (1:1) into a glass column (2 cm i.d.), and after 45 minutes, extracted with 250 mL of dichloromethane/hexane (1:1).

Method B involved accelerated solvent extraction of accurately weighed blubber samples (about 5 g) using dichloromethane/hexane (1:1) and toluene. The extracts from the two different solvents were combined, reduced to 10 mL, and spiked with the internal standard used in Method A.

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Figure 5.1 Marine mammal sampling locations.
Numbers refer to locations listed in Table 5.1

Table 5.1. Biological and sampling information on marine mammal samples analyzed for HDBPs.

Species	Location	Year collected	N, Sex ^b	Age class	Extraction method ^c
beluga (<i>Delphinapterus leucas</i>)	Beluga Chesterfield Inlet, Nunavut (1) ^a	1997	2 F, 1 M	1 adult, 2 immature	A
beluga (<i>Delphinapterus leucas</i>)	Igloodik, Nunavut (2)	1995	3 F, 6 M	adult	A
beluga (<i>Delphinapterus leucas</i>)	Svalbard, Norway (3)	1999	2 F, 6 M	adult	A
beluga (<i>Delphinapterus leucas</i>)	Pt. Lay, Alaska (4)	1998	5 M	immature	A
beluga (<i>Delphinapterus leucas</i>)	St. Lawrence River (5)	1997-8	7 M, 5 F	adult	A
bottlenose dolphin (<i>Tursiops truncatus</i>)	<i>Dolphins and porpoises</i>				
Dall's porpoise (<i>Phocoenoides dalli</i>)	Georgia / South Carolina (6)	1992-4, 1996, 1999	5 M	4 adult, 1 immature	C
Fraser's dolphin (<i>Lagenodelphis hosei</i>)	NW North Pacific Ocean (7)	1998	5 M	unk ^d	D
harbour porpoise (<i>Phocoena phocoena</i>)	Mindanao Sea, Philippines (8)	1996	2 M	unk	D
Hector's dolphin (<i>Cephalorhynchus hectori</i>)	Vancouver Island, British Columbia (9)	1996	3 F, 1 unk	3 adult, 1 immature	A
hump-backed dolphin (<i>Sousa chinensis</i>)	New Zealand (10)		5 M	immature	B
killer whale (<i>Orcinus orca</i>)	Bay of Bengal (11)	1992	1 M	unk	D
Risso's dolphin (<i>Grampus griseus</i>)	Vancouver Island, British Columbia (9)	1999	1 F	adult	A
Spinner dolphin (<i>Stenella longirostris</i>)	Taiji, Japan (12)	1991	1 M	adult	D
striped dolphin	Mindanao Sea, Philippines (8)	1996	3 M	unk	D
	Catalonia, Spain (13)	1990	10 M	adult	E

Species	Location	Year collected	N, Sex ^b	Age class	Extraction method ^c
<i>(Stenella coeruleoalba)</i>					
<i>Sea lions</i>					
California sea lion <i>(Zalophus californicus)</i>	California (14)	1994-5	5 M	4 adult, 1 immature	A
New Zealand sea lion <i>(Phocarcos hookeri)</i>	New Zealand (10)		5 M	immature	B
Steller sea lion <i>(Eumetopias jubatus)</i>	Hokkaido coast, Japan (15)	1997	1 M	unk	D
Steller sea lion <i>(Eumetopias jubatus)</i>	California (14)	1994	1 M	immature	A
Steller sea lion <i>(Eumetopias jubatus)</i>	S Alaska (16)	1995	3 M	unk	A
<i>Seals</i>					
harbour seal <i>(Phoca vitulina)</i>	Washington (17)	1992	4 F, 2 M	3 adult, 3 immature	A
harbour seal <i>(Phoca vitulina)</i>	Bering Sea (16)	1989-90	6 F, 9 M, 2 unk	12 adult, 5 immature	A
harbour seal <i>(Phoca vitulina)</i>	Sweden (18)	1982-3, 1985-9, 1992	9 F, 9 M	1 adult, 17 immature	A
harbour seal <i>(Phoca vitulina)</i>	St. Lawrence River (5)	1999	1 F, 4 M	3 adult, 2 immature	A
harbour seal <i>(Phoca vitulina)</i>	Svalbard, Norway (3)	1999	3 M	3 adult	A
harbour seal <i>(Phoca vitulina)</i>	Strathclyde coast, SW Scotland (19)	1988-9	5 M	5 adult	A
harbour seal <i>(Phoca vitulina)</i>	Wadden Sea (20)	1999-2000	5 F, 9 M	6 adult, 8 immature	A
Baikal seal <i>(Phoca sibirica)</i>	Lake Baikal, Russia (21)	1992	5 M	adult	D

Species	Location	Year collected	N, Sex ^b	Age class	Extraction method ^c
Largha seal (<i>Phoca largha</i>)	Japan (12)	1997	3 M	immature	D
New Zealand fur seal (<i>Arctocephalus forsteri</i>)	New Zealand (10)		5 M	2 adult, 3 immature	B
ringed seal (<i>Phoca hispida</i>)	Eureka, Nunavut (22)	1994	3 F, 3 M	4 adult, 2 immature	A
ringed seal (<i>Phoca hispida</i>)	western Greenland (23)	1999	4 F, 5 M	3 adult, 6 immature	A
ringed seal (<i>Phoca hispida</i>)	Kara Sea (24)	1995	5 M	adult	A
ringed seal (<i>Phoca hispida</i>)	Pangnirtung, Nunavut (25)	1999	4 F, 6 M	7 adult, 2 immature	A
ringed seal (<i>Phoca hispida</i>)	White Sea (26)	1999	4 F, 5 M	immature	A
bowhead whale (<i>Balaena mysticetus</i>)	Beaufort Sea (27)	<i>Mysticete cetaceans</i> 1997-8	16 F, 19 M	12 adult, 23 immature	F

^anumbers correspond to locations shown in Figure 5.1

^bM=male, F=female

^cextraction methods are described in "5.2 Experimental Section"

^dunk=unknown

Method C also used accelerated solvent extraction. Accurate amounts of blubber (about 2 g) were extracted using dichloromethane. The extracts were initially reduced to approximately 20 mL and dried over activated Na_2SO_4 . The Na_2SO_4 was removed by filtration, and washed with hexane. The hexane washings were combined with the extracts, spiked with the internal standard, and reduced to 10 mL.

In Method D accurately weighed blubber samples (about 5 g) were mixed with activated Na_2SO_4 and Soxhlet extracted with hexane. The extracts were reduced to 10 mL and spiked with the internal standard.

Method E also utilized Soxhlet extraction. As with the other methods, an accurately weighed amount of blubber (about 4 g) was mixed with activated Na_2SO_4 . The mixture was Soxhlet extracted with chloroform/hexane (1:1), reduced to 10 mL, and spiked with the internal standard.

The final extraction method, Method F, followed the technique outlined in Muir et al. (1988) with the following modifications: blubber samples were combined with dichloromethane and extracted using a Polytron mixer. After extraction, lipid determination by gravimetric methods, and removal of lipids, organohalogenes were separated into two fractions on a silica gel (100% activated) column. Fraction 1 was eluted with hexane, and Fraction 2 with dichloromethane/hexane (1:1). Fraction 2, containing the HDBPs, was reduced in volume and spiked with volume corrector ($^{13}\text{C}_{12}$ -CB 138) prior to analysis.

After extraction, all samples, aside from the bowhead whales which were worked up according to Method F, were subjected to the same procedure. The sample extracts were first filtered using pre-cleaned PTFE syringe filters. Next, the lipid content of the

samples was determined gravimetrically. Lipids were then removed by gel permeation chromatography (GPC) (Letcher et al. 1995). The GPC eluates were reduced to approximately 1 mL and applied to a Florisil column (8 g, 1.2% deactivated). Organohalogen compounds were eluted using 110 mL dichloromethane/hexane (1:1). The Florisil column eluate containing the organohalogens was reduced to 500 μ L and spiked with volume corrector ($^{13}\text{C}_{12}$ -CB 138) prior to analysis. A total of 25 method blanks containing no sample were used. One blank was carried through all steps of the sample preparation and analysis with each set of samples.

The samples and blanks were analyzed for PCB congener 153 (CB-153) using gas chromatography electron impact ionization in the single ion monitoring mode using the method outlined by Sandau et al. (2000). An external standard was used to quantitate CB-153. In addition, all Dall's porpoise samples were analyzed for total PCBs (taken as the sum of congeners corresponding to those present in an Aroclor mixture external standard), plus the following organochlorine compounds: 1,2,4,5-tetrachlorobenzene, 1,2,3,4-tetrachlorobenzene, pentachlorobenzene, hexachlorobenzene, α -HCH, β -HCH, γ -HCH, octachlorostyrene, heptachlor epoxide, oxychlordane, trans/cis-chlordane, trans/cis-nonachlor, p,p'-DDD/DDE/DDT, dieldrin, photomirex, mirex, and tris-(4-chlorophenyl)-methanol. An external standard was used to quantitate the 21 organochlorine compounds in the selected ion monitoring electron impact ionization mode.

HDBPs were analyzed using the method described in Tittlemier et al. (2001c) using gas chromatography electron capture negative ionization mass spectrometry in the single ion monitoring mode. Quantitation was performed using synthesized 1,1'-

dimethyl-3,3',4-tribromo-4',5,5'-trichloro-2,2'-bipyrrole (DBP-Br₃Cl₃), 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrole (DBP-Br₄Cl₂), 1,1'-dimethyl-3,3',4,4',5-pentabromo-5'-chloro-2,2'-bipyrrole (DBP-Br₅Cl), and 1,1'-dimethyl-3,3',4,4',5,5'-hexabromo-2,2'-bipyrrole (DBP-Br₆) as external standards (Tittlemier et al. 2001a).

5.2.2 Statistical analyses

Statistical analyses were performed using Statistica (v 5.5) or SAS (v. 8). SAS (v.8) GLM procedure was used for all ANOVAs and the model was considered significant if $p < 0.05$. Logarithmic transformation of both raw concentration data and ratio data was required to satisfy assumptions of normality and homoscedasticity. Specific geographic-species combinations which had $n=1$ had to be discarded from further analysis due to non-replication: this included Grey and killer whales from British Columbia, Canada, hump-backed dolphin from India, Steller sea lion from Japan and California, and Risso's dolphin from Japan.

In previous studies of organohalogens in marine mammals (Muir et al. 1988), age and sex have been shown to affect concentrations found in blubber. Data subsets containing samples from only adult males were assembled as much as possible to limit variability in the data from these possible confounding factors. To avoid discarding samples, the hypothesis that HDBPs would vary with sex and age of the animal using ANCOVA or 2-way ANOVA was tested using available data subsets. If no significant differences were detected, the data were pooled for analysis of geographical patterns.

However, when significant differences were encountered, they were accounted for before performing the geographical model.

To minimize confounding effects of feeding differences, metabolic potential, or other ecological factors, analyses examining geographical trends of HDBPs were performed by grouping animals into four groups: beluga, dolphins and porpoises, sea lions, and seals. In addition, where appropriate, animals were classified as originating from either a Pacific or non-Pacific Ocean influenced environment to test for further geographical differences in HDBP concentrations.

5.3 Results

Table 5.2 lists the geometric means and ranges of Σ HDBP and CB-153 concentrations on a lipid weight basis, and Σ HDBP/CB-153 ratios. Since recoveries of the $^{13}\text{C}_{12}$ -PCB internal standards were $80 \pm 20\%$, Σ HDBP and CB-153 concentrations were recovery corrected.

5.3.1 Effects of age and sex

St. Lawrence River beluga, Igloodik beluga, Wadden Sea harbour seal, Alaskan harbour seal, and bowhead whale sample subsets were used to test for the effects of age and sex on Σ HDBP and Σ HDBP/CB-153 concentrations. Length was used to estimate age class for the bowhead whale samples (George et al. 1999). These were the only subsets of the same species from a single location containing enough samples for valid statistical analyses.

There were no significant effects of age or sex on the Σ HDBP and Σ HDBP/CB-153 values in the Wadden Sea harbour seal, Alaskan harbour seal, and bowhead whale sample subsets. Sex had a significant effect on Σ HDBP concentrations in the St. Lawrence River and Igloolik beluga sample subsets. Males had significantly higher Σ HDBP concentrations than females ($p < 0.02$, $p < 0.008$) (Figure 5.2); there were no significant effects on Σ HDBP/CB-153 ratios. The lack of an observed effect of age on Σ HDBP and Σ HDBP/CB-153 concentrations may have been due to the limited age range since all samples were obtained from adults (7–41 years old).

Figure 5.2 Σ HDBP concentrations in male and female beluga from Igloolik and the St. Lawrence River.

Table 5.2. Σ HDBP, CB-153, and Σ HDBP/CB-153 concentrations in marine mammals.

Species	Location ^a	N	Lipid % geomean	Σ HDBP geomean (range), ng/g lipid weight	CB-153 geomean (range), ng/g lipid weight	Σ HDBP/CB-153 geomean (range)
<i>Beluga</i>						
beluga	Chesterfield Inlet, Nunavut (1)	3	94.0	17.8 (9.5-27.1)	543 (307-961)	0.0450 (0.028-0.072)
beluga	Iglolik, Nunavut (2)	9	86.9	16.6 (0.5-54.4)	528 (86.7-1890)	0.0310 (0.006-0.080)
beluga	Svalbard, Norway (3)	2	66.6	2.0 (0.6-6.1)	1270 (646-2480)	0.0016 (0.0002-0.0094)
beluga	Pt. Lay, Alaska (4)	5	83.3	14.4 (2.04-99.2)	1380 (1150-1670)	0.0100 (0.0025-0.070)
beluga	St. Lawrence River (5)	12	88.5	14.8 (2.4-52.7)	7990 (1450-53700)	0.0018 (0.0008-0.0049)
<i>Dolphins and porpoises</i>						
bottlenose dolphin	Georgia / South Carolina (6)	5	47.0	102 (24.9-235)	28800 (12800-51400)	0.0035 (0.0011-0.0083)
Dall's porpoise	NW North Pacific Ocean (7)	5	72.4	2540 (1430-4710)	1240 (959-1550)	2.04 (1.00-4.05)
harbour porpoise	Vancouver Island, British Columbia (9)	4	93.4	176 (6.2-977)	1470 (695-4390)	0.120 (0.0089-0.362)
Hector's dolphin	New Zealand (10)	5	50.6	59.0 (15.4-841)	709 (368-1770)	0.0832 (0.0133-1.53)
hump-backed dolphin	Bay of Bengal (11)	1	39.3	1890	269	7.03
killer whale	Vancouver Island, British Columbia (9)	1	87.9	584	4510	0.130
Risso's dolphin	Taiji, Japan (12)	1	76.1	210	4390	0.0479
Fraser's and	Mindanao Sea,	5	54.2	432	358	1.21

Species	Location ^a	N	Lipid % geomean	ΣHDBP geomean (range), ng/g lipid weight	CB-153 geomean (range), ng/g lipid weight	ΣHDBP/CB-153 geomean (range)
Spinner dolphin	Philippines (8)			(49.3-2300)	(262-679)	(0.129-7.1093)
striped dolphin	Catalonia, Spain (13)	7	38.4	516 (306-1480)	61000 (11200-119000)	0.0085 (0.0031-0.0574)
<i>Sea lions</i>						
California sea lion	California (14)	5	48.1	479 (92.5-9800)	7970 (914-90800)	0.0534 (0.0010-0.376)
NZ sea lion	New Zealand (10)	5	63.1	48.2 (27.5-84.4)	75.7 (49.0-173)	0.637 (0.298-1.70)
Steller sea lion	Hokkaido coast, Japan (15)	1	67.9	331	480	0.689
Steller sea lion	California (14)	1	68.8	177	4645	0.0380
Steller sea lion	S Alaska (16)	3	41.4	234 (82.7-1040)	457 (285-1110)	0.5133 (0.135-3.47)
<i>Seals</i>						
harbour seal	Washington (17)	6	69.0	16.2 (6.9-44.5)	1050 (380-3470)	0.0154 (0.00723-0.0451)
harbour seal	Bering Sea (16)	18	80.3	8.3 (1.0-526)	156 (65.0-785)	0.0531 (0.00466-7.51)
harbour seal	Sweden (18)	18	77.7	0.15 (0.02-22.4)	8280 (1790-110000)	0.00002 (0.00001-0.00100)
harbour seal	St. Lawrence River (5)	5	90.7	0.36 (0.02-2.5)	11100 (3400-46300)	0.00004 (0.00003-0.00064)
harbour seal	Svalbard, Norway (3)	3	56.2	4.3 (0.4-37.2)	1610 (1330-2240)	0.00268 (0.00030-0.281)
harbour seal	Strathclyde coast, SW Scotland (19)	5	55.7	9.7 (0.5-29.6)	14700 (2210-282000)	0.00066 (0.00010-0.00367)
harbour seal	Wadden Sea (20)	14	72.9	0.31	9870	0.00003

Species	Location ^a	N	Lipid % geomean	ΣHDBP geomean (range), ng/g lipid weight	CB-153 geomean (range), ng/g lipid weight	ΣHDBP/CB-153 geomean (range)
Baikal seal	Lake Baikal, Russia (21)	5	82.6	(0.02-15.8) 0.35	(1280-24100) 2950	(0.00001-0.00132) 0.00012
Largha seal	Japan (12)	3	84.6	(0.02-32.4) 14.3	(1250-7850) 249	(0.00001-0.0259) 0.0574
NZ fur seal	New Zealand (10)	5	67.4	(11.2-18.0) 126	(171-503) 57.0	(0.0290-0.0998) 2.21
ringed seal	Eureka, Nunavut (22)	6	89.3	(27.6-402) 1.2	(29.5-92.7) 237	(0.29786-13.60981) 0.00522
ringed seal	western Greenland (23)	9	89.3	(0.1-46.4) 0.8	(73.3-1770) 107	(0.00137-0.0412) 0.00717
ringed seal	Kura Sea (24)	5	83.4	(0.3-1.5) 1.6	(48.1-286) 235	(0.00232-0.0232) 0.00693
ringed seal	Pangnirtung, Nunavut (25)	9	93.1	(0.6-11.5) 1.4	(140-376) 109	(0.00156-0.0355) 0.0125
ringed seal	White Sea (26)	9	89.0	(0.4-17.2) 0.4	(44.2-402) 367	(0.00345-0.103) 0.00115
				(0.1-6.0) <i>Mysticete cetaceans</i>	(158-1070)	(0.00020-0.00560)
bowhead whale	Beaufort Sea (27)	35	70.5	0.6 (0.1-1.8)	29.7 (9-197)	0.0200 (0.00119-0.0794)

^anumbers correspond to locations shown in Figure 5.1

Due to the lack of age and sex effects, the Σ HDBP and Σ HDBP/CB-153 data from male, female, adult, and immature seals and bowhead whales were combined and treated as one group. Data for dolphins, porpoises, and sea lions were similarly combined since the majority of samples were male. Beluga Σ HDBP data were corrected for effects due to sex using a general linear model.

5.3.2 Beluga

After correcting for sex, it was found that the Svalbard belugas had lower absolute Σ HDBP concentrations than the other four beluga groups studied ($p < 0.006$). In addition, there were significant ($p < 0.001$) differences in Σ HDBP/CB-153 ratios. Ratios were lower in samples from the St. Lawrence River and Svalbard regions than those from Igloodik and Chesterfield Inlet. Figure 5.3 shows the percentage contribution of each HDBP congener to Σ HDBP for beluga samples.

5.3.3 Dolphins and porpoises

Fraser's and Spinner dolphins from Mindanao Sea, Philippines were treated as one group since these species have similar diets (Pauly et al. 1998). Absolute HDBP concentrations were significantly ($p < 0.005$) higher in the Dall's porpoise samples from the northwestern northern Pacific Ocean than samples from the other locations. Σ HDBP/CB-153 values were significantly ($p < 0.003$) greater in the Dall's porpoise from the Pacific Ocean and dolphins from the Philippines than the other dolphin and porpoise

samples. Figure 5.4 illustrates the relative amount of HDBPs in the most contaminated Dall's porpoise compared to other organochlorine compounds.

Figure 5.3 HDBP congener profiles (expressed as a percentage of Σ HDBP) for beluga.

Numbers in parentheses refer to locations shown on Figure 5.1.

Samples were then classified as having either a Pacific or non-Pacific origin. There was no significant difference in Σ HDBP concentrations, but Σ HDBP/CB-153 ratios were lower in the non-Pacific group ($p < 0.01$).

Figure 5.4 Relative contribution (by weight) of HDBPs to the total quantitated organohalogen body burden for adult male Dall's porpoises from the northwestern North Pacific Ocean.

Σ PCBs includes 92 congeners; Σ DDT includes *p,p'*-DDT, *p,p'*-DDE, and *p,p'*-DDD; Σ CHL includes trans/cis-chlordane, trans/cis-nonachlor, and oxychlordane; Σ HCHs includes $\alpha/\beta/\gamma$ -HCH; Σ CIBZs includes 1,2,4,5-tetrachlorobenzene, 1,2,3,4-tetrachlorobenzene, pentachlorobenzene, and hexachlorobenzene; Σ other includes dieldrin, mirex, photomirex, tris-(4-chlorophenyl)-methanol, and octachlorostyrene.

5.3.4 Sea lions

There were no significant differences in Σ HDBP concentrations among the sea lions. In addition, Σ HDBP/CB-153 ratios were not significantly different among locations, although the ratio was lower in the California samples due to higher CB-153 concentrations than the other areas.

5.3.5 Seals

Absolute Σ HDBP concentrations and Σ HDBP/CB-153 ratios were not significantly different among seal samples from the various locations, although samples from New Zealand, Washington, Japan, Alaska, and Scotland grouped together and were higher than the remaining locations.

In addition, harbour, New Zealand fur, Baikal, and Larga seals were separated into groups from Pacific and non-Pacific Ocean influenced areas. The samples originating from non-Pacific areas had significantly lower Σ HDBP and Σ HDBP/CB-153 levels ($p < 0.00002$, $p < 0.000001$) than samples obtained from Pacific environments. Figure 5.5 shows the percentage contribution of each HDBP congener to Σ HDBP for the sample groups used in the Pacific versus non-Pacific comparison.

Figure 5.5 HDBP congener profiles (expressed as a percentage of Σ HDBP) for seal samples excluding ringed seals.

Numbers in parentheses refer to locations shown on Figure 5.1.

5.4 Discussion

5.4.1 Significance of HDBPs as organohalogen contaminants

HDBPs are just one of many classes of organohalogen compounds that have been found in marine mammals. They are however the first classes of persistent bioaccumulative organohalogens hypothesized to have a natural origin. The presence of another halogenated compound, also proposed to be naturally-produced, at relatively high levels in marine mammal samples has recently been described (Vetter et al. 2000). This compound, $C_9H_3N_2Cl_7$, is thought to contain a methyl-bipyrrole moiety, and may be related to HDBPs. The majority of other organohalogen contaminants commonly analyzed are anthropogenic or biotransformation products of anthropogenic compounds.

A wide range of HDBP concentrations were observed across all of the sampling locations and species studied. Concentrations ranged from sub ng/g in Baikal seals and harbour seals from the St. Lawrence River, Wadden Sea, and Sweden, to $\mu\text{g/g}$ levels in Dall's porpoise from the northwestern Pacific Ocean. Similar to organochlorine compounds (O'Hara et al. 1999), HDBPs were at lower concentrations in baleen whales (bowhead whales) compared to odontocetes (beluga) from a nearby location. In addition to trophic level, metabolism may also affect interspecies differences in HDBP concentrations. Evidence from a food web study suggests that ringed seals may have the ability to metabolize HDBPs (Tittlemier et al. 2001c). Possible differential metabolic capabilities of pinnipeds and cetaceans towards HDBPs may account for the differences in HDBP concentrations observed in these groups. In all cases though, geometric mean total HDBPs (ΣHDBPs) were at levels lower than total PCBs or total DDT compounds.

the most abundant organochlorine compounds, reported for the same species at similar locations (Nakata et al. 1997; Blomkvist et al. 1992; Nakata et al. 1998; Muir et al. 2000; Tanabe et al. 1988; Tanabe et al. 1983; Westgate and Tolley 1999; Tilbury et al. 1999; Prudente et al. 1997; Kuehl et al. 1991).

However, there were some instances in which HDBPs made a significant contribution to an organism's organohalogen body burden. The highest Σ HDBP concentrations were found in male Dall's porpoises from the northwestern Pacific Ocean. The pie chart in Figure 5.4 depicts the percent contribution of each contaminant class to the amount of total organohalogens quantitated in the samples. HDBPs comprised approximately 11% of the total mass of quantitated organohalogens, and were the fourth largest contributor after PCBs (39%), DDT compounds (27%), and chlordanes (15%). For the remaining marine mammal samples, HDBPs were less than 3% of the total quantitated organohalogens, and did not constitute a significant portion of the organohalogen body burdens. It is likely that the actual percent contribution of HDBPs to the total organohalogen body burden is less than calculated here, since other organohalogens, principally toxaphene, were not analyzed.

5.4.2 Variation of Σ HDBP concentrations with age and sex

The majority of sample groups were of inadequate size and composition to confidently test for relationships between HDBP concentrations, age, and sex. Belugas of the St. Lawrence River and Igloodik were the only cases where significant relationships between Σ HDBP concentrations and sex were observed. The mean concentration in males was approximately five times higher than in females. There was a similar

difference in CB-153 concentrations between males and females in the same samples. This difference has been observed on other occasions with many persistent organochlorine compounds such as DDT and PCBs (Stern et al. 1994), and is attributed to lactational and placental transfer of contaminants in females to offspring.

5.4.3 Geographical distribution

The general variation of Σ HDBP concentration within cetacean and pinniped groups appears to be governed strongly by one factor - location. There is a clear distinction between samples obtained from Pacific and non-Pacific locations in the Northern Hemisphere and tropics. There are likely confounding factors present, such as species- and area-specific differences in feeding ecology, biotransformation capabilities, sample condition, and inter-individual variability, which contribute to variations in HDBP concentrations. Nevertheless, samples originating from a Pacific environment contained significantly higher concentrations of HDBPs than the same or similar species from other locations. The highest Σ HDBP concentrations among all species and sites were found in Dall's porpoise samples obtained from northwestern Pacific Ocean environments. This geographical difference has been noted previously for HDBPs in seabird eggs (Tittlemier et al. 1999a).

A differentiation in Σ HDBP levels in Pacific and non-Pacific samples is observed in the seal and dolphin/porpoise subsets, the only two groups of species with an adequate range of sampling locations to allow for such a comparison. The distinction between concentrations in Pacific and non-Pacific seal blubber is clear, but is less apparent with the dolphins and porpoises. Striped dolphin blubber from the Mediterranean Sea

contained relatively high concentrations of HDBPs compared to the other European samples. When these concentrations are normalized to CB-153 and compared to the other Σ HDBP/CB-153 ratios it is clear that the Mediterranean samples were much more contaminated with organohalogenes overall. These samples were collected during the 1990 Mediterranean epizootic, and were previously shown to contain high levels of PCBs when compared to healthy striped dolphins sampled before and after the epizootic (Aguilar and Borrel 1994). The high HDBP levels may therefore be due to mobilization of blubber reserves and subsequent inability to metabolize or excrete compounds at a comparable rate, rather than a higher level of HDBPs in the Mediterranean environment than elsewhere. This sequence of events has been proposed to explain differences in concentrations of PCBs and DDT compounds in grey seals of varying nutritional condition from Sweden and Scotland (Blomkvist et al. 1992).

One of the most abundant and widespread anthropogenic organohalogen compound in the environment is CB-153, a hexachlorinated PCB congener. CB-153 is not readily biotransformed and bioaccumulates in biota. The water solubility, vapour pressure, and octanol/water partition coefficient (K_{ow}) of CB-153 are similar to those estimated for HDBPs, indicating CB-153 is a good marker of the relative distribution of semi-volatile anthropogenic and natural organohalogen compounds (Tittlemier et al. 2001d). However, it must be noted that HDBPs are probably not as persistent in pinnipeds as CB-153 (Tittlemier et al. 2001c).

The geographical distribution of Σ HDBP concentrations does not resemble the distribution patterns of some anthropogenic organohalogenes, such as CB-153, and PCBs in general. A tendency for levels of PCBs in Pacific to be higher than in non-Pacific

organisms has not been observed. Studies monitoring these organohalogens in seals and marine fish have indicated that the highest levels of contaminants are in samples from the Atlantic Ocean, North, and Baltic Seas, followed by the Northwest Pacific Ocean, Arctic Ocean, and finally the Antarctic Ocean, as discussed in Muir et al. (1988).

In contrast to PCBs, HDBPs appeared to be distributed uniformly in beluga sampled from Arctic regions and the St. Lawrence River. The relatively uniform distribution of HDBPs is different from what was observed for CB-153 (Table 5.2) and total PCBs in beluga from western Greenland and the St. Lawrence River. The levels of CB-153 in St. Lawrence beluga ranged from 5 to 15 times greater than those from the other sites. St. Lawrence beluga also contained approximately 15 times greater amounts of total PCBs than those from western Greenland (Muir et al. 1996; Stern et al. 1994). In contrast, Σ HDBP concentrations were essentially identical in the Chesterfield Inlet, Igloodik, Pt. Lay, and St. Lawrence River beluga; the Svalbard group contained lower levels.

There was also no consistent difference between concentrations of HDBPs in biota from the Northern hemisphere and New Zealand, the sole sampling site in the Southern hemisphere. Hector's dolphin and New Zealand sea lion samples contained less HDBPs than ecologically similar species in the Northern hemisphere, but New Zealand fur seals contained significantly higher levels than northern seal species. The few available studies concerning anthropogenic organohalogens in Southern hemisphere marine mammals conclude that levels are lower than those found in the Northern hemisphere (Kemper et al. 1994; Bacon et al. 1992; Connell et al. 1999). However, a recent study by Vetter et al. (2000) found that levels of Q1, an organohalogen

hypothesized to be of biogenic origin, were higher in seal blubber samples from the Antarctic compared to those sampled from the Arctic.

CB-153 concentrations and ratios of Σ HDBP/CB-153 confirm that HDBPs have a different distribution than PCBs. The data in Table 5.2 illustrate that samples with high levels of HDBPs do not necessarily contain correspondingly high levels of CB-153. The geographical distribution of CB-153 generally follows the distribution of industrialization and other human activity. The samples that contain the highest levels of CB-153 within each group are from sites near intense human activity, such as the St. Lawrence River, the Mediterranean Sea, and the Southeastern U.S. coast. Yet these same samples do not have the highest Σ HDBP concentrations.

Similar physical properties of CB-153 and HDBPs suggest that these compounds have comparable capacities to move throughout the environment. However, the potential of these compounds to accumulate in marine mammals is likely different in pinnipeds due to their ability to metabolize HDBPs (Tittlemier et al. 2001c). The relatively high Σ HDBP concentrations observed in cetacean blubber samples combined with the proposed low metabolic capabilities of cetaceans towards non-planar organohalogens may suggest that cetaceans do not metabolize HDBPs, or do not metabolize them to the same extent, as pinnipeds (Boon et al. 1992). If the potential to move and accumulate in a food web is similar between CB-153 and HDBPs, as is likely the case with cetaceans, the difference in their distribution patterns may be attributed to different sources and exposures. Sources of PCBs are terrestrial and distributed in industrialized urban areas. However, HDBPs appear to be formed primarily in the north Pacific Ocean. This provides more evidence to support the hypothesis that HDBPs are naturally-produced.

5.4.4 Transport of HDBPs

It is very likely that the source of HDBPs exists predominantly in the northern Pacific Ocean since the highest Σ HDBP levels have been observed in biota from this area. If the HDBPs are produced mainly in this area, transport mechanisms must be at work, or else there would not be as wide a distribution of HDBPs as was observed in marine mammals. Two possible mechanisms regulating HDBP distribution are atmospheric and oceanic current transport.

Atmospheric transport of organohalogenes of marine origin is governed by Henry's Law constant, which describes the air-water partitioning behaviour of a substance (Wania and Mackay 1993). Compounds with high Henry's Law constants will tend to volatilize from aqueous solution to air, while those with low constants will tend to remain in solution. The Henry's Law constants of HDBPs have been estimated from the ratio of vapour pressure to aqueous solubility, and are in the range of 0.002 to 0.007 Pa m³/mol (Tittlemier et al. 2001d). These values place HDBPs in the same range as penta- to heptabrominated PBDE congeners, and thus are not likely to undergo extensive atmospheric transport (Tittlemier et al. 2001b). In a previous study, HDBPs were not detected in herring gull egg samples from the Great Lakes, supporting the hypothesis that they are not atmospherically transported to a great extent (Tittlemier et al. 1999a).

Evidence for extensive atmospheric transport does not exist in the HDBP congener patterns observed in the samples. Increasing proportions of more volatile congeners with an increase in latitude has been described as being indicative of atmospheric transport of low volatility compounds. For example, tri-, tetra-, and

pentachlorinated PCB congeners increased in proportion to higher chlorinated congeners in burbot (*Lota lota*) obtained from remote Canadian lakes and rivers at increasing latitudes (Muir et al. 1990). When HDBP congener patterns in harbour, Largha, Baikal, and New Zealand fur seals from locations at various latitudes are compared, no such increase in the more volatile congeners is observed (Figure 5.5). In fact, the relative amounts of the least volatile congener, DBP-Br₆, are higher in samples from Alaska and Svalbard, the two most northern latitudes. The same results are found when HDBP congener patterns in beluga are considered (Figure 5.4). Aside from the samples obtained from Pt. Lay, the location closest to the Pacific Ocean, the predominant congener is the least volatile DBP-Br₆.

It does appear, however, that limited atmospheric transport of HDBPs occurs, because HDBPs were found at low concentrations in Baikal seals obtained from the freshwater environment of Lake Baikal, Russia. The pattern of HDBP congeners was similar to Pacific Ocean marine samples, and unlike that found in the North Atlantic Ocean and adjacent seas. Previously, HDBPs were not detected in seabird eggs from the freshwater environment of the Great Lakes, thereby indicating that they were present at much higher levels in marine environments (Tittlemier et al. 1999a). The difference between the presence of HDBPs in the freshwater environment of Lake Baikal and its apparent absence in the Great Lakes may be explained by average surface wind flows. January and July average winds flow in an easterly direction over a considerably large distance across the North American land mass before reaching the Great Lakes. On the other hand, July average winds flowing towards Lake Baikal originate over the northeastern Pacific Ocean and cross a shorter section of land mass (Hasse and Dobson

1986). The low vapour pressure HDBPs would be more easily transported a shorter distance over land to Lake Baikal than the Great Lakes before removal from the air masses by deposition.

Overall, the evidence does not clearly indicate atmospheric transport as the major mode of HDBP global distribution. Indirect evidence for ocean current transport can be found in the congener patterns of HDBPs from Pacific and Atlantic Ocean influenced environments, assuming that the primary source is in the north Pacific Ocean. Figures 5.3 and 5.5 illustrate the differences between HDBP congener patterns in beluga and harbour seal samples from these two environments. Samples originating in areas influenced by the Pacific Ocean have DBP-Br₄Cl₂ as the predominant congener. This was similarly observed for seabirds (Tittlemier et al. 1999a). Samples from the Atlantic, or areas where there are inputs of Atlantic waters such as the eastern Canadian Arctic, have either DBP-Br₃Cl₃ or DBP-Br₆ as the most abundant congeners. These differences in HDBP congener patterns are not as clear in the dolphin and porpoise samples, presumably due to the number of diverse species sampled. HDBP congener patterns have been shown to be related to an organism's feeding habits, e.g. whether the food chain is benthic or pelagic based (Tittlemier et al. 2001c). There is also the possibility that differences in metabolism of HDBPs exists among the species.

One would not expect the congener patterns to be so distinct between, and conserved within, Pacific and Atlantic regions if atmospheric transport was the only mechanism of movement. Since atmospheric transport is governed in part by a compound's vapour pressure, it would be expected that the more volatile congeners would predominate the composition of total HDBPs in all northern locations irrespective

of their source's HDBP composition. This would tend to minimize differences in congener patterns between Pacific and Atlantic originating samples. As illustrated in Figures 5.3 and 5.5, this clearly does not occur. It cannot be ruled out that HDBPs are produced by different organisms distributed in various locations, which produce the congener pattern differences. However, it seems unlikely that organisms producing such unique organohalogen compounds would be distributed widely in such different marine environments.

In reality, HDBPs are likely transported by a combination of atmospheric and ocean current transport. Evidence indicative of the primary mode of movement has likely been obscured over time. If HDBPs are naturally-produced, they would have been present in the environment for numerous centuries, and have had the time to be transported great distances by both mechanisms even if the rate of one mode of transport is much less than the other.

5.5 Conclusions

HDBPs are globally distributed in the marine environment. They likely undergo extensive transport by a combination of atmospheric and ocean current mechanisms. However, the predominant mode of transport cannot be determined from the available evidence. HDBPs appear to be primarily produced in the northern Pacific Ocean, as indicated by relatively high concentrations in biota from this region. In some instances in this area, HDBPs contribute significantly to an organism's total organohalogen body burden. The variation of HDBP concentration with location is very different from that of anthropogenic organochlorines such as CB-153. This difference in distribution suggests

a biogenic rather than anthropogenic source of HDBPs. The widespread distribution of HDBPs, most likely occurring as a slow process as indicated by their low vapor pressure and water solubility, suggests that HDBPs have a long lifetime in the marine biosphere.

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CHAPTER 6.

EXAMINATION OF THE BIOACCUMULATION OF HALOGENATED DIMETHYL BIPYRROLES IN AN ARCTIC MARINE FOOD WEB USING STABLE NITROGEN ISOTOPE ANALYSIS*

6.1 Introduction

Halogenated dimethyl bipyrrroles (HDBPs) form a family of novel hexahalogenated heterocyclic compounds containing both chlorine and bromine. These compounds were first identified in marine seabirds from the Pacific coast of Canada (Tittlemier et al. 1999a). Their source is currently unknown, but evidence indicates that they are of biogenic origin. The mixed halogenation and predominance in marine rather than freshwater biota suggest that HDBPs do not have anthropogenic sources (Tittlemier et al. 1999a). The structures of the known HDBPs (shown in Figure 3.2) are also similar to that of a known natural marine product, hexabromo-2,2'-bipyrrrole (Andersen et al. 1974).

Few data presently exist on environmental concentrations of HDBPs. However, the most abundant HDBP congener in Pacific Ocean biota, DBP-Br₄Cl₂, has been found at ppm concentrations in higher trophic level organisms such as bald eagles (*Haliaeetus leucocephalus*) (Tittlemier et al. 1999a) and Dall's porpoise (*Phocoenoides dalli*)

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* adapted from Tittlemier et al. (2001c).

(Tittlemier et al. 2001e). These occurrences of high concentrations show that HDBPs are bioaccumulative, that is, they may be accumulated by an organism via uptake from food and water.

Physical property data for HDBPs, specifically high octanol/water partition coefficients (K_{ow} s), suggest that HDBPs may be transferred through aquatic food webs (Tittlemier et al. 1999b). Trophic transfer is the major exposure pathway of higher trophic level aquatic organisms to hydrophobic organohalogenes (Thomann et al. 1992). In fact, the high K_{ow} s of these compounds suggest that they have the potential to biomagnify, or increase in concentration from one trophic level to the next provided that they are not metabolized. The degree of biomagnification of hydrophobic organochlorines in invertebrates and fish has been shown to be related to K_{ow} in a freshwater food web (Kidd et al. 1998).

Stable isotope analysis is often used to provide a continuous variable with which to assess both trophic level (Michener and Schell 1994; Hobson et al. 1995) and trophic transfer of persistent organic pollutants (Kidd et al. 1998). In the case of stable nitrogen isotope analysis, the ratio of the heavier to lighter stable isotopes of nitrogen ($^{15}\text{N}/^{14}\text{N}$) is generally found to increase with trophic position in aquatic food webs. Trophic magnification factors (TMFs) can then be estimated from slopes of logarithmic concentration of contaminant vs. trophic level (Jarman et al. 1996). TMF differs from biomagnification factor (BMF) since it is a description of biomagnification over multiple, as opposed to just two trophic levels, of a food web.

In this study, three HDBP congeners of known structure – 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrole (DBP-Br₄Cl₂), 1,1'-dimethyl-3,3',4,4',5-pentabromo-5'-chloro-2,2'-bipyrrole (DBP-Br₅Cl) and 1,1'-dimethyl-3,3',4,4',5,5'-hexabromo-2,2'-bipyrrole (DBP-Br₆) – and the hypothesized 1,1'-dimethyl-3,3',4-tribromo-4,5,5'-trichloro-2,2'-bipyrrole (DBP-Br₃Cl₃) were analyzed in an aquatic food web from the Northwater (NOW) polynya (Figure 6.1). Polynyas are areas of year-round open water surrounded by sea ice. The NOW polynya supports a highly productive food web and provides an important habitat for marine mammals and seabirds, in some cases throughout the year. Trophic levels of the food web involving zooplankton, fish, seabirds, and marine mammals were characterized using stable nitrogen isotope analysis.

6.2 Experimental section

6.2.1 Sample Collection

All samples were collected in 1998. Sediment samples were taken in July from the sediment surface of box core samples. Zooplankton samples (*Calanus hyperboreus*, *Mysis oculata*, and *Sagitta sp.*) were collected between May and July using vertical tows of large zooplankton nets. Samples were sorted to species shortly after collection. In May, Arctic cod (*Boreogadus saida*) were opportunistically collected with hand-held nets. Seabirds [dovekie (*Alle alle*), black guillemot (*Cephus grylle*), black-legged kittiwake (*Rissa tridactyla*), and glaucous gull (*Larus hyperboreus*)] were collected from the NOW polynya in May and June by shotgun. Samples of ringed seal (*Phoca hispida*)

blubber were obtained from local hunters from Qaanaaq, Greenland during the same period. Seabirds and seals were dissected shortly after death, and muscle and liver samples were placed in Whirl Pak bags and frozen until analyzed isotopically.

Figure 6.1 Size and approximate location of the NOW polynya in May/June.

6.2.2 Stable Isotope Analysis

Nitrogen stable isotope analysis was carried out according to the method described in Moisey et al. (2001). Prior to analysis, all tissue samples were washed in distilled water and then freeze dried, powdered and treated with a 2:1 (v/v)

chloroform/methanol solution to remove lipids. Whole body homogenates were the tissue substrates used for zooplankton, whereas muscle was analyzed for birds, fish, and seals.

Isotope assays were performed on subsamples of homogenized materials by loading into tin cups and combusting at 1,800°C in a Robo-Prep elemental analyzer. Resultant N₂ gas was then analyzed using an interfaced Europa 20:20 continuous-flow isotope ratio mass spectrometer with every 5 unknowns separated by 2 laboratory standards (albumen).

Stable isotope abundances were expressed in δ notation as the deviation from a standard in parts per thousand (‰) according to the following equation:

$$\delta^{15}\text{N} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000 \quad (1)$$

where R is the ratio ¹⁵N/¹⁴N. The R_{standard} values were based on atmospheric N₂ (AIR).

Trophic levels were determined using equations modified slightly from those reported in Hobson et al. (1995). Trophic level was determined relative to the copepod *Calanus hyperboreus*, which was assumed to occupy trophic level 2 since it is an obligate herbivore. Trophic level (TL) was determined for each individual sample of zooplankton, fish and marine mammal using the relationship:

$$\text{TL}_{\text{organism}} = 2 + \left(\frac{\delta^{15}\text{N}_{\text{organism}} - \delta^{15}\text{N}_{C. \text{hyperboreus}}}{3.8} \right) \quad (2)$$

where TL_{organism} is trophic level of the organism, $\delta^{15}\text{N}_{C. \text{hyperboreus}}$ is equal to 9.0 ± 0.2 ‰, and 3.8 is the isotopic trophic enrichment factor in parts per thousand (Hobson et al. 2001).

For birds, there is evidence that trophic enrichment values differ from other marine organisms. Avian captive-rearing studies suggest an isotopic enrichment factor of +2.4‰ for muscle tissue to account for this difference (Hobson and Clark 1992). Thus equation (2) was modified using the relationships in (3) and (4) to result in an equation which describes the trophic level of seabirds in the NOW polynya (5):

$$TL_{bird} = TL_{prey} + 1 \quad (3)$$

$$\delta^{15}N_{bird} = \delta^{15}N_{prey} + 2.4 \quad (4)$$

$$TL_{bird} = 3 + \left(\frac{\delta^{15}N_{bird} - 11.4}{3.8} \right) \quad (5)$$

Equations (2) and (5) are somewhat arbitrary, but are based on current knowledge of how $\delta^{15}N$ values behave in Arctic marine food webs and adequately describe seabird trophic level in the NOW (Hobson et al. 2001).

6.2.3 Organohalogen Analysis

The following tissues were analyzed for organohalogens: zooplankton (whole body), Arctic cod (whole body), seabirds (liver), and ringed seal (blubber). Sediment and zooplankton samples were freeze-dried, spiked with an internal standard, and extracted with 1:1 (v/v) dichloromethane/hexane using a Dionex ASE 200 accelerated solvent extractor. All cod, seabird, and seal samples were extracted according to a similar method described in Norstrom et. al. (1988). Briefly, a known amount of tissue was ground with anhydrous Na_2SO_4 , spiked with an internal standard (mixture of $^{13}C_{12}$ -PCBs) and extracted with 1:1 dichloromethane/hexane. Lipid amounts were determined gravimetrically, and the lipids were subsequently removed from the sample by GPC.

Sulfur containing compounds were removed from the sediment samples by the addition of reduced copper. The lipid-free (biota) and sulfur-free (sediment) extracts were reduced and applied to a Florisil column (8 g, 1.2% deactivated). For the sediment, zooplankton, cod, and seabird samples, organohalogens were recovered by consecutive elution with 35 ml hexane [Fraction 1 (F1)], 38 ml of 85% hexane: 15% dichloromethane (F2), and 52 ml of 1:1 dichloromethane/hexane (F3). For seals, organohalogens were eluted by a single elution of 100 ml of 1:1 dichloromethane/hexane. All fractions were rotary evaporated, solvent exchanged into isooctane, and reduced under a gentle N₂ stream. Volume correction standard (¹³C₁₂-CB 138) was added prior to analysis.

HDBPs were analyzed as described in Tittlemier et. al. (1999a) using a Hewlett Packard 5987B mass spectrometer coupled via a 5988 GC/MS direct interface to a 5890 Series II GC. The GC was equipped with a 30 m x 0.25 mm i.d. DB5-MS column with a film thickness of 0.25 µm. HDBPs were analyzed in the electron capture negative ionization single ion monitoring mode. Methane (99.97%) was used as the moderating gas. The sediment, zooplankton, cod, and seabird F2 Florisil fractions, and the combined ringed seal Florisil fractions, were analyzed for HDBPs. Synthesized HDBPs (Tittlemier et al. 2001a) were used as external standards to quantitate the four congeners in the samples.

6.3 Results

6.3.1 HDBP Concentrations

Concentrations of HDBPs were normalized to the average $^{13}\text{C}_{12}$ -PCB recovery since average recoveries of the internal standards ranged from 60-110%. The recovery corrected and lipid normalized concentrations of the HDBP congeners and results of the ^{15}N stable isotope analysis are given in Table 6.1. Relative amounts of each HDBP congener in each species are illustrated in Figure 6.2.

6.4 Discussion

6.4.1 HDBP concentrations

HDBPs were detected in all of the samples studied from the NOW polynya. As shown in Table 6.2, levels of DBP- Br_4Cl_2 were generally similar to those recorded in seabird samples occupying similar ecological niches from Atlantic Canada (Tittlemier et al. 1999a). For this comparison, the DBP- Br_4Cl_2 egg wet weight concentrations were transformed to lipid normalized liver concentrations using an egg-to-liver organochlorine ratio calculated from Braune and Norstrom (Braune and Norstrom 1989). Such a comparison cannot be made for the other congeners since the present study contains the first reported concentration data for these congeners in seabirds.

Table 6.1. HDBP congener concentrations and stable isotope analysis results for NOW polynya food web, arithmetic mean $\pm$ standard deviation.

Biota concentrations are on a lipid weight basis, ng/g.

organism ^a	n	$\delta^{15}\text{N}$ (‰)	TL ^b	DBP-Br ₃ Cl ₃	DBP-Br ₄ Cl ₂	DBP-Br ₅ Cl	DBP-Br ₆
SED	2	na ^c	na	<0.006 ^d	0.0020 $\pm$ 0.0002	<0.002	0.028 $\pm$ 0.028
CH	3	9.0 $\pm$ 0.2	2.0 $\pm$ 0.05	<0.003	0.021 $\pm$ 0.02	<0.001	<0.001
MO	3	10.8 $\pm$ 0.4	2.5 $\pm$ 0.09	0.11 $\pm$ 0.10	0.22 $\pm$ 0.03	0.017 $\pm$ 0.002	0.58 $\pm$ 0.23
SS	3	10.9 $\pm$ 0.2	2.5 $\pm$ 0.04	<0.003	0.057 $\pm$ 0.027	<0.001	<0.001
DOVE	3	11.9 $\pm$ 0.9	3.1 $\pm$ 0.2	0.55 $\pm$ 0.15	1.8 $\pm$ 0.3	0.16 $\pm$ 0.04	0.69 $\pm$ 0.33
COD	5	14.1 $\pm$ 0.5	3.3 $\pm$ 0.1	0.17 $\pm$ 0.28	0.38 $\pm$ 0.39	0.05 $\pm$ 0.05	0.53 $\pm$ 0.60
BLKI	5	13.2 $\pm$ 0.4	3.5 $\pm$ 0.1	3.3 $\pm$ 1.5	15 $\pm$ 6	1.1 $\pm$ 0.4	13 $\pm$ 23
BLGU	6	14.9 $\pm$ 0.7	3.9 $\pm$ 0.2	0.62 $\pm$ 0.44	1.9 $\pm$ 1.5	0.18 $\pm$ 0.07	6.9 $\pm$ 4.7
GLGU	4	16.0 $\pm$ 0.8	4.2 $\pm$ 0.2	11 $\pm$ 7	42 $\pm$ 28	1.8 $\pm$ 0.9	13 $\pm$ 4
SEAL	10	17.4 $\pm$ 0.5	4.2 $\pm$ 0.2	0.027 $\pm$ 0.022	0.017 $\pm$ 0.008	<0.02	0.046 $\pm$ 0.044

^aSED=sediment, CH=*Calanus hyperboreus*, MO=*Mysis oculata*, SS=*Sagitta* sp., DOVE=dovekie, COD=Arctic cod, BLKI=black-legged kittiwake, BLGU=black guillemot, GLGU=glaucous gull, SEAL=ringed seal

^btrophic level

^cnot applicable

^destimated method detection limit

Concentrations of DBP-Br₄Cl₂ in the offshore surface and omnivorous feeders from the NOW were on the same order of magnitude as similar species from Atlantic Canada. It should be noted that the diet of the glaucous gulls in the NOW likely reflects a more carnivorous feeding strategy than that of the herring gulls from Newfoundland and Nova Scotia (Nettleship et al. 1990). This would account for the higher DBP-Br₄Cl₂ levels observed in the glaucous gulls. Dovekies provide the single clear exception to the similarities between the NOW and previously published Atlantic seabird data (Tittlemier et al. 1999a). Dovekie DBP-Br₄Cl₂ concentrations (1.76 ppb) are approximately ten times lower than those of the Atlantic puffin (*Fratercula artica*, 20 ppb), a species which has a similar winter habitat but a more piscivorous diet. The difference in concentrations would be driven by the dovekies feeding at a lower trophic position than the puffins. A change in dovekie diet just prior to their collection in early summer (Fisk et al. 2001a) may also contribute to the differences in DBP-Br₄Cl₂ concentrations. During late spring, as dovekies return to the NOW polynya from the St. Lawrence Estuary and Atlantic Canada, they feed exclusively on lipid-rich relatively uncontaminated zooplankton, rather than fish (N. Karnovsky, unpublished data).

Table 6.2. Comparison of DBP-Br₄Cl₂ concentrations in seabird species from the NOW polynya and Atlantic Canada (Tittlemier et al. 1999a).

Species ^a	n (n in pool)	Location	mean ± std. deviation DBP-Br ₄ Cl ₂ lipid wt. (ng/g) in liver
Offshore Surface Feeders			
BLKI	5	NOW polynya	15 ± 6
LSP	(10)	Gull Island, NF	55
LSP	(10)	Kent Island, NB	58
Offshore Subsurface Feeders			
DOVE	3	NOW polynya	1.8 ± 0.3
ATPU	(10)	Gull Island, NF	20
ATPU	(10)	Machias Seal Island, NB	32
Omnivores			
GLGU	4	NOW polynya	42 ± 28
HERG	(4)	Gull Island, NF	11
HERG	(10)	Sable Island, NS	28

^aBLKI=black-legged kittiwake, LSP=Leach's storm-petrel, DOVE=dovekie, ATPU=Atlantic puffin, GLGU=glaucous gull, HERG=herring gull

6.4.2 HDBP congener patterns

Relative amounts of each HDBP congener varied among species (Figure 6.2). In the majority of the bird species, *C. hyperboreus*, and *Sagitta sp.* DBP-Br₄Cl₂ was the predominant congener. These species are primarily pelagic, and therefore likely to reflect the HDBP pattern in the water column. The apparent absence of the other congeners in the zooplankton is probably due to concentrations being very near the detection limit. DBP-Br₄Cl₂ was also the major congener in seabird samples analyzed previously (Tittlemier et al. 1999a). The exception occurred with black guillemots, where DBP-Br₆ was the most abundant congener. This may be due to black guillemots feeding on

Figure 6.2 Abundances of HDBP congeners relative to DBP-Br₄Cl₂ in NOW polynya food web.

Species data labels are the same as those used in Table 6.1.

benthic organisms during part of the year (Gaston and Jones 1998). Benthic organisms are more likely to contain DBP-Br₆ as the predominant congener since it was the most abundant in sediment samples. Assuming that predominance of DBP-Br₆ indicates a benthic contribution, the HDBP congener pattern for *M. oculata* suggests that those zooplankton feed on benthic organisms as well. *M. oculata* is considered a suprabenthic organism, feeding on the sediment surface but also spending some time in the water column. Therefore it is likely that there is a benthic component to its diet (Hobson et al. 2001).

DBP-Br₃Cl₃ and DBP-Br₆ were the predominant congeners in the cod and seal samples. The patterns in the two species resembled each other, which is consistent with the importance of Arctic cod in the ringed seal diet (Welch et al. 1992). The HDBP congener patterns found in the cod and seal samples appeared more similar to those in the benthic than the pelagic species, apart from the dominance of the DBP-Br₃Cl₃.

It is interesting to speculate why there are such differences in patterns of HDBPs in such a closely integrated ecosystem as found in the NOW polynya. It is very unlikely that any HDBP congener can be converted to another by any of the species in this study. The bipyrrole rings are fully halogenated in all four congeners, so such a conversion would require replacement of chlorine by bromine, or the reverse. *C. hyperboreus* is one of the most abundant herbivorous zooplankton species in the polynya, and therefore is representative of the bottom of the pelagic food web. Concentrations of anthropogenic organochlorine compounds in *C. hyperboreus* appear to be governed by water concentrations (Fisk et al. 2000). It is therefore expected that species such as Arctic cod ca. 1-2 trophic levels higher than *C. hyperboreus* would have a similar HDBP pattern, assuming that cod do not significantly metabolize HDBPs. This is the case for PCB congeners (Fisk et al. 2001b). Most cod samples followed the ln concentration vs. trophic level relationship (Figure 6.3), suggesting that metabolism is slow if it occurs at all. One plausible hypothesis is that cod are more connected to the benthic food web than has been assumed, which would account for the relatively high concentration of DBP-Br₆. The high relative level of DBP-Br₃Cl₃ in cod compared to *M. oculata* and dovekie may be due to greater efficiency of absorption of this congener from the diet by fish, since it has the lowest log K_{OW} and smallest molecular size of the four congeners.

6.4.3 Trophic Magnification of HDBPs

In general, the natural logarithm of concentrations of HDBPs were found to increase linearly with a corresponding increase in trophic level (Figure 6.3). However, ringed seals did not follow this trend. Concentrations of OCs and PCBs quantitated in the same samples also deviated from the ecosystem trophic magnification relationship. This was found to be due to the concentration differences between male and female samples (Fisk et al. 2001b). Females generally have lower concentrations of OCs and PCBs because they have increased elimination capacities through maternal transfer and lactation (Weis and Muir 1997). There was no significant difference ($p < 0.06$) between HDBP concentrations in the male and female seals, indicating that lactation is not the most important route of excretion. A more plausible explanation is that ringed seals are more proficient than birds at metabolizing HDBPs. Seals generally have a higher capability than zooplankton, fish, and cetaceans, but lower than birds and terrestrial mammals, for metabolism of organohalogen pesticides and *m,p*-substituted PCBs (Boon et al. 1989). Metabolism of these compounds is mediated by CYP2B-like enzymes, therefore it is unlikely that these enzymes are responsible for HDBP metabolism in seals. Metabolic indices have suggested that seals have potentially higher CYP1A-type enzyme activities than birds and cetaceans, but less than terrestrial mammals, depending on level of exposure to compounds that are Ah receptor agonists, such as 2,3,7,8-TCDD and CB-126 (Nakata et al. 1998; Tanabe et al. 1988). However, since the Arctic ringed seals are not expected to contain high levels of

Figure 6.3 Relationship between HDBP congener concentration and trophic level in NOW polynya food web.

Seal data was not included in the linear regressions. Species data labels are the same as those used in Table 6.1.

Figure 6.3 (continued)

dioxin-like CYP1A inducers (Norstrom et al. 1990), metabolism of HDBPs by CYP1A is also an unlikely scenario. Differences in HDBP concentrations between birds and ringed seals could be due to mediation of HDBP metabolism by constitutive enzymes which are not involved in metabolism of persistent xenobiotic organohalogen compounds.

Trophic magnification factors (TMFs) were calculated over all trophic levels of the food web excluding the seals. TMFs are defined as e^{slope} of the linear regressions of the natural logarithm of concentration versus trophic level. The linear regressions for HDBPs are shown in Figure 6.3. Slopes for the HDBPs were not significantly different, but it does appear that DBP-Br₄Cl₂ has a higher TMF (14.6 ± 1.5) than DBP-Br₃Cl₃ (5.2 ± 1.6), DBP-Br₃Cl (6.9 ± 1.6), and DBP-Br₆ (7.0 ± 1.5). DBP-Br₄Cl₂ would thus be expected to biomagnify through the NOW food web to a greater extent than the other congeners. The TMF values were similar to those for recalcitrant compounds such as CB-153 (TMF= 9.8) and *p,p'*-DDE (TMF= 13.9) determined for the same food web (Fisk et al. 2001b). This similarity suggests that, with the exception of seals, HDBPs are metabolized slowly or not at all in this food web.

It has been demonstrated that TMFs tend to increase with an increase in recalcitrance and K_{ow} (Kidd et al. 1998). Feeding studies with rainbow trout have also shown such a relationship between TMF and K_{ow} (Fisk et al. 1998). However, in this case differences in HDBP K_{ow} s may not be large enough to show differences in TMFs and therefore a relationship between K_{ow} and TMF. Log K_{ow} s of the four HDBP congeners were determined to be 6.5 for DBP-Br₃Cl₃, 6.5 for DBP-Br₄Cl₂, 6.6 for DBP-

Br₅Cl, and 6.7 for DBP-Br₆ using the slow-stirring method (Chapter 4), indicating a narrow range of values for the four congeners.

6.5 Conclusions

This study reports the presence of HDBPs in a number of species inhabiting the Arctic marine ecosystem. Their presence in plankton, fish, seabirds, and seals provides more evidence for their widespread occurrence and expands their known distribution to include Pacific, Atlantic, and Arctic Ocean biota. The distribution of HDBPs indicates that they are formed in widely ranging marine environments, or are subject to long range transport from regions of formation. Neither possibility rules out a biogenic source. However, the first suggests that the source is a common organism with a wide occurrence. The second scenario implies HDBPs are formed by an organism whose range is limited. There is evidence from analysis of seabird eggs that HDBP concentrations are higher in the North Pacific Ocean than elsewhere indicating the source may be located here. Absence in Great Lakes seabirds suggests that atmospheric transport is not their major mode of movement. HDBPs may therefore be circulated globally via ocean currents. In some cases HDBP congener patterns in NOW polynya biota were consistent with known predator – prey relationships, such as for ringed seal and cod. However, there appeared to be significant differences in HDBP congener patterns in benthic- and pelagic-based food webs, which were dominated by DBP-Br₆ and DBP-Br₄Cl₂, respectively. Benthic and pelagic signatures appeared to be mixed in some species at higher trophic levels.

In addition, results showed that the four, possibly naturally-produced, organohalogen compounds biomagnify in a marine food web. All HDBP congeners were found to magnify with trophic level as determined by stable nitrogen isotope analysis, except in ringed seals, which appear more capable than birds of metabolizing HDBPs. This order of metabolic capability is reversed to what is usually observed with xenobiotic organohalogens. The TMFs of HDBPs determined from the slope of the $\ln$ concentration vs. trophic level regressions ranged from 5 to 15, which is the same range as recalcitrant organochlorine compounds such as 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153). It is conceivable that due to their metabolism, HDBPs will not be found to bioaccumulate in other mammals to the relatively high levels found for recalcitrant xenobiotic organohalogens.

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CHAPTER 7.

INDUCTION OF CYTOCHROME P4501A AND PORPHYRIN ACCUMULATION BY HALOGENATED DIMETHYL BIPYRROLES IN CHICKEN EMBRYO HEPATOCYTES

7.1 Introduction

Biochemical changes resulting from exposure to environmental contaminants are often used as indicators, or biomarkers, to determine the qualitative or quantitative exposure of organisms to such compounds. Changes monitored include the increase in gene transcription, subsequent production of proteins, metabolic activity, or accumulation of metabolites. Two specific examples of biomarkers used to monitor exposure to aromatic organohalogenes are the induction of the cytochrome P4501A gene (CYP1A), measured as ethoxyresorufin-*o*-deethylase (EROD) activity (Stegeman et al. 1992), and the accumulation of porphyrins (Fox et al. 1988). Inferences concerning the toxicity of contaminants can be drawn from these biochemical changes since they are often correlated to toxicity (van den Berg et al. 1998).

Generally, CYP1A induction is mediated by binding of an inducer to the aryl hydrocarbon receptor (AhR), a protein found in a range of mammalian, avian, reptilian, amphibian, and piscivorian species (Hahn 1998; Karchner et al. 2000). Binding of the inducer to unliganded AhR initiates a series of events which, in part, culminates in the activation of gene transcription and the subsequent production of enzyme cytochrome P4501A (Whitlock and Denison 1995; Denison and Whitlock 1995). The mechanism of

porphyrin accumulation is not as well characterized as the induction of CYP1A, but there is evidence that it may be regulated by binding to the AhR and the induction of CYP1A5 enzyme in chickens (Sinclair et al. 2000). Chicken CYP1A5 preferentially catalyzes oxidation of uroporphyrinogen, which prevents the conversion of uroporphyrinogen to coproporphyrinogen, and results in the accumulation of uroporphyrin.

The prototypical AhR ligand is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD is also the compound which exerts the strongest negative effects in *in vivo* toxicological assays (Safe 1990). Other planar halogenated anthropogenic compounds, considered to be "classical" AhR ligands, such as non-ortho polychlorinated biphenyls, tetrabrominated dibenzodioxins, and tetrachlorinated dibenzofurans, have been shown to bind to AhR with affinities on the order of 0.1 to 0.5 times the strength of TCDD (McKinney et al. 1985; Poland et al. 1976). These compounds elicit correspondingly weaker *in vivo* effects than TCDD (Safe 1990). Even though exposure to TCDD and other classical AhR ligands results in the activation of a detoxification mechanism, the resistance of these compounds to metabolism is thought to contribute to their toxicity (Denison and Heath-Pagliuso 1998).

In addition, many naturally-produced non-planar compounds also bind to the AhR. Examples of such non-classical AhR ligands include the flavonoids quercetin and kaempferol (Ciolino et al. 1999), the polyether brevetoxin-6 (Washburn et al. 1997), the heme breakdown product bilirubin (Phelan et al. 1998), and the non-aromatic lipoxin A₄ (Schaldach et al. 1999).

Halogenated dimethyl bipyrrroles (HDBPs) are a group of mixed brominated chlorinated aromatic compounds (Gribble et al. 1999; Tittlemier et al. 2001a), somewhat

similar in structure to aromatic organohalogen AhR ligands and CYP1A inducers. There is a paucity of HDBP toxicological data which inhibits the evaluation of their effects on biota.

This study examined the ability of environmentally-relevant HDBP congeners and mixtures to bind to the AhR, induce CYP1A, and cause porphyrin accumulation in chicken embryo hepatocytes. The results obtained provide the first data on the biochemical effects of HDBPs in an *in vitro* assay.

7.2 Experimental section

7.2.1 Halogenated dimethyl bipyrrroles

The HDBP congeners shown in Figure 3.2 were synthesized as described in Gribble et al. (1999) and Tittlemier et al. (2001a). Only three congeners could be isolated to greater than 99% purity: 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrole (DBP-Br₄Cl₂); 1,1'-dimethyl-3,3',4,4',5-pentabromo-5'-chloro-2,2'-bipyrrole (DBP-Br₅Cl); and 1,1'-dimethyl-3,3',4,4',5,5'-hexabromo-2,2'-bipyrrole (DBP-Br₆). The remaining tribrominated congeners were thought to be formed as byproducts in the synthesis of DBP-Br₄Cl₂. The structures of these HDBPs have not been fully characterized, but are proposed to be 1,1'-dimethyl-3,3',4,4'-tribromo-5,5'-trichloro-2,2'-bipyrrole (DBP-Br₃Cl₃a) and 1,1'-dimethyl-3,3',4,4'-tribromo-5,5'-trichloro-2,2'-bipyrrole (DBP-Br₃Cl₃b) based on knowledge of the synthetic mechanisms.

Five stock dosing solutions were made in DMSO, consisting of: (1) DBP-Br₄Cl₂; (2) DBP-Br₅Cl; (3) DBP-Br₆; (4) "Br₃/Br₄ Mix": 49.5% DBP-Br₄Cl₂, 23.2% DBP-

Br₃Cl₃a, and 27.3% DBP-Br₃Cl₃b; (5) "HDBP Mix": 21.9% DBP-Br₃Cl₃a, 25.7% DBP-Br₃Cl₃b, 46.7% DBP-Br₄Cl₂, 3.4 % DBP-Br₅Cl, and 2.3% DBP-Br₆. The HDBP Mix solution was a mixture of HDBPs in similar relative amounts as those observed in bald eagle and seabirds from British Columbia, Canada (Tittlemier et al. 1999a).

7.2.2 Chicken embryo hepatocyte (CEH) assay

The assay outlined in Kennedy et al. (1995) was used to measure the induction of CYP1A and accumulation of porphyrins in chicken embryo hepatocytes dosed with HDBPs. Chicken embryo hepatocyte cultures were prepared in 48-well plates (Kennedy et al. 1993) and dosed with HDBP solutions ranging from 61 nM to 2.02 mM. Four replicate plates were dosed with each of the five HDBP solutions. Two plates were also dosed with TCDD at concentrations of 0.0202 nM to 2.02 µM and used as standards in the assay. One TCDD and two HDBP replicate plates of each solution were incubated for 24 and 48 hours.

The induction of CYP1A was measured as EROD activity. Concentrations of resorufin, the product of EROD reaction, and total protein were monitored by spectrofluorometry using a fluorescence plate reader (Kennedy et al. 1995). Total porphyrin accumulation was measured by spectrofluorometry immediately after the quantitation of resorufin and total protein.

7.3 Results

7.3.1 CYP1A induction

Results from the CEH EROD assays are given in Table 7.1 and Figure 7.1. Listed are the effective doses of TCDD, HDBP congeners, or HDBP mixtures required to produce half the maximum EROD activity (ED_{50}). ED_{50} s were calculated as arithmetic means of the two replicate plates exposed to HDBPs for periods of 24 and 48 hours. Since the CYP1A inducing capabilities of TCDD and the HDBP Mix were evaluated on only one plate each, no mean is given. The ED_{50} s obtained after 48 hours of exposure were all greater than those calculated after 24 hours. There did not appear to be any significant difference in ED_{50} s among the HDBP congeners and mixtures.

Induction equivalency factors (IEFs) were determined to assess the effectiveness of HDBPs to induce CYP1A relative to TCDD. IEFs were calculated as the ratio of $ED_{50,TCDD}$ to $ED_{50,HDBP}$ for a specific exposure period. This method of estimating IEF may overestimate the potencies of the HDBPs since they did not elicit the same maximal response as TCDD. IEFs for exposure periods of 24 and 48 hours are listed in Table 7.1. Unlike the ED_{50} s, the IEFs remained fairly constant over the two exposure periods.

Table 7.1. Effective doses of halogenated dimethyl bipyrroles required to produce half maximum EROD activity (ED₅₀) and induction equivalency factors (IEFs) relative to TCDD after incubation with chick embryo hepatocytes for periods of 24 and 48 hours.

Test Compound / Mixture	ED ₅₀ , 24 hours (nM)	ED ₅₀ , 48 hours (nM)	IEF, 24 hours	IEF, 48 hours
TCDD	0.0302	0.104	1	1
Br ₃ /Br ₄ Mix	22.5	85 ± 70	0.001	0.002 ± 0.001
HDBP Mix	6.67	54	0.005	0.002
DBP-Br ₄ Cl ₂	13.1	105 ± 23	0.002	0.0010 ± 0.0002
DBP-Br ₅ Cl	69 ± 9	203 ± 12	0.00044 ± 0.00006	0.00051 ± 0.00003
DBP-Br ₆	58.5	127 ± 23	0.0005	0.0008 ± 0.0001

7.3.2 Porphyrin accumulation

Figure 7.1 illustrates the accumulation of porphyrins that occurred after CEH cell cultures were exposed to various concentrations of HDBPs for 24 hours. Concentrations of accumulated porphyrins were measured per milligram of protein in each plate well. Figure 7.2 shows the net concentrations of porphyrins accumulated after exposure to the maximum dose of HDBPs for 24 and 48 hours. Net concentrations were calculated as the difference between the porphyrin concentrations in the maximum dose wells and the blank DMSO wells. Minor amounts of porphyrins were accumulated in the blank wells (27 ± 17 pmol/mg protein), and there was no significant difference between porphyrin accumulation in the blank wells between the 24 and 48 hour incubation periods. Arithmetic means $\pm$ standard deviations were determined for the two replicate plates, except for HDBP Mix B, for which there was no replicate. For all HDBPs and mixtures, porphyrin accumulation was greater in the cell cultures which had been exposed for the longer time period.

Figure 7.1 EROD activity (closed triangles) and porphyrin (open squares) accumulation dose-response curves obtained after exposing chicken embryo hepatocytes to halogenated dimethyl bipyrrole (HDBP) congeners and mixtures for 24 hours.

Figure 7.1 (continued)

Figure 7.1 (continued)

Figure 7.2 Gross porphyrin concentrations observed after exposure of chicken embryo hepatocytes to the maximum dose (2.02 mM) HDBPs.

7.4 Discussion

7.4.1 CYP1A induction

Results of the EROD and AhR binding assays suggest that HDBPs are another group of non-classical AhR ligands. All three pure HDBP congeners and two HDBP mixtures induced CYP1A and promoted cytochrome P4501A1 production, measured as EROD activity. Dose-response curves illustrating the HDBP induced EROD activity are shown in Figure 7.1. The ability of the HDBPs to induce CYP1A after 24 hours of exposure was comparable to that of such mono-ortho PCBs as CB-105 ($ED_{50}=3.3$), CB-118 ($ED_{50}=19.1$), and CB-70 ($ED_{50}=37$) (Kennedy et al. 1996). These mono-ortho PCBs and HDBPs had similar IEFs as well.

As noted in previous studies (Kennedy et al. 1995; Lorenzen et al. 1997; Hahn et al. 1993), the EROD activity defined by the dose-response curve decreased at high dose concentrations after reaching a maximum. Proposed reasons for this decrease include cytotoxicity and inhibition of the deethylase reaction by dose compound. Exposure time also affected the HDBP maximal EROD activity. The maximum HDBP induced EROD activity observed decreased and the ED_{50} s increased between the 24 and 48 hour exposure periods. This decrease in EROD activity is opposite to what was observed for CB-77 (Lorenzen et al. 1997) and TCDD using the same assay. It may be possible that a portion of the HDBPs were metabolized over this period, resulting in a deregulation of CYP1A and subsequent decrease in EROD activity. Degradation of the cytochrome P4501A enzyme, or production of another protein not involved in EROD activity but

measured in the total protein analysis may also have caused the decline in EROD activity observed on the dose-response curves.

7.4.2 Porphyrin accumulation

Porphyrin accumulation was observed after dosing cell cultures with all HDBP congeners and mixtures. Significant accumulation of porphyrins only occurred in cells which received the two highest doses (Figure 7.1). The onset of porphyrin accumulation always coincided with the return of EROD activity to basal levels, suggesting that the decrease in EROD activity may be a prerequisite for detectable porphyrin accumulation. The process by which porphyrins accumulate is not clear, and is complicated by the possibility that different mechanisms exist. Work has shown that porphyrin accumulation caused by planar compounds may be regulated by CYP1A and/or the AhR, and that accumulation associated with non-planar PCBs does not seem to involve the induction of CYP1A (Lorenzen et al. 1997).

In contrast to EROD activity, porphyrin accumulation increased as the incubation times increased (Figure 7.2). This is consistent with the apparent correlation with decreased EROD activity observed in the dose-response curves. An increase in accumulation over time was also observed in cells dosed with CB-77 (Lorenzen et al. 1997). The net accumulation of porphyrins in the cells receiving the highest dose (105 to 254 pmol/mg protein) after 48 hours of exposure was somewhat less than that observed for two porphyrinogenic PCB congeners CB-136 (500 pmol/mg protein) and CB-153 (550 pmol/mg protein) (Lorenzen et al. 1997). However, the dose-response curves for these PCB congeners reached a maximum at a dose of approximately 2 mM, whereas the

curves for HDBPs generally did not appear to reach a maximum. Higher doses of HDBPs were not tested due to difficulties in making HDBP solutions at higher concentrations. The lack of maxima on the HDBP dose-response curves does not rule out the possibility that HDBPs cause greater accumulation of porphyrins at higher doses than previously tested PCB congeners.

The net accumulation of porphyrins in the cells exposed to the highest dose for 48 hours significantly increased with the bromine content of the test compound or mixture ($p < 0.029$). There does not seem to be an analogous relationship for porphyrin concentrations after 24 hours of exposure to HDBPs, ED_{50} , or maximum EROD activity values. It is not clear what the ramifications of this trend are on the proposed mechanism and structure activity relationships of porphyrin accumulation. Kawanishi et al. (1978) and Sassa et al. (1986) suggested that PCBs were required to be coplanar and substituted in the meta and para positions to mediate porphyrinogenic activity in cultured CEH cells. HDBPs are fully substituted, and are predicted to adopt non-planar configurations (Mandernach 2001). Their conformation is not expected to vary to a substantial degree with changes in bromine content since the 3/3' positions (those positions where substituents may contribute to steric hindrance and affect coplanarity) remain completely brominated in four of the five congeners studied. A change in HDBP induced porphyrin accumulation with increasing bromine content appears to occur without coplanarity, suggesting that coplanarity may not be the a major factor governing porphyrinogenic activity.

7.5 Conclusions

The results of this work indicate that HDBPs have the ability to affect AhR mediated processes, such as CYP1A induction. The ability of HDBPs to induce CYP1A and porphyrin accumulation has implications on the use of IEFs to calculate TCDD-equivalent (TCDD-EQ) concentrations during risk assessments. Even though the HDBP IEFs were approximately 3 to 4 orders of magnitude less than that of TCDD in the CEH assay, HDBPs may still contribute significantly to the TCDD-EQ if they are present in large enough amounts. This is most likely to occur with biota from the North Pacific Ocean, since observed HDBP levels in marine mammals and seabirds from this area were higher than samples from the Great Lakes, Arctic, and Atlantic environments (Tittlemier et al. 1999a, 2001e).

If HDBPs are proven to have a biogenic origin and are shown to have the ability to bind to the AhR, they can be added to the growing list of naturally-produced AhR ligands. It becomes interesting to consider the implications of HDBP-AhR binding on the existence of the AhR. HDBPs are structurally similar to a known marine bacterial product (Andersen et al. 1974). It has been hypothesized that many halogenated marine natural products are formed for use as chemical defense agents against predators (Field et al. 1995; Kinnel et al. 1977; Paul 1987; Paul et al. 1993; Pennings et al. 1994). The fact that the AhR is present in a wide variety of marine vertebrates (Hahn et al. 1994), mediates metabolizing enzymes, and binds to compounds which may be a feeding deterrent suggests that the AhR may have evolved in part to overcome the chemical defenses of prey organisms.

7.6 Acknowledgment

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CHAPTER 8.

REPRODUCTIVE AND MORPHOLOGICAL EFFECTS OF HALOGENATED DIMETHYL BIPYRROLES ON CAPTIVE AMERICAN KESTRELS (*FALCO SPARVERIUS*)

8.1 Introduction

Many organohalogen compounds bioaccumulate in avian species. Bioaccumulative organohalogen compounds that are routinely monitored (Hebert et al. 1999) are anthropogenic, or biotransformation products of anthropogenic compounds, which are generally resistant to metabolism and excretion. Such compounds include recalcitrant PCB and dioxin congeners, and metabolites of organohalogen compounds such as *p,p'*-DDE.

Certain bioaccumulative anthropogenic organohalogens have been shown to have deleterious effects on the reproductive success of wild birds. One of the most well known instances is egg shell thinning in raptors caused by *p,p'*-DDE, a metabolite of the insecticide *p,p'*-DDT. The effects of *p,p'*-DDE on egg shell thickness were implicated in the population decline of peregrine falcons (*Falco peregrinus*) in the 1950s and 1960s (Peakall 1993).

Other effects of anthropogenic organohalogens on avian reproduction have been demonstrated during studies using captive American kestrels (*F. sparverius*), a common raptor species in North America. In addition to egg shell thinning, adverse effects have

included feminization of male embryos and increases in eggs and hatchlings lost after exposure of breeding kestrels to *o,p'*-dicofol (MacLellan et al. 1996), declines in sperm concentrations in kestrels exposed to mirex and Aroclor 1254 (Bird et al. 1983), and suppression of egg laying, delays in clutch initiation, and reduction in hatching success after exposure to a mixture of Aroclors 1248, 1254, and 1260 (Ferne et al. 2001a, 2001b).

In spite of physical and behavioural similarities to toxic anthropogenic organohalogenes (Tittlemier et al. 2001c), nothing is known about the ability of halogenated dimethyl bipyrroles (HDBPs) to elicit adverse effects in biota. HDBPs have been shown to induce CYP1A and cause porphyrin accumulation in chicken embryo hepatocytes *in vitro* (Chapter 7). However, *in vitro* assays may not fully account for processes which occur *in vivo* and affect toxicity. This study examined the effects of a mixture of HDBP congeners on reproductive and morphological parameters of adult and juvenile captive American kestrels. American kestrels were used as a model species in this study since the highest levels of HDBPs have been observed in bald eagles (*Haliaeetus leucocephalus*), another raptor and high trophic level consumer (Tittlemier et al. 1999a).

8.2 Experimental Section

8.2.1 Birds

Thirty-two pairs of captive-bred American kestrels from the Avian Science and Conservation Centre of McGill University were randomly assigned to four dose groups.

The birds, all experienced breeders, were exposed to either low, medium, or high concentrations of HDBPs, or a corn oil control, in their diet. Canadian Animal Care Guidelines were adhered to throughout the entire experiment.

8.2.2 Dosing

Beginning April 20, 2000, kestrels were fed cockerels dosed with a solution of the four major HDBPs found in biota to give a congener distribution similar to that found in bald eagle liver from British Columbia [unpublished data]. Dose solutions were prepared from synthesized HDBPs as follows (percent of Σ HDBP by weight in parentheses): 1,1'-dimethyl-3,3',4-tribromo-4',5,5'-trichloro-2,2'-bipyrrole (DBP-Br₃Cl₃a, 22.1%), 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrole (DBP-Br₄Cl₂, 46.9%), 1,1'-dimethyl-3,3',4,4',5-pentabromo-5'-chloro-2,2'-bipyrrole (DBP-Br₅Cl, 3.1%), and 1,1'-dimethyl-3,3',4,4',5,5'-hexabromo-2,2'-bipyrrole (DBP-Br₆, 2.0%). There was an additional tribrominated trichlorinated 1,1'-dimethyl-2,2'-bipyrrole (DBP-Br₃Cl₃b, 25.9%) in the dosing solution. This congener was present as an impurity in the synthesized DBP-Br₃Cl₃a, but the precise placement of the halogen atoms is unknown. Crystals of each congener were dissolved in a known mass of diethyl ether. Corn oil was added to this high dose HDBP solution and stirred. The diethyl ether was removed using gentle heating under a N₂ stream. Medium and low dose solutions were made by diluting the initial high dose solution with corn oil. The control solution was prepared using only corn oil and diethyl ether in the same fashion as the high dose solution.

Dose and control solutions (100 μL) were injected intracranially into thawed day-old cockerels. Resulting ΣHDBP concentrations in the kestrel diet were 0 $\mu\text{g/g}$ (control), 0.018 $\mu\text{g/g}$ (low), 0.60 $\mu\text{g/g}$ (medium), and 20 $\mu\text{g/g}$ (high). The medium dose was estimated to reflect the exposure of bald eagles from the Pacific coast of Canada to HDBPs (Tittlemier et al. 1999a). The adult kestrels were fed 1.5 dosed cockerels per day throughout the entire experiment. For the first three weeks after hatching, 1 additional dosed cockerel was included per hatchling. This was increased to 1.25 dosed cockerels per young kestrel after three weeks. Exposure continued until the end of the experiment to mimic the continuous exposure experienced by wild birds. The average exposure period was 95 ± 5 days for adults, and 31 ± 3 days for juveniles.

Kestrels were paired randomly (with the exception that there was no common ancestor for at least six generations) on May 4, 2000, two weeks after dosing began. Pairs were housed in outdoor breeding pens (2.5 x 1.2 x 2.5 m).

8.2.3 Eggs

After pairing, nest boxes were checked daily for eggs, and laying dates noted. The first egg of each clutch was immediately removed, weighed, and its length and width measured. All first-laid eggs were stored at -20°C until further analysis. Subsequent eggs were marked to indicate laying sequence and left to develop normally. Eggs were candled 10 days after laying and classified as either fertile, infertile, or addled.

Collected first-laid eggs were rinsed in water to remove debris and air-dried. Cleaned eggs were scored around the midline, emptied, and their contents homogenized. The contents were placed into chemically cleaned glass jars and stored at -40°C until

HDBP analysis. Five eggshell thickness measurements (eggshell plus inner membrane) were taken using a Mitutoyo digital micrometer at different positions around the circumference of the air cell half of the egg.

8.2.4 Morphology

Adult kestrels were weighed on the first day of dosing, the day of pairing, and the final day of the experiment. Antebrachium (Olendorff 1972), whole body mass, central rectrix, tarsus, ninth primary, and wing cord lengths of 28 day-old juveniles were determined according to established methods (Baldwin et al. 1931).

8.2.5 Kestrel tissue sampling

All birds (n=63 adults, 67 juveniles) were sacrificed over a period of four days at the end of the experiment, approximately 95 days after the first dosing for adults. Birds were weighed and approximately 3 mL of whole blood was taken from the right jugular vein using heparinized disposable needles and syringes. The blood was stored on ice for approximately 30 minutes until it was centrifuged at 5000 rpm for 7 minutes. The plasma fraction was filtered using a serum filter and then placed in cryovials and stored in liquid N₂.

After blood sampling, birds were killed by cervical dislocation and the liver removed and weighed. A vertical slice about 5 mm thick was taken from the longest part of the left lobe for histological analysis. This slice was preserved in 10% buffered

formalin solution and stored at room temperature. The remaining liver was placed in a pre-cleaned glass vial and stored at -40°C until analysis for HDBPs.

The kestrel carcass was placed in a Whirl-Pak bag and stored at -20°C until processing. Prior to HDBP analysis, the beak, feet and primary flight feathers were removed. The trimmed carcass was then homogenized and stored in a pre-cleaned glass jar at -40°C until HDBP analysis.

8.2.6 HDBP analysis

Carcass, liver, and egg samples and aliquots of each dosing corn oil solution were analyzed according to the method and instrumentation outlined in Tittlemier et al. (2001c). Briefly, each sample was mixed with anhydrous Na_2SO_4 , spiked with an internal standard solution of ^{13}C -labelled organochlorines ($^{13}\text{C}_6$ -pentachlorobenzene, $^{13}\text{C}_6$ -hexachlorobenzene, $^{13}\text{C}_{12}$ -CB118, $^{13}\text{C}_{12}$ -CB153, $^{13}\text{C}_{12}$ -CB180, $^{13}\text{C}_{12}$ -CB194), wet packed into a glass column, and extracted using 1:1 dichloromethane/hexane (v/v). Lipids were determined gravimetrically, and removed using GPC. The lipid-free extract was further cleaned by passing through a Florisil column. Volume corrector ($^{13}\text{C}_{12}$ -CB138) was added to all samples prior to analysis. One method blank containing no tissue was run through the same procedure with every seven tissue samples.

Plasma samples were processed using solid phase extraction (SPE) to isolate HDBPs for analysis. An accurate amount of plasma (approximately 0.8 g) was weighed out into a pre-cleaned 50 mL glass centrifuge tube. The sample was spiked with ^{13}C -labelled organochlorine mixture to act as a recovery standard. The plasma solution was mixed well and left to stand for 30 minutes. An equal mass of formic acid was added to

the sample to deproteinate the plasma, mixed well, and left for 20 minutes. HDBPs were isolated on Supelco ENVI-C18 6 mL SPE cartridges. The cartridges were activated using 2 x 6 mL methanol and rinsed with 2 x 6 mL deionized water. After addition of the sample and adsorption of the HDBPs, the cartridges were dried under vacuum with N₂ for 5 hours. A drop of acetone was added at this time to further remove any remaining liquid. The cartridges were air-dried overnight. HDBPs were eluted using 3 x 2 mL 1:1 dichloromethane/hexane. The SPE eluate was further cleaned on a Florisil column (1.2 cm id., 8 g Florisil, 1.2% deactivated) and eluted with 110 mL 1:1 dichloromethane/hexane. The Florisil eluate was reduced in volume on a rotary evaporator, and spiked with volume corrector (¹³C₁₂-CB138) prior to analysis. One blank of distilled water was processed alongside the plasma samples. Total plasma lipids were determined from an archived plasma aliquot using the colourimetric sulpho-phospho-vanillin reaction method (Frings et al. 1972). Standards for the plasma lipid determination were made up using olive oil.

HDBPs were analyzed using gas chromatography – electron capture negative ionization mass spectrometry in the selected ion monitoring mode. Instrumentation consisted of a Hewlett Packard 5987B mass spectrometer coupled via a 5988 direct interface to a 5890 Series II gas chromatograph (GC). The GC was equipped with a 30 m x 0.25 mm i.d. DB5-MS column with a film thickness of 0.25 µm. Methane (99.97%) was used as the moderating gas. Congener specific quantitation was performed using external standards of the five synthesized HDBPs.

8.2.7 Statistical analyses

Proc GLM (SAS v8.0) was used to test for differences in HDBP residue concentration, egg parameters, HDBP congener contribution and some morphological measurements within and among dose groups. HDBP concentrations were log transformed to satisfy the assumptions of homoscedasticity and normality; all other variables did not require transformation.

A principal components analysis (PCA, SAS v8.0) was performed on five morphometric measurements (antebrachium, tarsus, central rectrice, 9th primary, and wing cord lengths) of the juveniles to obtain a surrogate for body size. An ANOVA was then performed on the factor scores to examine differences between dose groups and sex.

Body conditions of the juveniles were estimated as the residuals of a linear regression of Day 28 mass and the PCA factor which described the most variation in size. Body conditions were normalized by calculating body condition as the ratio of the residual from the linear regression and Day 28 mass. The relationship between normalized body condition and dose of HDBP administered was examined using an ANOVA.

8.3 Results

8.3.1 HDBP residues

Total concentrations of the five HDBP congeners (Σ HDBPs) in the kestrel tissues are listed in Table 8.1. As expected, Σ HDBP concentrations in tissues from the various dose groups were significantly different from each other. The mean ratio of Σ HDBP

concentrations in carcasses was 1:39:826 (low:medium:high dose groups), close to that of the dosing solutions, 1:33:1111. The nominal daily intakes of HDBPs for adults, based on the dosing solution concentrations and 1.5 cockerels supplied per day as feed, were calculated to be 0.0027 µg/day, 0.09 µg/day, and 3 µg/day, for the low, medium, and high dose groups respectively. The effective doses received per day for adults were estimated as the mean total mass of HDBPs accumulated in carcasses divided by the average number of days the birds were dosed. The effective doses were estimated to be 0.002 and 0.004 µg/day, 0.07 µg/day, and 1 µg/day, for the female and male low dose groups, medium, and high dose groups, respectively.

Table 8.1. Geometric mean $\pm$ standard error (n) Σ HDBP concentrations (ng/g, lipid wt) in kestrel tissues after approximately 95 days of dosing.

An asterisk denotes value is significantly different ($p < 0.05$) from control.

Tissue	Control	Low dose	Medium dose	High dose
egg	30 $\pm$ 10 (15)	9 $\pm$ 5* (14)	250 $\pm$ 60* (12)	12 000 $\pm$ 2 000* (17)
liver	na ^a	na	na	7 000 $\pm$ 1 000 (7)
plasma	na	na	na	14 000 $\pm$ 3 000 (7)
carcass	5 $\pm$ 3 (15)	17 $\pm$ 5* female (8) 40 $\pm$ 20* male (8)	900 $\pm$ 80* (16)	17 000 $\pm$ 2 000* (32)

^ana = not analyzed

Some samples from the egg and carcass control groups contained HDBPs, probably due to cross contamination during the feeding study, since HDBPs were not found in three undosed cockerels analyzed. Carcass controls contained significantly lower amounts of HDBPs than the low dose samples, indicating that there was low HDBP contamination in the control diet. Mean Σ HDBP levels in the egg control group were four times higher than control group carcass. Since egg concentrations were consistently lower than carcass concentrations in dose groups, this is strong indication that the control egg samples were cross-contaminated during handling or processing. Even if the Σ HDBP levels in the egg control group were real, they were 10 and 467 times lower than those found in medium and high dose samples, which would not compromise interpretation of effects found in these groups.

In the low dose group, female carcasses contained lower Σ HDBP concentrations than males (2-way ANOVA, $p < 0.03$). There were no other significant effects of age or sex on Σ HDBPs in the kestrel carcasses, thus data from male and female juveniles and adults from the other dose groups were combined.

8.3.2 Egg parameters

Egg parameters that were monitored are listed in Table 8.2. Of the physical egg parameters measured, only egg shell thickness means significantly varied among dose groups ($p < 0.02$). The high dose egg shells were thicker than those from the other three groups. Masses of the first-laid eggs were not significantly different among dose groups. There were also no significant differences in the volumes of all eggs laid, estimated using the following equation from Hoyt (1979).

$$\text{volume} = \text{width}^2 \times \text{length} \times 0.51 \quad (1)$$

The total number of eggs laid in each clutch (clutch size) and the percentage of fertile eggs in each clutch were not significantly different among dose groups. Only one pair of kestrels, in the control dose group, did not produce any eggs. Aside from the medium dose group, each group had one pair which produced a clutch with no fertile eggs.

Table 8.2. Kestrel egg measurements and clutch parameters.

Mean $\pm$ standard deviation (n). An asterisk denotes value is significantly different ($p < 0.05$) from control.

Parameter	Control	Low Dose	Medium Dose	High Dose
egg mass (g)	15 $\pm$ 2 (7)	15 $\pm$ 1 (8)	15.0 $\pm$ 0.6 (8)	15 $\pm$ 1 (8)
egg volume (mm ³)	14 000 $\pm$ 1000 (38)	14 600 $\pm$ 900 (39)	14 000 $\pm$ 1000 (43)	14 400 $\pm$ 900 (42)
egg shell thickness (mm)	0.17 $\pm$ 0.02 (10)	0.17 $\pm$ 0.01 (9)	0.17 $\pm$ 0.01 (9)	0.19 $\pm$ 0.01* (14)
clutch size	5 $\pm$ 2 (8)	5.3 $\pm$ 0.7 (8)	5.5 $\pm$ 0.8 (8)	5.4 $\pm$ 0.5 (8)
% fertile eggs in clutch	80 $\pm$ 40 (8)	60 $\pm$ 40 (8)	80 $\pm$ 30 (8)	60 $\pm$ 40 (8)
% eggs hatched in dose group	65	94	77	93
laying lag of first egg (days)	12 $\pm$ 3 (7)	12 $\pm$ 3 (8)	15 $\pm$ 6* (8)	15 $\pm$ 8* (8)
time taken to complete clutch (days)	9.0 $\pm$ 0.8 (7)	10 $\pm$ 1 (8)	8 $\pm$ 1 (8)	10 $\pm$ 3 (8)

The only other parameter to show a significant difference among dose groups was the number of days from pairing of the kestrels until the laying of the initial clutch egg (designated as “laying lag”). The laying lag was significantly higher in the medium and high dose groups than the low and control groups. There was no associated effect on the time taken to complete a clutch, measured as the number of days between laying the initial and final eggs of the clutch.

8.3.3 Morphology

Relationships among dose groups and the morphological measurements taken for the juveniles on Day 28 associated with size were studied using PCA. The five physical measurements – antebrachium, tarsus, central retrace, 9th primary, and wing cord lengths – were reduced to two factors. The first factor explained 52% of the data variation, and was significantly correlated with central retrace, 9th primary, and wing cord lengths – variables all associated with feather growth. Factor 2 accounted for 25% of the variability and was significantly correlated with antebrachium and tarsus lengths. A 2-way ANOVA using dose and sex as independent variables was performed on the factor scores, and a significant difference in factor 2 among the dose groups was found ($p<0.04$). The control group factor 2 mean was higher than the low dose group (Figure 8.1). The variation of factor 2 with dose did not follow a dose-dependent trend since the control group was not significantly different from the high and medium groups. The whole body mass at Day 28 was larger for females than for males in all groups, but statistically significant only in the control group ($p<0.04$). However, there was no

significant effect of dose on Day 28 mass ($p=0.3$). In addition, there was no significant effect of dose on the normalized body condition of juveniles ($p=0.5$).

Figure 8.1 Variation of PCA factor 2 (positively correlated with antebrachium and tarsus lengths of juvenile kestrels) with dose groups.

Asterisk denotes a significant difference ($p<0.05$) from control group.

As opposed to the juveniles, only whole body mass measurements were monitored for adult kestrels. There were no significant differences in adult kestrel whole body masses, taken at any of the three time points (initial, pairing, final), among dose groups. Females and males were analyzed separately due to mass differences associated with sex. Figure 8.2 illustrates the distinction between the final adult male and female

kestrel whole body masses. Females were significantly larger than males in the control ($p<0.03$), low ($p<0.02$), and medium ($p<0.03$) dose groups. This sexual dimorphism was however not significant in the high ($p=0.26$) dose group.

Figure 8.2 Variation of adult male and female kestrel final whole body masses with dose groups.

P-values given refer to differences between female and male masses within a dose group.

Within a dose group there were often differences in adult whole body masses as the experiment progressed (Figure 8.3). Whole body mass tended to decrease from the beginning to the end of the study in the high dose males and females ($p < 0.001$, $p < 0.004$), medium dose males and females ($p < 0.03$, $p < 0.03$), and low dose males ($p < 0.03$). The decrease was not significant in the male and female control groups ($p = 0.6$, $p = 0.1$) plus the female low dose group ($p = 0.3$).

8.3.4 HDBP congener patterns

Figure 8.4 compares the fraction of each HDBP congener in the dosing solution to the fractions observed in the eggs, liver, plasma, and carcasses of the high dose group. The general trend that was observed was the depletion of the lower and enrichment of the higher molecular weight congeners compared to the dosing solution.

Congener patterns in carcasses from the different dose groups were fairly consistent. Only the fraction of DBP-Br₃Cl₃b in the low dose group was significantly lower than in the dosing solution and other groups. This congener was also depleted relative to the dosing solution in the low dose eggs. DBP-Br₄Cl₂ was found to be enriched in the low dose eggs, and DBP-Br₆ enriched in the medium and high dose eggs when congener fractions were compared between dose groups and the original dosing solution.

Figure 8.3 Change in adult kestrel whole body masses during the feeding experiment.

P-values given refer to differences among masses over time within a dose group. Significant differences are indicated by unique letter designations.

Figure 8.4 HDBP kestrel congener fractions in analyzed tissues from the high dose group.

Horizontal lines refer to the congener fraction in the dosing solution. Asterisks denote a significant difference ($p < 0.05$) from the dosing solution.

8.4 Discussion

8.4.1 Bioaccumulation of HDBPs

HDBPs were detected in all tissues at all dose levels, illustrating that these compounds are bioaccumulative and undergo maternal transfer to eggs. Since the nominal daily intakes were similar to the estimated effective daily doses calculated from body burdens measured at the end of the experiment, it appears that the HDBPs are absorbed efficiently, and slowly excreted from the kestrel. The greatest discrepancy – approximately a three-fold difference – occurred between the nominal daily intake and effective dose of the group receiving food containing the highest concentration of HDBPs. This difference may indicate decreased absorption efficiency, increased clearance, overestimate of the actual dose, or a combination of these processes occurring in the high dose group.

Concentrations measured in the three dose groups were comparable to the range observed in avian samples from the Pacific, Atlantic, Great Lakes, and eastern Arctic regions of Canada. The major HDBP congener, DBP-Br₄Cl₂, was found in seabird eggs at levels ranging from 0.61 to 140 ng/g wet weight (Tittlemier et al. 1999a). This corresponds to egg Σ HDBP concentrations of approximately 19 to 4400 ng/g lipid weight, using an average lipid content of 8% and assuming DBP-Br₄Cl₂ comprises about 40% of total HDBPs. Carcass Σ HDBP concentrations, estimated from liver concentration data (Tittlemier et al. 2001c), ranged from 8 to 160 ng/g lipid weight in seabird livers from the eastern Canadian Arctic. It is clear that the resulting Σ HDBP concentrations observed in this experiment are within an environmentally relevant range.

Even though HDBPs were present in the control samples, Σ HDBP concentrations were significantly different than those of the medium and high dose groups. These differences should thus not obscure any possible dose-dependant effects elicited by the HDBPs.

8.4.2 Reproductive and morphological effects of exposure to HDBPs

There were few significant observed dose-dependent effects of HDBPs on the morphology and reproduction of kestrels. The only detected effects concerned with reproduction – an increase in laying lag and egg shell thickness – were somewhat similar to effects observed after exposure to anthropogenic organohalogens. A larger increase in laying lag (6, as opposed to 3 days) has been observed in another study dosing kestrels with technical mixtures of PCBs at a similar concentration (Ferne et al. 2001a). Changes in kestrel egg shell thickness after exposure to 20 $\mu\text{g/g}$ *o,p'*-dicofol (MacLellan et al. 1996) have also been reported. However, these changes were decreases, as opposed to increases, in thickness.

It is unlikely that these effects associated with exposure to HDBPs would have a serious negative impact on kestrel reproductive success. The slight increase in laying lag did not result in smaller clutch sizes, as found in the past (Perrins 1996). In addition, most adverse consequences from changes in egg shell thickness would be restricted to decreases in thickness and shell strength. An increase in egg shell thickness would increase the shell strength and reduce premature breakage and embryo death. There is the possibility that an increase in egg shell thickness could impede hatching. However, evidence of this was not observed, since there was no correlation between percent of hatched eggs and egg shell thickness. The lack of deleterious effects of HDBPs on

overall kestrel reproductive success also leads to the conclusion that mating behaviour was not affected.

Once hatched, the juvenile kestrels' growth appeared to be marginally affected by HDBPs. The birds receiving HDBP contaminated food in the low dose group were significantly smaller than those in the control group. Depression of growth has been noted in other cases as a result of exposure to organohalogenes. Great blue heron (*Ardea herodias*) chicks exhibited decreased body weight and skeletal growth with increased exposure to TCDD (Hart et al. 1991). However, there does not seem to be a dose-dependent relationship between growth and HDBP exposure since the size of the control birds most closely resembled those in the high dose group. If the alteration of growth observed is a real effect, it appears to be the most pronounced at low doses.

Exposure to HDBPs did not seem to have an effect on adult whole body mass. There were no differences between different dose groups in the initial, pairing, or final masses of the birds. However, as opposed to between dose groups, there were some changes in body mass associated with HDBP exposure within groups. HDBP exposure appeared to result in lower female body masses and therefore diminished differences between the masses of males and females in the high dose group. It also magnified the decrease in whole body mass over the course of the experiment in the groups receiving higher doses of HDBPs.

The changes in masses that occurred were only minor, since no significant absolute differences were observed among dose groups. However, if decreased body mass in adults results in less energy reserves for feeding and rearing behaviour, development and growth of the young may be affected. The subtle changes in mass may

also affect mating opportunities. Female lesser kestrels (*F. tinnunculus*), a species related to the American kestrel which also exhibits reversed sexual size dimorphism, showed a preference for lighter males with shorter tarsi (Hakkarainen et al. 1996).

8.4.3 Maternal transfer of HDBPs

Maternal transfer of organohalogen contaminants via deposition of lipids to eggs is of interest, due to the purported sensitivity of embryos to such compounds (Barron et al. 1995). Ratios of lipid normalized Σ HDBP concentrations in eggs to carcasses suggest that HDBPs are transferred to a similar extent as recalcitrant anthropogenic organochlorine compounds. These ratios are only estimates though, since carcass Σ HDBP concentrations reflect HDBP levels at the time of sacrifice, rather than the time of egg laying. Lipid normalized individual egg/carcass ratios averaged 0.59 ± 0.31 and varied over a wide range of values (0.004 to 1.7). The average Σ HDBP lipid normalized egg/carcass ratio is similar to that obtained for total PCBs, polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) for herring gulls (0.60 ± 0.11) (Braune and Norstrom 1989). A ratio in the range of 0.3-0.7 is expected for altricial and semi-precocial species, such as American kestrels, which invest low amounts of maternal lipids in yolk development (Drouillard and Norstrom 2000). The majority of egg lipids will therefore originate directly from the female's diet.

The egg HDBP congener pattern shown in Figure 8.4 is somewhat depleted in the highly brominated congeners compared to other tissues. A similar phenomenon was observed for the accumulation of PCDDs in herring gull eggs (Braune and Norstrom 1989). The egg/carcass lipid concentrations of the octachlorinated dibenzodioxin, which

has physical properties similar to that of DBP-Br₆ (Tittlemier et al. 2001d) were lower than those of tetrachlorinated dibenzodioxins and other organochlorine compounds in herring gulls. Relative concentrations of most organochlorines in herring gull egg lipid were more similar to those in liver than carcass. There is a tendency for this to be the case for HDBP accumulation in kestrel eggs (Figure 8.4), but the relationship is not significant. The carcass HDBP congener pattern may not be representative of the maternal congener pattern at the time of egg laying since the adult females consumed dosed food for approximately 60 days after egg laying. Changes in the congener pattern due to metabolism or differential elimination might have occurred during the 60 days of exposure after laying, yet prior to sacrificing, especially if the adult females had not reached equilibrium with the food at the point of egg laying.

8.4.4 Metabolism of HDBPs

It is unlikely that extensive metabolism of HDBPs occurred during the experiment since the nominal daily intakes of HDBPs were generally similar to the estimated effective doses of HDBPs received. HDBPs have similar biomagnification factors as recalcitrant PCB congeners in an Arctic food web including various species of seabirds (Tittlemier et al. 2001c), indicating they are likely recalcitrant themselves. In herring gulls, susceptibility of PCBs to metabolism is related to the availability of adjacent unsubstituted positions. Those congeners lacking adjacent open positions underwent little or no metabolism (Braune and Norstrom 1989). HDBPs do not appear structurally vulnerable to metabolism, since there are no unsubstituted positions on the aromatic rings, and the nitrogen is protected by a methyl group.

The variations in congener patterns between the dose solution and tissues are likely due to differential excretion of congeners in feces. Fecal excretion is a significant mechanism in the elimination of compounds resistant to metabolism (Barron et al. 1995; Drouillard et al. 2001). It has also been shown that larger molecules with large octanol/water partition coefficients ($\log K_{ow} > 6.5$) demonstrate greater diffusion resistance across the boundary layers of the intestinal wall to feces (Moser and McLachlan 1999). This resistance results in a decrease in fecal excretion efficiency, and a subsequently greater accumulation of the larger molecules. Such a scenario is observed in the kestrel carcass, liver, and plasma tissues. The larger DBP-Br₅Cl and DBP-Br₆ are enriched, and the smaller tri- and tetrabrominated congeners are generally depleted, relative to the dose solution.

8.5 Conclusions

Captive American kestrels exposed to a dietary source of HDBPs accumulated these compounds in carcass tissue, plasma, and liver. HDBPs were also transferred to yolks during egg development. Alterations in the HDBP congener patterns observed in the various tissues were presumed to be a result of a decrease in the fecal excretion of the larger HDBP congeners as opposed to metabolism.

The exposure and subsequent accumulation of HDBPs did not result in any acute effects in the kestrel study population. The reproductive success of the kestrels as measured by the percent of fertile and hatched eggs within each clutch was also not affected, even though egg shell thickness and length of time taken to initiate egg laying appeared to be influenced by exposure to HDBPs. In addition, normal changes in kestrel

body mass over the course of the experiment only seemed to be slightly amplified by HDBP exposure. No extreme changes in variables associated with size were observed. The lack of serious effects in this study suggests that HDBPs are not likely to affect raptor populations.

8.6 Acknowledgment

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CHAPTER 9.

GENERAL CONCLUSIONS

9.1 Summary

Research described in this thesis has demonstrated that the group of unknown halogenated compounds, initially observed in 1988 are halogenated dimethyl bipyrroles (HDBPs). The fundamental physical properties of HDBPs – aqueous solubilities, liquid sub-cooled vapour pressures, Henry's Law constants, octanol/water partition coefficients – indicate that HDBPs have a low mobility. HDBPs are predicted by a simple Level I equilibrium distribution model to be associated mainly with soil and sediments. In addition, as bromine atoms are substituted for chlorine atoms aqueous solubilities and liquid sub-cooled vapour pressures decrease, which may result in reduced mobility.

HDBPs were found to be globally distributed in marine mammal blubber and present in seabird eggs from the Atlantic and Pacific coasts of Canada. Highest concentrations were observed in biota from Pacific as opposed to Atlantic-influenced environments. In some instances concentrations of total HDBPs were at levels comparable to ubiquitous anthropogenic organohalogens such as chlordanes. The distribution of HDBPs did not resemble the distribution of the recalcitrant PCB congener CB-153. CB-153 was at the highest concentrations in samples obtained from areas near industrialized areas such as the St. Lawrence River, and the Mediterranean, Baltic, and

Wadden Seas. HDBP concentrations in samples from these areas were among the lowest observed.

Examination of an Arctic marine food web determined that HDBPs biomagnify to a similar extent as recalcitrant anthropogenic compounds such as CB-153. However, ringed seals appeared to be able to metabolize HDBPs.

HDBPs displayed some dioxin-like activity in a chicken embryo hepatocyte *in vitro* bioassay. Their ability to induce the production of metabolizing enzyme cytochrome P450 1A was comparable to PCB congeners containing one chlorine atom occupying an ortho position. However, a mixture of HDBPs did not elicit any negative effects on reproductive or morphological endpoints in American kestrels exposed through their diet.

9.2 Evaluation of the hypothesis

The results of this research confirm that HDBPs are widely distributed compounds which biomagnify similarly to persistent PCBs such as CB-153 in a marine food web, including seabirds. HDBPs also appeared to bioaccumulate without significant metabolism in the American kestrel. HDBPs may not be as persistent as CB-153 in pinnipeds, since evidence from the Arctic marine food web indicates that ringed seals have the ability to metabolize HDBPs. HDBPs are transferred from kestrel females to eggs. It is highly probable that transfer also occurs from beluga female to calf via lactation, based on the large differences in Σ HDBP concentrations observed in males and females.

The limited toxicological studies performed do not implicate HDBPs as toxic compounds. HDBPs exhibited neither acute nor chronic toxicity in the kestrel feeding study. However, HDBPs had the ability to induce CYP1A in chicken hepatocytes. Even though the induction of CYP 1A has been correlated to dioxin-like effects, this is not a clear endpoint of toxicity. Furthermore, the chicken is hypersensitive among bird species for AhR mediated toxicity, and therefore a poor model for assessment of risk to wild bird species. It must be kept in mind that since an organism's susceptibility to toxic effects vary with sex, age, and species, the results of the two studies performed cannot be directly applied to evaluate the effects of HDBPs in other systems.

No research was performed to directly assess the hypothesis that HDBPs are naturally-produced. However, a few strong pieces of circumstantial evidence were uncovered which indicated that HDBPs may have a natural origin. The first was a structural resemblance between HDBPs and the marine chromobacterial product hexabromo-2,2'-bipyrrole. The second piece was an absence of any reference to the chemical formulae or structures of the four environmentally relevant HDBPs in the chemical literature. The final piece of evidence is the difference in geographical distribution of HDBPs and the anthropogenic CB-153. The highest concentrations of HDBPs were found in biota sampled from Pacific Ocean influenced environments. HDBP concentrations, as opposed to CB-153, were much lower in samples obtained from industrialized areas such as the Great Lakes, Baltic, Wadden, and Mediterranean Seas. These results all lend support to a biogenic origin of HDBPs.

9.3 Implications of research

There are two general implications of the research outlined in this thesis. The main conclusion is that there are other POPs present in the environment other than commonly analyzed PCBs, chlordanes, dioxins, et cetera. Sometimes these novel POPs behave in a similar fashion as persistent bioaccumulative anthropogenic organohalogenes, and are present in biota at significant concentrations. The novel POPs may also elicit effects which can obscure or confound the analysis of common analytes, for example, in EROD bioassays.

The second main conclusion is that organohalogen POPs are not all necessarily anthropogenic in origin. As described in the introduction, this is contrary to some beliefs which hold that organohalogenes are unnatural. This result also has implications on proceedings concerned with the regulation of production and release of organohalogenes into the environment. In addition to industrial and other anthropogenic sources, biogenic sources must be considered as well when compounds display toxicological activity.

9.4 Future work

One of the most interesting paths of future research on HDBPs is the investigation into their origins. It is clear that confirmation of a biogenic source of HDBPs will have implications on organohalogen research. Work has begun on using radiocarbon (^{14}C) to determine whether or not HDBPs are naturally-produced. This research will examine the amount of ^{14}C isotope present in DBP- Br_4Cl_2 extracted from biota using accelerator mass spectrometry (Eglinton et al. 1996), and effectively date the DBP- Br_4Cl_2 . Values showing an input of recent carbon to the synthesis of DBP- Br_4Cl_2 may indicate a

biosynthetic process, whereas an absence of ^{14}C would indicate a very old carbon source, such as petrochemicals, and likely anthropogenic synthesis.

Another avenue of research into determining the origin of HDBPs would be to examine temporal trends of HDBP concentrations in biota or other environmental compartments, such as sediment. A trend mirroring the industrial output of anthropogenic organohalogens such as PCBs would suggest an anthropogenic source. On the other hand, no apparent correlation between HDBP concentrations and industrial outputs of organohalogens over time would suggest a biogenic source. No matter which path is taken, they would both address one of the most interesting aspects of HDBPs remaining to be examined.

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