ABSTRACT

Title of Thesis:	BIOMONITORING ORGANOCHLORINE COMPOUNDS USING BALD EAGLES (HALIAEETUS LEUCOCEPHALUS) IN VOYAGEUR'S NATIONAL PARK 2011-2017 AND DEVELOPING NEW BIOMONITORING TECHNIQUES
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Wildlife are used to monitor the presence and persistence of legacy organochlorine contaminants in the environment. In this study, bald eagles (*Haliaeetus leucocephalus*) were utilized as an indicator of exposure to organochlorine compounds at Voyageur's National Park, Minnesota from 2011-2017. This demonstrated decreasing concentration trends and a lack of recent inputs of organochlorine compounds. However, the use of organochlorine compounds continues in other parts of the world. Therefore, a technique for using solid phase extraction to quantify organochlorine analytes in avian plasma was developed in order to facilitate international biomonitoring of these compounds. Using this method, organochlorine compounds are extracted from plasma and stored within extraction cartridges during transport from collection site to analysis site. This has important implications for international wildlife

biomonitoring. If organochlorine analytes are separated from their matrix at the site of collection, sensitive or hazardous biological materials do not need to be transported or stored.

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by

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2018

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Preface

This thesis was organized into four chapters. The first chapter was written as a literature review of topics discussed in the following three chapters and repetition among all four chapters may be present. Chapters two and three are intended for publication and are comprised of an introduction, methods, results, discussion, and conclusion. Tables and figures directly follow the chapter in which they are referenced. Chapter four is a brief synopsis of the overall findings of this research. Formatting of this thesis follows the University of Maryland Electronic Thesis and Dissertation (ETD) Style Guide.

Dedication

I would like to dedicate this thesis to my dog, Kipper Jeffurry Allan Eberius. He is a

very good boy.

Acknowledgements

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Chapter 1: A Review of Legacy Organochlorine Contaminants and Biomonitoring using Bald Eagles (Haliaeetus Leucocephalus) as Biosentinels

1: Sources, fate, and effects of organochlorine compounds

Organochlorine (OC) compounds, also known as chlorinated hydrocarbons, are a broad class of synthetic molecules which includes both OC pesticides and polychlorinated biphenyls (PCBs). The only structural commonality among all OC compounds is the presence of one or more chlorine atoms attached to a hydrocarbon molecule (Kamrin 1997). Chlorine is one of the most abundant elements occurring in nature, and many chemical products contain chlorine atoms or chlorine is utilized for their production (Beyer and Biziuk 2009). Organochlorine compounds became widely popular in the mid-twentieth century and were used for flame retardation, electrical stability, insulation, hydraulic lubricants and insect pest control (Hutzinger et al. 1974; Loganathan and Kannan 1994; Giesy and Kannan 1998). Concern for the human and environmental health impacts of chlorinated compounds emerged when OCs were found to resist degradation and elimination, be able to bioaccumulate and biomagnify, and cause toxicity to non-target organisms (National Academy of Science 1971; Loganathan and Kannan 1994; Metcalfe and Haffner 1995). While in use, OC compounds were released into the environment through spills, direct spraying, agricultural runoff, and during manufacturing (Loganathan and Kannan 1994; Karami-Mohajeri and Abdollahi 2011). It has since been demonstrated that the main mode of transport of OC compounds is through the atmosphere (Jayaraj et al. 2016) which helps explain the global distribution of OCs (Loganathan and Kannan 1994). Despite being removed from use in much of the developed world in the 1970s, OC compounds

continue to persist in the environment and in biota today, demonstrating their long-lasting effects (Loganathan and Kannan 1994; Beyer and Biziuk 2009).

The environmental fate of a chemical is dependent on compound-specific properties including biochemical structure, stability, historic location and volume of use, and mode of release into the environment (Beyer and Biziuk 2009; Jayaraj et al. 2016). From the 1940s to 1970s OC compounds were released rapidly at a rate much greater than they were degraded or removed, resulting in accumulations of OCs in the environment (Loganathan and Kannan 1994). Once released, compounds will settle into an environmental compartment: air, water, soil or sediment, or biota and each compound has unique properties that are predictive of its environmental fate (Howard and Meylan 1997). Relative environmental partitioning of specific compounds is commonly estimated using the compound's log transformed octanol-water partitioning coefficient (log K_{ow}) which captures the ratio of a compound dissolved in the octanol and water phases of a two-phase system (Lyman et al. 1982; Howard and Meylan 1997). Organochlorine chemicals tend to be hydrophobic, having higher log K_{ow} values than hydrophilic molecules, which indicates that they are more likely to partition into non-polar compartments, sorb to soils, and bioconcentrate and less likely to be soluble in aqueous or hydrophilic compartments (Karickhoff et al. 1979; Howard and Meylan 1997). Thus, concentrations of hydrophobic OC compounds dissolved in water can be orders of magnitude less than concentrations in soil, sediment, suspended particulate matter, or biological tissues. Other physical-chemical properties that determine environmental fate of a compound are water solubility, boiling point, dissociation constant, Henry's Law constant, hydrolysis rate, biodegradation and bioconcentration

(Howard and Meylan 1997). Each of these properties are distinct for individual compounds but are often generalized for chemical classes (such as the 209 PCB congeners) (Howard and Meylan 1997).

Heavy usage of OC compounds in industrial and agricultural areas resulted in direct discharge into neighboring bodies of water from terrestrial runoff (Loganathan and Kannan 1994). Water clearance in large bodies of water is much slower than in rivers, which causes extended residence time of historic chemicals (Loganathan and Kannan 1994). Non-target aquatic organisms can be exposed to OC compounds from the water column, their diet, and direct contact with contaminants in the sediment (Metcalfe and Haffner 1995; Schäfer et al. 2015; Costa et al. 2008). However, lipophilic compounds are more likely to biomagnify and be spread through the food web than hydrophilic compounds, which can explain the differences in total body burden at different trophic levels (Metcalfe and Haffner 1995).

The fate of each xenobiotic compound dictates the range of effects that the substance can elicit. The tendency for OCs to accumulate in aquatic systems and biomagnify in biota has important implications on aquatic food webs and tertiary predators.

1.1: Organochlorine pesticides

Organochlorine pesticides were introduced in the 1940s and used heavily through the early 1970s for agricultural pest management and vector-borne disease control in the United States (Costa et al. 2008). The original Stockholm Convention on Persistent Organic Pollutants identified nine OC pesticides as persistent organic pollutants (POPs). The treaty designated POPs for either global elimination (8 of 9

designated OC pesticides) or restriction (1 of 9 OC pesticide) (Stockholm Convention 2013). Some OC pesticides, such as well-known Dichlorodiphenyltrichloroethane (DDT), continue to be used in developing nations, especially those in the Southern Hemisphere, to control vector populations (Mowbray 1988; Goldberg 1991; Stockholm Convention 2013). Aldrin, Chlordane, Dieldrin, Endrin, Heptachlor, Hexachlorobenzene, Mirex, and Toxaphene are other OC pesticides classified as POPs (Stockholm Convention 2013). These compounds were used for agricultural seed treatments, direct crop treatment, commercial and private termite control, and wood treatment and additives (Karami-Mohajeri and Abdollahi 2011; Jayaraj et al. 2016). However, it has been demonstrated that the majority (99.7%) of agriculturally applied OC pesticides are released into the surrounding environment, while only 0.3% elicit effects on the target pest (Pimentel 1995). Furthermore, OC pesticides used today in the warm southern hemisphere can rapidly volatilize and are selectively deposited in colder climates (Gregor and Gummer 1989; Loganathan and Kannan 1994).

Organochlorine pesticides exhibit a wide range of chemical structures. Despite their structural dissimilarities, OC pesticides bear many common toxic effects (Karami-Mohajeri and Abdollahi 2011; Jayaraj et al. 2016). Most pesticides are designed to disrupt physiological pathways of a target organism, with the aim of reducing fitness and reproductive success of the pest (Jayaraj et al. 2016; Costa et al. 2008). Acting on pathways that are heavily conserved, OC pesticides often lack target selectivity and frequently cause adverse effects to non-target organisms (Costa et al. 2008; Jayaraj et al. 2016). OC pesticides are known to cause, among other things, neurological damage, alteration of ion channel function and firing, cancer, decreased body weight, reduced

fertility, and developmental deformities in non-target organisms (Costa et al. 2008; Karami-Mohajeri and Abdollahi 2011; Jayaraj et al. 2016).

The stability and metabolism of OC pesticides are important determinants of their environmental fate and effects. OC pesticide half-lives range from one day to fifteen years (Jayaraj et al. 2016). Some OC pesticides degrade biotically or abiotically into daughter compounds called metabolites, with biochemical properties that are distinct from the parent compound (Aguilar 1984; Hitch and Day 1992; Muñoz-Arnanz and Jiménez 2011). Relative concentrations of parent compounds to metabolites have been used to estimate the time since the release of the parent compound (Tavares and Costa 1999; Harner et al. 1999; Qiu et al. 2004; Holoubek et al. 2009; Muñoz-Arnanz and Jiménez 2011). Decreasing ratios of parent compound to metabolite can indicate more recent environmental exposure or emerging sources of contamination (Holoubek et al. 2009; Muñoz-Arnanz and Jiménez 2011). In some cases, metabolites are more persistent and harmful than the parent compound. For example, the detrimental egg-shell thinning effects that resulted from widespread use of DDT are attributed to one of its metabolites, dichlorodiphenyldichloroethylene (DDE) (Wiemeyer et al. 1984; Bowerman et al. 1998; Donaldson et al. 1999). Dichlorodiphenyldichloroethylene is of additional concern because of its long half-life (Loganathan and Kannan 1994). Thus, the metabolism and degradation of OC pesticides into novel compounds has profound effects on the distribution and consequences of OCs in the environment.

Together, the substantial release, atmospheric transport, bioaccumulative tendency, and potential for adverse effects of OC pesticides makes the use of these compounds of global concern.

1.2: Polychlorinated biphenyls

Polychlorinated biphenyls were manufactured in the United States from 1929 to 1979 and used as industrial coolants, flame retardants, and plasticizers before being banned by the U.S. Environmental Protection Agency (EPA) (Hutzinger et al. 1974; U.S. EPA 1979). Unlike OC pesticides, all PCBs share a common organic structural backbone (Hutzinger et al. 1974). Polychlorinated biphenyls are synthesized by chlorinating interconnected benzene rings, or biphenyl, and the product contains a mixture of compounds with varying degrees of chlorination on the biphenyl core (Mullin et al 1984; Giesy and Kannan 1998). There are 209 PCB congeners, or discrete structures, each with unique chlorine substitutions (Loganathan and Kannan 1994; Giesy and Kannan 1998; Beyer and Biziuk 2009). Of the 209 possible PCB structures, only 130 appear in commercial PCB products and mixtures in proportions ≥0.05% (Giesy and Kannan 1998). The stability and resonance of the delocalized biphenyl structure gives PCBs high thermal capacity and low electrical conductivity, which are ideal qualities for many industrial materials (Hutzinger et al. 1974; Loganathan and Kannan 1994; Giesy and Kannan 1998; Beyer and Biziuk 2009). However, these qualities also cause PCBs to persist in the environment and resist degradation (Giesy and Kannan 1998; Beyer and Biziuk 2009). Differences in chlorine substitution ultimately result in variability among PCB appearance, toxicity, and stability (Hutzinger et al. 1974; Giesy and Kannan 1998; Beyer and Biziuk 2009). In general, highly chlorinated PCBs tend to be less water soluble, less bio-degradable, more lipophilic, and more rapidly accumulated (Loganathan and Kannan 1994; Metcalfe and Haffner 1995). A small subset of PCB congeners, known as dioxin-like PCBs, are

known to elicit toxic effects on biota (Metcalfe and Haffner 1995; Giesy and Kannan 1998). Dioxin-like, or coplanar, PCBs have chlorine substitutions on the biphenyl ring at the meta- and para- positions or have only one ortho- substitution, which give them structural resemblance to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Metcalfe and Haffner 1995; Giesy and Kannan 1998). Polychlorinated biphenyls with no ortho-substitutions are considered non-ortho PCBs and PCBs with one ortho substitution are considered mono-ortho PCBs (Metcalfe and Haffner 1995). Biota exposed to non- and mono-ortho PCBs can experience endocrine disruption, dermal lesions, weight loss, enzyme induction and inhibition, decreased reproductive health, immune dysfunction and suppression, and teratogenicity (Metcalfe and Haffner 1995; Giesy and Kannan 1998). These responses are known to be mediated largely by binding of a cellular protein known as the aryl hydrocarbon receptor (AhR) (Metcalfe and Haffner 1995; Giesy and Kannan 1998). This receptor protein is partially conserved among vertebrates, and species-specific sensitivity can be characterized based on protein structure (Whitlock 1990; Metcalfe and Haffner 1995). Because of these adverse effects on biota, PCBs were also identified as POPs by the Stockholm Convention in 2001 (Stockholm Convention 2013).

Although PCBs are introduced into the environment as mixtures of many congeners, each congener within a mixture will not have an identical environmental fate (McFarland and Clarke 1989). Microbes are able to de-chlorinate (biodegrade) PCBs, but the rate of metabolism is insignificant and variable based on number and orientation of chlorine substitutions (Bopp 1986; Quenses III et al. 1988; Borja et al. 2005). The most important determinant of PCB persistence in the environment is the

orientation of chlorine atoms around the biphenyl moiety (Beyer and Biziuk 2009). Non-ortho substituted congeners are less readily degraded than other PCBs (Tanabe et al. 1987). The relative congener contributions to total PCB concentration may change as the mixture moves through environmental compartments or if some PCB congeners are selectively bioaccumulated by biota (Beyer and Biziuk 2009). The dynamic, long-lasting, and detrimental impacts to the biota of PCBs parallel those of OC pesticides, and emphasizes the importance of monitoring and mediating OC contaminants in the environment.

2: Biomonitoring organochlorines in bald eagles (Haliaeetus leucocephalus)

Populations of bald eagles (*Haliaeetus leucocephalus*) have been used as a sentinel of environmental contamination throughout the species' geographic range and the effects of OC compounds on bald eagles are well documented (Cromartie et al. 1975; Anthony et al. 1993; 2007; Donaldson et al. 1999; Bowerman et al. 2002; Dykstra et al. 2001; 2005). Three decades of bald eagle biomonitoring efforts have identified long-term spatial and temporal trends of OC compounds in the Great Lakes using nestling plasma collected for the state of Michigan's Bald Eagle Biosentinel Program since 1986 (Bowerman et al. 1995; 1998; 2003; 2005; Weirda et al. 2014). Reference concentrations have been monitored simultaneously using nestlings in Voyageur's National Park (VNP), Minnesota, USA since 1989 (Bowerman 1993; Pittman et al. 2015). The Great Lakes were heavily polluted with legacy OC compounds because of the high volume of agriculture and industrialization surrounding the freshwater system. As a result, the Great Lakes are the most frequently studied bodies of freshwater for OC contamination (Loganathan and Kannan 1994).

Bald eagles were identified as an ideal indicator of environmental contamination and health of the subsequent food web in the Great Lakes region (International Joint Commission 1991). Their life history has been well documented, thus their maturation, dietary patterns, and responses to numerous stressors are well defined (Wiemeyer et al. 1984; Elliot et al. 1995; Bowerman et al. 2002; Cesh et al. 2008). As an apex predator of the aquatic food web, bald eagles consume a diet comprised primarily of fish (Buehler 2000), which puts individuals at an elevated risk of exposure to POPs, due to the tendency of these compounds to bioaccumulate and biomagnify in aquatic food webs (Bowerman et al. 1995). Additionally, bald eagles prey on turtles, gulls and waterfowl (Clark 1982; Donaldson et al. 1999) and will opportunistically hunt terrestrial mammals and reptiles (Ewins and Andress 1995; Mabie et al. 1995; Marr et al. 1995). However, it is unlikely that terrestrial prey significantly contributes to total OC body burden (Donaldson et al. 1999). Therefore, concentrations of organochlorine compounds recovered from bald eagle plasma are a reflection of the organism's dietary exposure to these compounds from the aquatic food web (Anthony et al. 1993; Bowerman et al. 2003).

Bald eagle populations were critically endangered in the mid-twentieth century and the decline has been attributed to the adverse effects of legacy halogenated compounds (Postupalsky 1978; Grier 1982; Bowerman et al. 1998; Watts et al. 2008; Baldwin et al. 2012). Bald eagle productivity is heavily influenced by human disturbance to nestlings and nesting pairs, habitat availability and quality, and anthropogenic contaminant loads (Grier 1982; Hansen 1987; Dykstra et al. 2001; Bowerman et al. 1995; Grim and Kallemeyn 1995; Grubb et al. 2002; Cruz et al. 2017).

Most notably, the egg-shell thinning effects of the DDT-metabolite DDE have been cited as root causes of the decline (Wiemeyer et al. 1984; Bowerman et al. 1998; Donaldson et al. 1999). Organochlorine compounds are associated with a variety of other adverse effects in bald eagles including endocrine disruption, edema, teratogenesis, and neurotoxicity (Wiemeyer et al. 1984; Gilbertson et al. 1991; Bowerman et al. 2000). Unable to produce viable eggs or young, populations fell precipitously and bald eagles were categorized as endangered in 1967 (Wiemeyer et al. 1984; Anthony et al. 1993; Best et al. 1994; Bowerman et al. 1995; 1998; 2000). Following the ban of OC pesticides and PCBs in the United States, bald eagle populations began to recover (Bowerman et al. 1998), and the species was removed from the endangered species list in 2007. Bald eagle population recovery was closely and continuously monitored by the state of Michigan from 1961 to 2017 (Best et al. 1994; Bowerman et al. 1995; unpublished data). Similar population monitoring programs were employed in other areas such as VNP in Minnesota (Grim and Kallemeyn 1995), Everglades National Park, Florida (Baldwin et al. 2012), the Chesapeake Bay (Watts et al. 2008), and the Aleutian Islands (Anthony et al. 1999). In 1961 there were just 34 successful fledged young from 21 mating pairs in Michigan (Michigan Department of Natural Resources unpublished data). Fifty-six years later, in 2017 there were 843 fledged young from 550 successful breeding territories (Michigan Department of Natural Resources unpublished data). Bald eagle population monitoring in Michigan is unique because the comprehensive productivity monitoring has been complemented with OC residue quantification in nestlings since 1986. Together, these data were used to identify effect level concentrations and estimate contaminant level

targets. The no observable adverse effect concentrations (NOAEC) of the 4,4'-DDE enantiomer and total PCBs and for bald eagle productivity were identified by Bowerman et al. (11.4 and 36.4 µg kg^{-1} respectively) (2003). These effect limits are useful for assessing potential population level effects, but limitations must be recognized as they were developed using field collected (rather than laboratory controlled) data.

The ability to collect accurate population counts and localized contaminant measurements makes the use of the bald eagles as a sentinel of environmental contamination highly informative. Bald eagles build large nests in super-canopy trees that are easily visible from aerial observation (Fraser et al. 1983; Wood et al. 1989; Bowerman et al. 2002; Steenhof and Newton 2007; Baldwin et al. 2012). Because of their nesting behavior, population level reproductive success can be counted, rather than estimated by an index (Fraser et al. 1983; Bowerman et al. 2002; Baldwin et al. 2012). Bald eagle nestlings are flightless and dependent on adults for food for until they are approximately 12 to 14 weeks of age (Gerrard and Bortolotti 1988), and adults forage for food within a home range of approximately 4.9 km^2 to feed the nestlings (Watson 2002). Therefore, compounds accumulated within nestling tissues are indicative of contamination in the environment proximate to the nest site (Anthony et al. 1993; Bowerman et al. 2003).

Biomonitoring OC contamination in of the Great Lakes has indicated that OC levels are declining both in fish and bald eagles (Amant et al. 1984; Baumann and Whittle 1988; MacKay and Gilbertson 1991; Carlson et al. 2010; Weirda et al. 2016). Collectively, it has been demonstrated that PCBs have been gradually but consistently

declining since the 1970s, while DDT concentrations initially declined rapidly in the 1980s and have been slow to decline since (Loganathan and Kannan 1994; Kannan et al. 2005; Carlson et al. 2010; Weirda et al. 2016). Specifically, PCBs and 4,4'-DDE declined significantly in bald eagle nestlings in Michigan from 1987 to 2008 (Weirda et al. 2016) and in lake trout from 1970-2003 (Carlson et al. 2010). Despite these declines, residues in Michigan bald eagles remain elevated compared to reference levels in VNP; the lowest DDE levels reported in eagles nesting on Lake Erie (2004-2008 geometric mean 46 μg kg^{-1}) were more than four times greater than those reported in VNP (Weirda et al. 2016; Pittman et al. 2015). During this time period, more than 30% of eagle nestlings in Michigan had DDE and PCB residues at levels greater than the NOAECs for bald eagle productivity (Weirda et al. 2016). Additionally, these low concentrations of OCs can cause sub-lethal and adverse effects on wildlife at the individual and population levels (Best et al. 1994; Bowerman et al. 1995; 2003; Dykstra et al. 2001; 2005; Elliot et al. 1995; Elliott and Norstrom 1998). Therefore, despite the decreasing trends of OC compounds in the Great Lakes and elsewhere, consistent monitoring of legacy OC compounds is invaluable for characterizing and identifying areas of concern and potential risk.

3: Monitoring Organochlorine Concentrations in the Environment

Hydrophobic OC compounds accumulate in fatty tissues such as brain, liver, subcutaneous fat, and plasma of biota. Long-term or chronic exposure is best quantified in fat stores and liver tissue where these compounds tend to bioaccumulate (Kumar et al. 2002). Recent dietary exposure can be estimated by quantifying residue concentrations in plasma (Bowerman et al. 2002; Kumar et al. 2002). Blood sample

collection is preferable for biomonitoring because it is less invasive and can be done without sacrificing study subjects. Contaminant loads and body burden are dependent on a species' diet, home range, life history, and position within the food web. Characterizing exposure within different niches, trophic levels, and spatial regions provides more insight into the way contaminants persist in ecosystems and partition to environmental compartments. Due to the inherent variability associated with environmental samples (Hebert et al., 1997, Elliott and Norstrom, 1998, Morrissey et al. 2004; Elliot et al. 2009), long term monitoring is necessary to differentiate between trends and minor fluctuations.

Tracking the occurrence of POPs on a global scale provides a more complete understanding of the fate of legacy and emerging compounds. However, biomonitoring internationally can be complicated and slow. Transporting sensitive biological samples long distances can threaten the quality of samples, and bringing materials across international borders can be hindered by international travel and transport requirements. Furthermore, traveling with chemical extracts that are dissolved fluid is not only hazardous due to the flammability of liquids, but is prohibited by air-travel safety restrictions. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an agreement among 183 governments that was organized in 1973 to protect and monitor the trade of products and specimen from wild animals and plants. The international trade of over 35,000 wild animal and plant species is protected and regulated by CITES. While member parties (countries) are required to implement CITES designated regulations, these regulations do not replace national laws (CITES 1983). Furthermore, states and regional organizations adhere to

regulations within the agreement voluntarily (CITES 1983). Therefore, national laws and enforced regulations implemented by each nation may vary slightly among member parties. These differences can result in complications and hardships associated with transporting research samples across international borders.

Modern solid phase extraction (SPE) methods for separating OC compounds from plasma are fast, effective, and require minute volumes of blood (Sundberg et al. 2006). Solid phase extraction is a technique for separating specific compounds dissolved in a liquid from other compounds in a mixture based on physical and biochemical differences among the molecules. Oasis® hydrophilic-lipophilic balance (HLB) SPE cartridges contain a stable and versatile polymer filter designed for SPE of a variety of compounds of all pHs. The polymer is a combination of one hydrophilic and one lipophilic monomer (divinylbenzene-N-vinylpyrrolidone and divinylbenzene respectively) (Waters Corporation 2014). The use of Oasis HLB cartridges has been described in methods for SPE of a wide range of target molecules including OC pesticides, PCBs, cadmium, lead, and pharmaceutical compounds (Sundberg et al. 2006; Doušaa et al. 2006; Buchberger 2007; Anthemidis et al. 2011). Hydrophobic analytes from various biological media sorb to the HLB polymer with 3-fold greater capacity than traditional silica based sorbents (Dias and Poole 2002; Water's Corporation, 2014). Due to the small volume of sample necessary, the total quantity of OC compounds in the cartridges after extraction is very small. The small volume of sample and strong retention of hydrophobic OC compounds create an opportunity to optimize this extraction method to advance international biomonitoring in avian species. It is unknown however, for what length of time OC analytes can remain stable

within Oasis HLB SPE cartridges while maintaining analytical validity. If OC analytes could be separated from their matrix at the site of sample collection, sensitive or hazardous biological materials would not need to be transported or stored. If analytes could be separated from their biological material at the location at which they were collected, international researchers would not need to travel with sensitive, hazardous, or CITES restricted specimens, or chemical extracts in flammable liquids.

4: Project Overview and Objectives

4.1: Investigating SPE Parameters for Optimizing Field Collection of Avian Plasma for Contaminant Analysis

The length of time that chemical analytes can be stored within Oasis HLB SPE cartridges, prior to elution and chromatographic quantification, without losing analytically validity had not been explored prior to this study. Previous studies have evaluated the sorption capabilities of Oasis® HLB cartridges relative to other polymer sorbents, and the passive sampling capabilities of these extraction cartridges in aquatic environments (Dias and Poole 2002; Mazzella et al. 2010). Although this extraction sorbent is commonly used for SPE, an empirical analysis of the stability of OC compounds sorbed to the polymer filter over time is unknown. Previous research has suggested that OC compounds may be stable when stored in Oasis® HLB SPE cartridges (Sundberg et al. 2006). However, this conclusion was based on a limited number of storage intervals (14 and 21 days of storage only), number of analytes (11), and sample size (n=1 per treatment). The goal of this study was to fill the gap in our understanding of the capabilities of this extraction tool in order to build the foundation of new methods for field extraction and transport of contaminants from plasma

samples. If analytes are extracted from internationally-collected plasma samples at the site of sample collection, investigators can more easily transport the analytes back to their labs for chemical analysis, and can minimize travel-related complications. This would hasten the analysis process and allow for expedited response to environmental concerns.

The objectives of this study were to: (1) determine the length of time OC compounds extracted from avian plasma can be stored in Oasis® HLB SPE cartridges without statistically significant analytical loss, (2) assess the precision and accuracy of measurements of OC compounds stored in Oasis® HLB SPE cartridges, and (3) validate the method with a field simulation. It was hypothesized that if analyte retention in Oasis® HLB SPE cartridges is inversely related to storage interval length, then as storage interval length increases analyte retention will decrease. By extracting chemical analytes from their biological matrix and transporting analytes within an extraction sorbent, international researchers would no longer need to travel with sensitive samples, CITES restricted material, or chemical extracts in flammable liquids. Understanding the stability of OC compounds within Oasis HLB SPE cartridges will help to build the foundation of new practices for extraction and transport of chemical analytes in plasma samples.

4.2: Spatial Temporal Monitoring of Organochlorines in Bald Eagles (*Haliaeetus leucocephalus*) of Voyageur's National Park

Bald eagles have been used as bioindicators of environmental contaminants in VNP since 1989. The eagle population in VNP is stable and the concentrations of many POPs have decreased. Within the park, total PCBs and DDTs have decreased from

1995-2010, park-wide significant decreasing trends have been detected (Pittman et al. 2015). Dieldrin concentrations were detected in 61.1% of 203 nestlings and increased 50.25% during the time interval (Pittman et al. 2015), but the cause of this increase is unknown. In the current study, OC compounds were measured in bald eagle nestling plasma collected from 2011 to 2017 in order to better understand POPs trends over time and across the VNP spatial region.

The objective of this study was to assess long-term trends of legacy organochlorine residue concentrations in bald eagle nestling tissues collected within VNP, Minnesota, USA and the immediate area from 2011 to 2017. The goals of this research were to determine if emerging sources of Dieldrin are in the VNP area, if legacy OC contaminants have significantly decreased, and if OC compounds are a result or historic or recent use. The data collected for this study will be used in combination with previously collected and reported data to evaluate changes in OC levels in an ongoing longitudinal monitoring program.

Chapter 2: Investigating Solid Phase Extraction Parameters for Optimizing Field Collection of Avian Plasma for Contaminant Analysis

1: Introduction

Anthropogenic activities have generated a seemingly infinite number of non-naturally-occurring compounds. When xenobiotic chemicals or synthetic analogues of natural compounds are introduced into environmental systems, there is a potential for adverse effects on biota. The field of ecotoxicology is dedicated to characterizing and quantifying these effects. However, before toxicological endpoints can be addressed and assessed, the nature and extent of a xenobiotic compound's existence and persistence in the environment must be understood. Therefore, monitoring the concentrations of anthropogenic chemicals in the environment is a crucial starting point for environmental and ecological risk assessment.

A historically important class of environmental contaminants are organochlorine (OC) compounds. Hydrophobic OC chemicals accumulate in fatty tissues such as liver, brain, subcutaneous fat, and plasma of biota. Long-term or chronic exposure to OCs is best quantified in fat stores and liver tissue where these compounds tend to bioaccumulate (Kumar et al. 2002), while recent dietary exposure can best be estimated by quantifying residue concentrations in plasma (Bowerman et al. 2002; Kumar et al. 2002). Blood sample collection is preferable to other tissues for biomonitoring because it is less invasive and can be done without sacrificing study subjects. Exposure is dependent on a species' diet, home range, life history, and trophic level. Characterizing exposure within different niches and spatial regions provides more insight to the way compounds persist in ecosystems and within environmental

compartments. Monitoring OC residues in wildlife has helped to define and identify the spatial and temporal trends of these compounds and areas that are of concern for human and environmental health (Bowerman et al. 2002; Roe et al. 2003). Due to the inherent variability associated with environmental samples (Hebert et al. 1997; Elliott and Norstrom, 1998, Morrissey et al. 2004; Elliot et al. 2009), long term monitoring is crucial for differentiating between trends and minor fluctuations (Bowerman et al. 2003). Additionally, ongoing monitoring is necessary because of the rapid development of new compounds and the emergence of new pathways of exposure of legacy contaminant compounds, such as long-range atmospheric transport and deposition.

Investigating the distribution of persistent organic compounds globally provides a more complete understanding of the fate of legacy and emerging compounds. However, collecting and transporting biological material internationally can be complicated and slow. Traveling with sensitive biological samples long distances can threaten the quality of samples, and bringing materials across international borders can be hindered by international travel and transport requirements. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an agreement among 183 governments that was organized by a subset of these governments in 1973 to protect and monitor the trade of products and specimen from wild animals and plants. The international trade of over 35,000 wild animal and plant species is protected and regulated by CITES. While member parties (countries) are required to implement CITES designated regulations, these regulations do not replace national laws (CITES 1983). Furthermore, states and regional organizations adhere to regulations within the agreement voluntarily (CITES 1983).

Therefore, national laws and enforced regulations implemented by each nation may vary among member parties. Research material are not exempt from these requirements, and inconsistences in enforcement between member nations can result in complications when transporting research samples transnationally. Together, these factors can create hardships for researchers collecting plant and animal samples internationally.

Solid phase extraction (SPE) is a technique for separating specific compounds dissolved in a liquid from other compounds in a mixture based on physical and biochemical differences among the molecules, and can be used to separate OCs from blood. Oasis® hydrophilic-lipophilic balance (HLB) SPE cartridges contain a stable and versatile polymer sorbent designed for SPE of a variety of compounds of all pHs. The polymer is a combination of one hydrophilic and one lipophilic monomer (divinylbenzene-N-vinylpyrrolidone and divinylbenzene, respectively) (Waters Corporation 2014). The use of Oasis HLB cartridges has been described in methods for SPE of a wide range of target molecules including OC pesticides, PCBs, cadmium, lead, and pharmaceutical compounds (Sundberg et al. 2006; Doušaa et al. 2006; Buchberger 2007; Anthemidis et al. 2011). Hydrophobic analytes from various biological media sorb to the HLB polymer with 3-fold greater capacity than traditional silica based sorbents (Dias and Poole 2002; Water's Corporation 2014). Modern SPE of OC pesticides and PCBs from avian plasma require minimal volumes (approximately 100 μL) of plasma (Sundberg et al. 2006). Due to the small volume of sample necessary, the total quantity of OC compounds in the cartridges after extraction is very small. Organochlorine compounds bind tightly to the sorbent while biological

material passes through and can be disposed of as biological waste. The small volume of sample and strong retention of hydrophobic OC compounds create an opportunity to further optimize this extraction method to advance international biomonitoring in avian species.

Previous research has suggested that OC compounds may be stable when stored in Oasis® HLB SPE cartridges (Sundberg et al. 2006). However, this conclusion was based on a limited number of storage intervals (14 and 21 days of storage only), number of analytes (11), and sample size (n=1 per treatment). The present study investigates a technique for extracting OC compounds from avian plasma that modifies SPE methods previously reported (Sundberg et al. 2006). This technique was designed to aid international toxicological monitoring of environmental pollutants. The objectives of this study were to determine the stability, precision, and accuracy of measurements of OC compounds (Table 1) in avian plasma when analytes are stored in Oasis® HLB SPE cartridges for an extended period of time, rather than being eluted immediately after extraction for quantitative analysis (Figure 1). This was accomplished through a preliminary method development analysis using lab-manipulated plasma, and validated in a field simulation using field-collected plasma. It was hypothesized that if analyte retention in Oasis® HLB SPE cartridges is inversely related to storage interval length, then as storage interval increases analyte retention will decrease.

If analytes could be separated from their biological material at the location at which they were collected, international researchers would not need to travel with sensitive or hazardous CITES restricted specimens or chemical extracts in flammable liquids. Understanding the long-term stability of OC compounds within Oasis HLB

SPE cartridges will help build the foundation of new practices for extraction and transport of chemical analytes in plasma samples.

2: Methods

2.1: Sample Preparation and Extraction

Pooled, mixed-gender domestic chicken (*Gallus domesticus)* plasma in sodium heparin was obtained from BioChemed Services (Winchester, VA). Chicken plasma was selected because individuals lack exposure to environmental sources of OC contaminants. Thus, any compounds extracted from the chicken plasma are assumed to originate from laboratory manipulation. Samples were prepared for extraction following methods described by Sundberg et al. (2006). In short, frozen plasma was thawed, placed in sterile glass test tubes in 100-μL aliquots, and treated with 400 μL of 8M urea to desorb analytes from the matrix and denature and dilute plasma proteins (Sundberg et al. 2006). A surrogate standard solution with known concentrations was added to all plasma samples to confirm extraction efficiency. A standard solution with known concentrations of PCBs and OC pesticides was added to experimental samples as described in section 2.2. A schematic of the general extraction and quantification procedures are depicted in Figure 1.

Organochlorine compounds were extracted from plasma following SPE described by Sundberg et al. (2006). Oasis® HLB SPE cartridges were conditioned for extraction using 4 mL aliquots of methanol and equilibrated with 4 mL aliquots of nanopure water (Waters Corporation 2014). Samples were loaded onto SPE cartridges using a 12-point vacuum manifold with glass serum pipettes. Glass test tubes were rinsed with 3 mL of nanopure water and the rinse was loaded onto the filter to ensure

complete transfer of the sample. Extraction cartridges were rinsed with 3 mL of nanopure water for matrix clean-up after sample loading.

2.2: Study Design

Due to apparatus restrictions, samples were run in batches of 24. Within each run set, a standard solution with known concentrations of PCBs and OC pesticides was added to 20 of the denatured chicken plasma samples (positive control; "spikes"). The remaining four samples in each batch were used as blanks (negative controls).

The organization of a batch is illustrated in Figure 2. In each run-set, two spikes and one blank served as positive and negative controls, respectively, by allowing no storage-time treatment to confirm extraction efficiency of the PCB and OC pesticide standard solution and inter-batch homogeneity. The remaining 18 spiked samples were experimental units. They were divided into three groups of six samples, and each group was paired with one of the three remaining blanks (henceforth referred to as "treatment spikes" and "treatment blanks" respectively). Each of the experimental groups ("treatment groups") were randomly assigned one of the nine storage-time treatments and served as one of three repetition groups of a storage-time treatment (for a total of 18 treatment spikes and three treatment blanks per storage-time treatment) with the exception of the 14-day treatment for which one treatment group was destroyed (leaving only 12 treatment spikes and two treatment blanks for this storage-time treatment) (Table 2). To capture potential inter-batch variation, treatment groups were distributed randomly among the batches so that the means of the groups within a treatment could be assessed for variability. Nine batches were generated for a total of 26 treatment groups, 26 treatment blanks, and 156 treatment spikes (excluding the

destroyed treatment group) (Table 3). Additionally, there were a total of 18 untreated control spikes and nine untreated control blanks (Table 3).

2.3: Storage-Interval Treatment

After extraction, loaded SPE cartridges were collected, labeled, wrapped in aluminum foil, and sorted by treatment group in plastic bags. Cartridges were stored at room temperature with a LogTag TRIX-8 Temperature Logger to confirm temperature range during the storage interval. Samples were stored for 0, 1, 3, 7, 14, 21, 28, 42, 63, or 84 days (Table 2) in order to simulate potential time intervals between field collection and lab extraction.

2.4: Elution and Chemical Quantification

After each sample's predetermined storage-time treatment elapsed, chemicals were eluted from the SPE cartridges into sterile glass test tubes using 2 mL volumes of pesticide grade dichloromethane. The extracts were dried using a low flow of nitrogen gas through a sample concentrator. An internal standard solution of known concentration was added to each extract prior to chemical analysis to verify analyte concentrations.

Extracts were reconstituted in hexane and pipetted into 2-mL amber screw-top gas chromatograph (GC) vials with glass conical inserts. A vehicle blank and a calibration solution (hexane with known volumes of PCB and OC pesticide standard solution, internal standard, and surrogate solution) were analyzed after each batch in an Aligent Technologies 7890A GC with electron capture detection (ECD) to identify potential sample carry over, contamination, and to assure proper calibration.

2.5: Analyte Residue Quantification

PCB and OC pesticide chemical standards were obtained for 51 compounds (22 PCBs, 24 OC pesticides, 2 compounds used as internal standards, and 3 compounds used as surrogate standards) from Ultra Scientific (North Kingstown, RI) and Accustandard (New Haven, CT) (Table 1).

A calibration curve was generated for each analyte and compound-specific method detection limits and limits of quantitation were calculated. Twenty-two PCB congeners and 24 OC pesticides were quantitatively analyzed in each sample (Table 1) using an Agilent 7890 GC/ECD with split injection and duel columns, following U. S. Environmental Protection Agency (EPA) Methods 8081 and 8082 (2007a; 2007b).

Dual column analysis was used to confirm all results; Extracts were analyzed simultaneously using two different copper columns and the results from each column must agree within 40% for results to be reportable. Percent recovery of standards in the untreated control spikes and blanks were required to average between 70 and 130% of the nominal concentration of analytes as a quality assurance and quality control (QAQC) acceptance criteria as suggested by EPA method 8000 (2018). If control spikes and blanks of a given batch did not meet the EPA QAQC standard, the batch was repeated. Statistical analysis was only performed for OC compounds that were recovered in greater than 50% of samples at quantities greater than the method detection limit (MDL).

2.6: Data Analysis

Extraction efficiency was evaluated for each batch by confirming that untreated control residues were within the QAQC guidelines. In order to standardize GC-

quantified residue concentrations in each experimental sample, concentration values were converted to percent recovery using two methods: (1) calculating the percent recovered from the theoretical concentration in the standard solution ("nominal percent recovery")

$$\frac{Sample\ residue\ concentration}{standard\ solution\ concentration}$$

(2) calculating the recovery as a percent of the residue concentrations in the untreated controls of each run ("run-normalized percent recovery").

$$\frac{Sample\ residue\ concentration}{Untreated\ control\ residue\ concentration}$$

Nominal recovery provides insight to what was recovered based on the theoretical (nominal) dose of the standard solutions. Run-normalized recovery provides insight on whether quantified concentrations varied from those of samples eluted and analyzed the day of extraction (untreated controls). Thus, method two accounted for differences due to storage interval treatment and standardized values based run-specific variability.

2.7: Analysis Criteria for Assessment of Storage Capabilities

Due to the large suite of analytes used, analyses were done by grouping similar compounds. Group recovery was calculated for $\sum$DDTs, $\sum$OC pesticides, and $\sum$PCBs. Three proxies were used to assess stability of OC compounds in Oasis® HLB SPE cartridges through time. Each criteria assessment was repeated for each compound group. Together, these results were assessed for each storage time point in a successive-chronological order to determine the latest time point where analytical validity is preserved. This defined the "successful" range of storage times. Statistical analyses were conducted in Statistical Analysis System (SAS) software, version 9.4 (SAS

Institute Inc., Cary, NC). A power analysis was conducted using the observed within- and between-group variance with 10 groups of n=18 and a 0.05 significance level. A second power analysis was conducted with the group n=12 to account for the 14-day treatment group with only 12 samples.

2.7.1 Analysis of Variance of Percent Recovery

Normality of each recovery response variable ($\sum$PCBs, $\sum$DDTs, and $\sum$OC pesticides) was assessed using the Shapiro-Wilk test. Homogeneity of variance among storage-time treatments was tested using Levene's test for homogeneity of squared deviations from group means. Analysis of variance of both nominal and run-normalized percent recovery were assessed using a general linear model with treatment, treatment group, and their interaction as independent variables and percent recovery as the dependent variable (percent recovery = treatment + treatment group + treatment*treatment group). Repetition group was included to determine if mean recovery varied among samples of the same treatment that were extracted in different batches. If batch, treatment group, and their interaction were not significant predictors of recovery (i.e., there was no significant difference between the treatment group means within a specific storage-time treatment), then batch, treatment group, and the interaction were removed from the model and all samples of a given treatment were assessed as one group. If variables (treatment repetition, storage-time treatment or their interaction) were significant predictors of recovery in the analysis of variance, post hoc pair-wise comparisons were made to assess differences of least square mean recovery using the Tukey-Kramer adjustment to establish if mean recovery was significantly lower than that of the untreated controls.

2.7.2 Agreement with QAQC Guidelines

The lower limit of the 95% confidence interval of the mean recovery at each storage-time point was compared to the lower permissible level (70%) of the EPA suggested QAQC standard for OC quantification using GC/ECD. This was used to determine the size of the difference detected, rather than just statistical significance (Gardner and Altman 1986). If the 95% confidence interval for the mean percent recovery of a storage-time point fell outside of the 70-130% QAQC range, that storage treatment was considered not to comply with EPA QAQC standards.

2.7.3 Agreement with lab-specific precision

The range of the 95% confidence interval of the mean of each time point was compared to that of the untreated control spikes to determine if the measurements in the treated samples were similar in precision to untreated measurements. While the 70-130% QAQC range is a good indicator of overall extraction performance, it does not necessarily represent the precision of measurements in our laboratory using our instrumentation and procedures. The EPA suggested 70-130% QAQC guideline is only recommended as an interim and conservative acceptance criterion and suggests that in-house limits are developed (Environmental Protection Agency 2018). Therefore, the range of the confidence interval of the untreated spiked samples, or the natural variation of the data, was used to a provide another perspective for analysis and to account for the laboratory specific conditions.

2.8: Field Extraction Simulation

Based on the findings of the method development using chicken plasma, this storage-treatment technique was validated using field-collected bald eagle (*Haliaeetus*

leucocephalus) plasma. Plasma was collected and preserved for analysis of OCs by the University of Maryland's (UMD) Joint Analytical Services Laboratory under methods approved by the UMD Institutional Animal Care and Use Committee Protocols 1154158-1 and 744587-1. Experimental methods used for method development with fortified chicken plasma were repeated using bald eagle plasma with minor modifications (Sundberg et al. 2006). Rather than spiking plasma samples before extraction, we instead assessed recovery based on the authentic concentrations within the wild-collected plasma samples (non-storage treated residue data). The storage time treatments analyzed were 1, 3, 7, 14, 21, and 28 days. Ten analyte-loaded extraction cartridges were used for each storage interval. Sample replicates at each time point were derived from two different bald eagle samples from contaminated areas. For accuracy assessment, the 95% confidence interval of the mean bald eagle recovery at each storage-time point was compared to the lower permissible level (70%) of the EPA suggested QA standard for OC quantification using GC/ECD. For precision analysis, mean bald eagle recovery at each storage-time point was analyzed for variance from the untreated bald eagle samples. These data were used to validate the extraction technique.

3: Results

Based on the intergroup and within-group variance observed, the power (or probability) to reject a false null hypothesis with n=18 for each group is 1. The power to reject a false null hypothesis with n=12 for each group is 0.995.

3.1: Assessment of Storage Capabilities

3.1.1 ∑DDTs

Run-normalized ∑DDTs percent recovery were normally distributed and had homogeneity of variance among groups (p=0.1191 and 0.1710, respectively). Treatment was a significant predictor of percent recovery (p<0.0001) but treatment group was not (p=0.6066) so all units of each treatment were pooled as one group. Though there were significant differences among the means of storage time points, no time points had a mean ∑DDTs recovery that was significantly lower than the untreated control spikes (Table 4). The lower limit of the 95% confidence interval of the mean of run-normalized ∑DDTs was not lower than the lower permissible level of the QAQC guidelines (70%) at any time point (Figure 3), and the range of the 95% confidence intervals of the means of each time point were similar to the 95% confidence interval of untreated controls (Table 4). Nominal recovery of total DDTs ranged from 45.5% to 118.4%.

3.1.2 ∑OC Pesticides

Run-normalized ∑OC pesticides percent recovery were normally distributed and had homogeneity of variance among groups (p=0.6062 and 0.3395 respectively). Treatment was a significant predictor of percent recovery (p<0.0001) but treatment group was not (p=0.1041) so all units of each treatment were pooled as one group. Though there were significant differences among the means of storage time points, no time points had a mean ∑OC pesticides recovery that was significantly lower than the untreated control spikes (Table 4). The lower limit of the 95% confidence interval of the mean of run-normalized ∑ OC pesticides was not lower than the lower permissible

level of the QAQC guidelines (70%) at any time point (Figure 3), and the range of the 95% confidence intervals of the means of each time point were similar to the 95% confidence interval of untreated controls (Table 4). Nominal recovery of total OC pesticides ranged from 53.3% to 116.7%.

3.1.3 ∑PCBs

Run-normalized ∑PCBs percent recovery were normally distributed and had homogeneity of variance among groups (p=0.1464 and 0.1117 respectively). Treatment was a significant predictor of nominal percent recovery (p<0.0001) but treatment group was not a significant predictor of nominal percent recovery (p=0.0922) so all units of each treatment were pooled as one group. Though there were significant differences among the means of storage time points, no time points had a mean ∑PCBs recovery that was significantly lower than the untreated control spikes (Table 4). The lower limit of the 95% confidence interval of the mean of run-normalized ∑PCBs was not lower than the lower permissible level of the QAQC guidelines (70%) at any time point (Figure 3), and the range of the 95% confidence intervals of the means of each time point were similar to the 95% confidence interval of untreated controls (Table 4). Nominal recovery of total PCBs ranged from 48.0% to 118.1%.

3.2: Field Extraction Simulation

The only individual compound that was detected at levels greater than the method detection limit in all of the field-collected eagle samples used for this validation was 4,4'-DDE, therefore this was the only individual compound that could be validated. Sum PCBs for each sample were calculated, however the composition of compounds varied among eagle samples. The mean 4,4'-DDE and mean total PCBs percent

recovery from field-collected bald eagle plasma fell within the 70-130% QAQC permissible range for all time points assessed (1, 3, 7, 14, 21 and 28 days) (Figure 4). Residues of 4,4'-DDE recovered at all time points did not vary significantly from untreated samples (p= 0.9784, 0.8703, 0.9995, 0.4313, 0.7610, and 1.000 respectively). Total PCBs did not vary significantly from untreated samples at any time point (p= 0.7835, 1.0000, 0.6880, 1.000, 1.000, and 1.000 respectively).

4: Discussion

Based on run-normalized percent recovery, the stability of OC analytes in Oasis ® HLB cartridges is not impacted by the length of time that analytes are stored in the sorbent matrix for at least 84 days. This is consistent with the findings of Sundberg et al. (2006) where OC-fortified serum samples (n=1) stored for 14 and 21 days in Oasis ® HLB cartridges had similar recoveries to recovery at day 0. Our results demonstrate the precision and accuracy of measurements over an extended time interval and validate this storage-methodology with field collected samples. No compound groups in fortified chicken plasma samples were statistically significantly different from the untreated control samples, which implies that analytical measurements using this extraction method are highly precise. The 95% confidence limits of the run-normalized means of all compound groups at all time points fell within the 70-130% QAQC recovery range, which implies the accuracy of the measurements (Figure 3). However, the suite of compounds addressed here (Table 1) is not inclusive of all OC compounds, and further analysis should be conducted if chemicals not included are to be monitored using this technique.

The lesser recovery of all OC compounds at the 28 and 42 days (relative to other time points) suggests that there may have been a batch effect on these samples (Figure 3). Laboratory observation notes indicated that gravity flow of run-set six (which included 28- and 42-day samples) (Table 2) was slower than usual during the extraction. Laboratory observation notes on the extraction of run-set seven indicated that the cartridges designated for 28-days of storage had considerably slower than usual gravity flow during the extraction. Longer loading times into Oasis ® HLB cartridges with water samples is associated with higher analytical readouts (Anthemidis et al. 2001), but here we see the opposite effect. The causes of these extended loading times for some samples and batches is unknown but may have to do with the lipid particulate content in the plasma sample, or ambient conditions (temperature or humidity) on the day of the extraction. If analytes are not successfully or completely desorbed from lipids, recoveries are lower (Sundberg et al. 2006). Additionally, humidity has been known to interfere with analyte sorption in SPE (de Fátima Alpendurada 2000). It is notable that the nominally-computed recoveries of samples fell outside the 70-130% QAQC recovery range for total DDTs and PCBs at 28 and 42 days and for total OC pesticides at 42 days (Appendix Figure 1 and Appendix Table 1). Although nominal recovery does not have implications on the effect of storage, it does suggest that overall extraction efficiency was reduced. It is known that OC recovery using Oasis ® HLB cartridges is not uniform (Sundberg et al. 2006) and that differences in extraction efficiency are related to molecular mass and polarity of the analyte (Dias and Poole 2002). Using the same fortification and extraction method (with no storage-treatment), Sundberg et al. (2006) found recovery below 70% for Aldrin (62.1%), Alpha-BHC

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(65.4%), 4,4'-DDE (68.3%). Therefore, although the mean recovery of compounds at 28 and 42 days are lower than other time points in this study, they are more accurate than other studies and within the 70-130% QAQC recovery range (Table 4).

When evaluating storage stability over time exclusive of the 28- and 42-day time points (which may be influenced by less-effective extraction), the run-normalized mean recovery of compounds is highly precise. When excluding 28- and 42-day means, total DDTs, OC pesticides, and PCBs recovery range from 91-107%, 94-104%, and 88-111% respectively (Table 4). This suggests that our laboratory-specific conditions are more precise than the 70-130% QAQC range.

Other variation of quantified residues could be a result of deterioration of the extraction matrix. After elution of time-treated cartridges, a white film was frequently noted in the extracts. The earliest time point that this abnormality was noted was at 14 days of storage. This was not observed at earlier time-points or in the untreated control extracts. Previous method optimization demonstrated that Oasis ® HLB filers can remain reliable for 500 extraction/elution cycles (Anthemidis et al. 2001), suggesting that the sorbent is highly resilient to degradation. However, this was concluded using a different eluent (methanol) than used here (dichloromethane). While analyte concentrations are consistent over time, these observations suggest there may be underlying changes in the extraction sorbent.

Field collection simulation with bald eagle plasma further supports the use of this method and demonstrates the accuracy of measurements when extracts are stored in the HLB cartridges. Like the fortified chicken plasma, PCBs and an OC pesticide (4,4'-DDE) were not significantly different when measured after being stored for 1, 3,

7, 14, 21 or 28 days and the means were within the 70-130% QAQC recovery range. Since sample replicates at each time point were derived from only two bald eagle samples, further analysis should be conducted with more field collected bald eagle plasma samples to confirm the precision of measurements after storage-time treatments.

When using this method to extract OC compounds from field collected plasma samples, it is advised that blood samples are collected, centrifuged, and preserved (frozen) during the course of sampling until all samples have been collected. In the final days before international travel, investigators should extract all samples at once and prepare samples for transport and storage as described here. Analytes should be eluted and quantitatively analyzed within two weeks of extraction for additional quality assurance.

5: Conclusions

A simple modification to a SPE extraction technique for quantifying OC compounds in avian plasma has been reported here. We have demonstrated that OC extracts from avian plasma stored in commercially available Oasis® HLB cartridges retain analytical validity for at least 84 days. The accuracy and precision of measurements of stored-extracts was validated in both lab-manipulated chicken plasma and field-collected bald eagle plasma. Additionally, it appears that OC pesticides and PCBs have similar stability when stored in the HLB cartridges. Laboratories utilizing this technique should determine their lab-specific precision of measurements of standards without storage-time treatment before analyzing stored samples as an

additional quality assurance procedure. Replication of this study, and modification with other analytes should be completed to further explore this extraction technique.

Table 1: Organochlorine analytes assessed for storage stability in Oasis® HLB SPE cartridges.

PCBs	OC Pesticides
PCB 8	Methoxychlor
PCB 18	Heptachlor Epoxide
PCB 28	Heptachlor
PCB 44	Hexachlorobenzene
PCB 52	gamma-Chlordane
PCB 66	alpha-Chlordane
PCB 77	Endrin Ketone
PCB 101	Endrin Aldehyde
PCB 105	Endrin
PCB 110	Endosulfan Sulfate
PCB 118	Endosulfan II
PCB 126	Endosulfan I
PCB 128	Dieldrin
PCB 138	Aldrin
PCB 153	delta-Benzene hexachloride (BHC)
PCB 156	beta-BHC
PCB 170	gamma-BHC
PCB 180	alpha-BHC
PCB 187	2,4'- Dichlorodiphenyltrichloroethane (DDT)
PCB 195	4,4'-DDT
PCB 206	2,4'- Dichlorodiphenyldichloroethane (DDD)
PCB 209	2,4'-DDD
	2,4'- Dichlorodiphenyldichloroethylene (DDE)
	4,4'-DDE

Table 2: Organization of storage-time treatment repetitions within batches for storage interval analysis. Each batch was comprised of 3 groups of 6 samples that represented one treatment group of a treatment. Each treatment was repeated 3 times within the batches. Treatment repetitions were placed into batches randomly.

Batch	Treatments (days)	Treatment Group Number
	1	1
1	3	1
	7	1
	14	NA*
2	21	1
	42	1
	1	2
3	63	1
	84	1
	14	1
4	28	1
	63	2
	84	2
5	84	3
	63	3
	42	2
6	42	3
	28	2
	28	3
7	21	2
	21	3
	7	2
8	7	2
	3	2
	1	3
9	3	3
	14	2

* group repetition was destroyed prior to chemical quantification

Table 3: Number of spikes, blanks, and repetitions groups, for each storage-time treatment.

Treatment (days)	Number of Treatment Groups	Spikes	Blanks
0 (untreated controls)	9	18	9
1	3	18	3
3	3	18	3
7	3	18	3
14	2	12	2
21	3	18	3
28	3	18	3
42	3	18	3
63	3	18	3
84	3	18	3
Total (Experimental Only)	26	156	26
Total	35	174	35

Table 4: Run-normalized percent recovery and 95% confidence interval for the mean total DDTs, OCs, and PCBs at each storage time point (N=18 for all except 14 days where N=12).

Treatment (days)	Mean run-normalized percent recovery (95% confidence interval of the mean)		
	DDTs	OCs	PCBs
0	100% (92-107%)	100% (92-106%)	100% (93-108%)
1	107% (98-115%)	104% (98-110%)	105% (96-114%)
3	91% (85-97%)	94% (89-99%)	88% (81-95%)
7	101% (98-105%)	99% (95-103%)	95% (89-100%)
14	93% (89-97%)	97% (93-102%)	90% (85-95%)
21	107% (103-112%)	102% (98-107%)	103% (98-107%)
28	87% (78-96%)	86% (81-92%)	82% (73-89%)
42	85% (78-92%)	85% (78-92%)	82% (73-91%)
63	107% (100-114%)	104% (98-109%)	110% (103-116%)
84	102% (97-107%)	99% (95-103%)	111% (106-117%)

Traditional SPE and Analytical Quantification Method

Proposed Novel SPE and Analytical Quantification Method

Step 1: Load plasma sample onto extraction filter

Plasma Sample → SPE Filter → Waste

Step 1: Load plasma sample onto extraction filter

Plasma Sample → SPE Filter → Waste

Step 2: Dispose of biological waste

SPE Filter **Chemical Analytes** → Bio Waste → Waste

Step 2: Dispose of biological waste

SPE Filter **Chemical Analytes** → Bio Waste → Waste

Step 3: Elute chemical analytes and quantify concentrations

SPE Filter **Chemical Analytes** → Chemical Extracts → [instrument]

Step 3: Store analyte-loaded filters

SPE Filter **Chemical Analytes** [clock]

Step 4: Elute chemical analytes and quantify concentrations

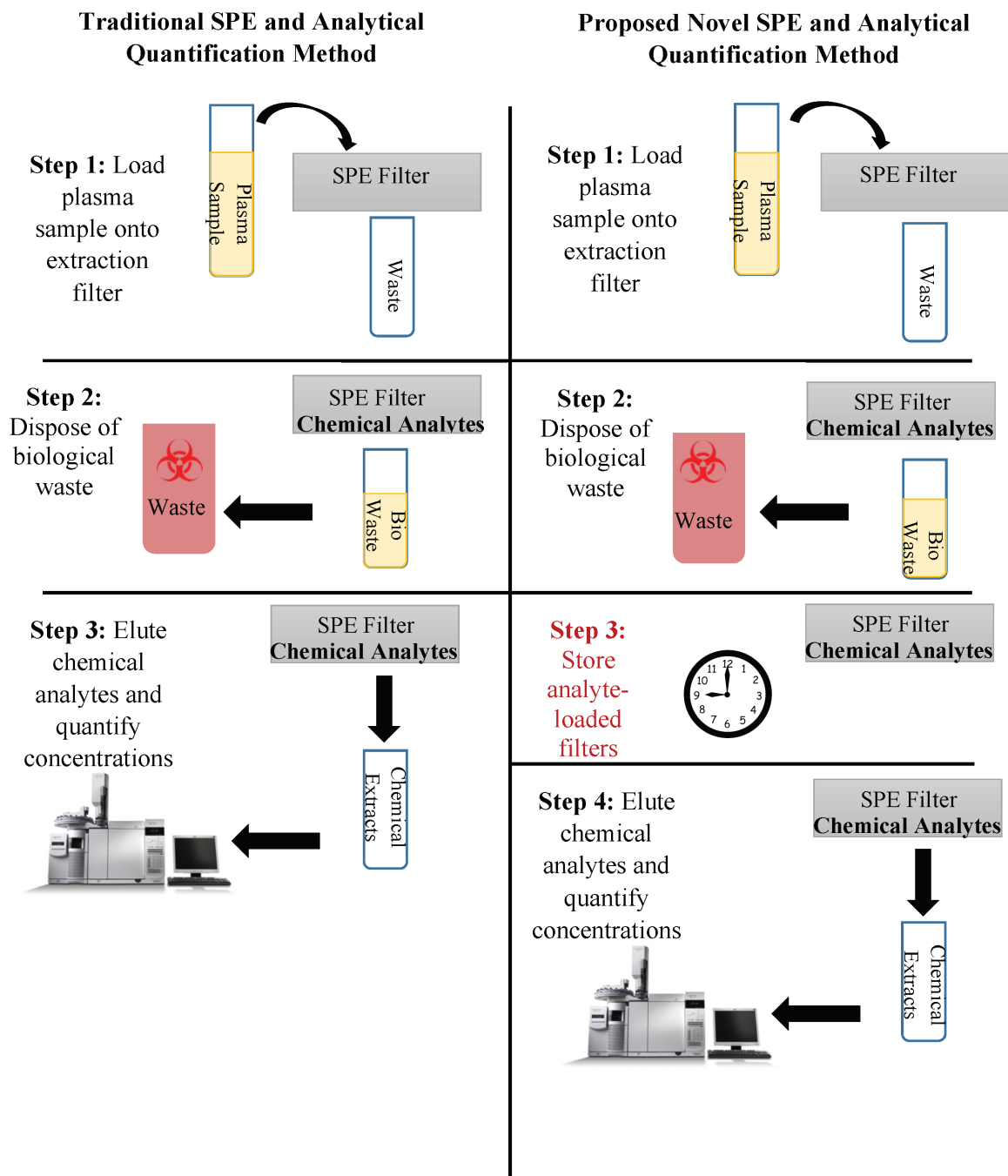SPE Filter **Chemical Analytes** → Chemical Extracts → [instrument]

Figure 1: Operational sequences for extraction and quantification of organochlorines compounds using classic and novel technique.
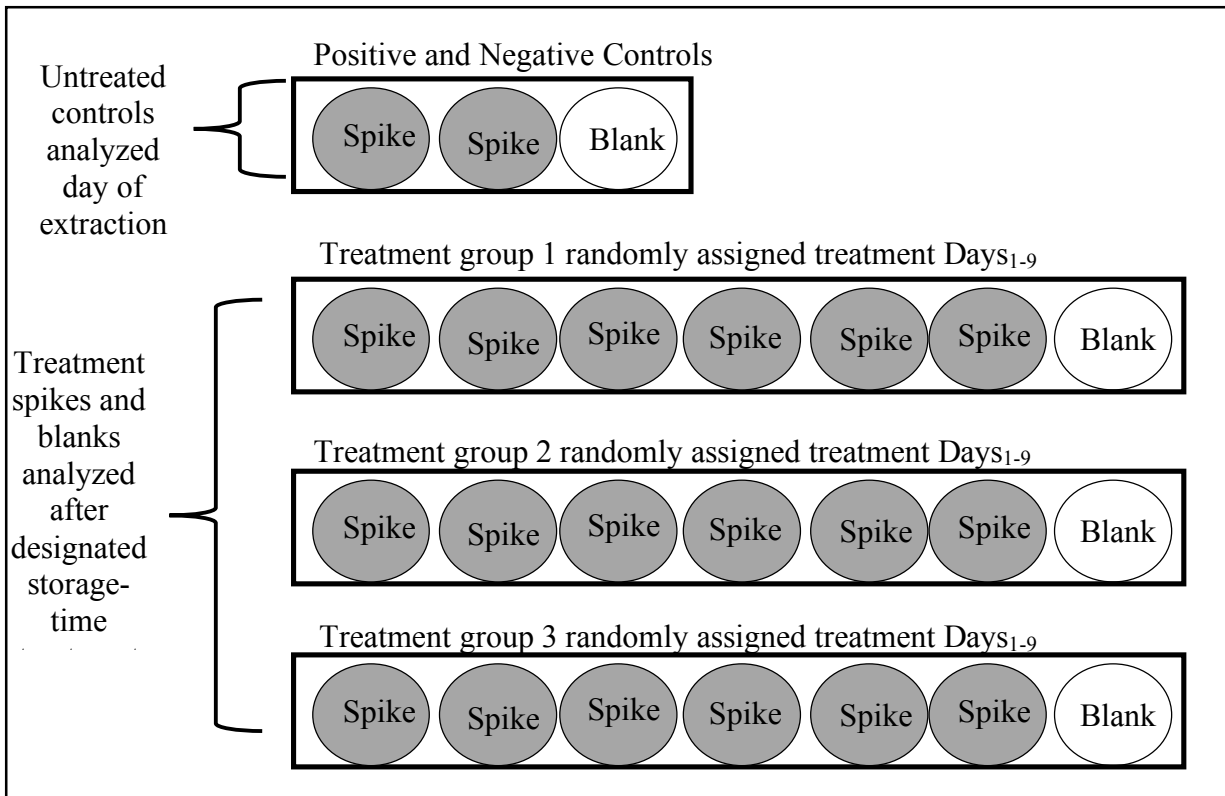
Figure 2: Organization of samples within each batch. Untreated (no storage) spikes and blank were eluted and analyzed the day of the extraction. The remaining 18 spikes and 3 blanks were assigned storage-interval treatments and not eluted and analyzed the day of extraction.
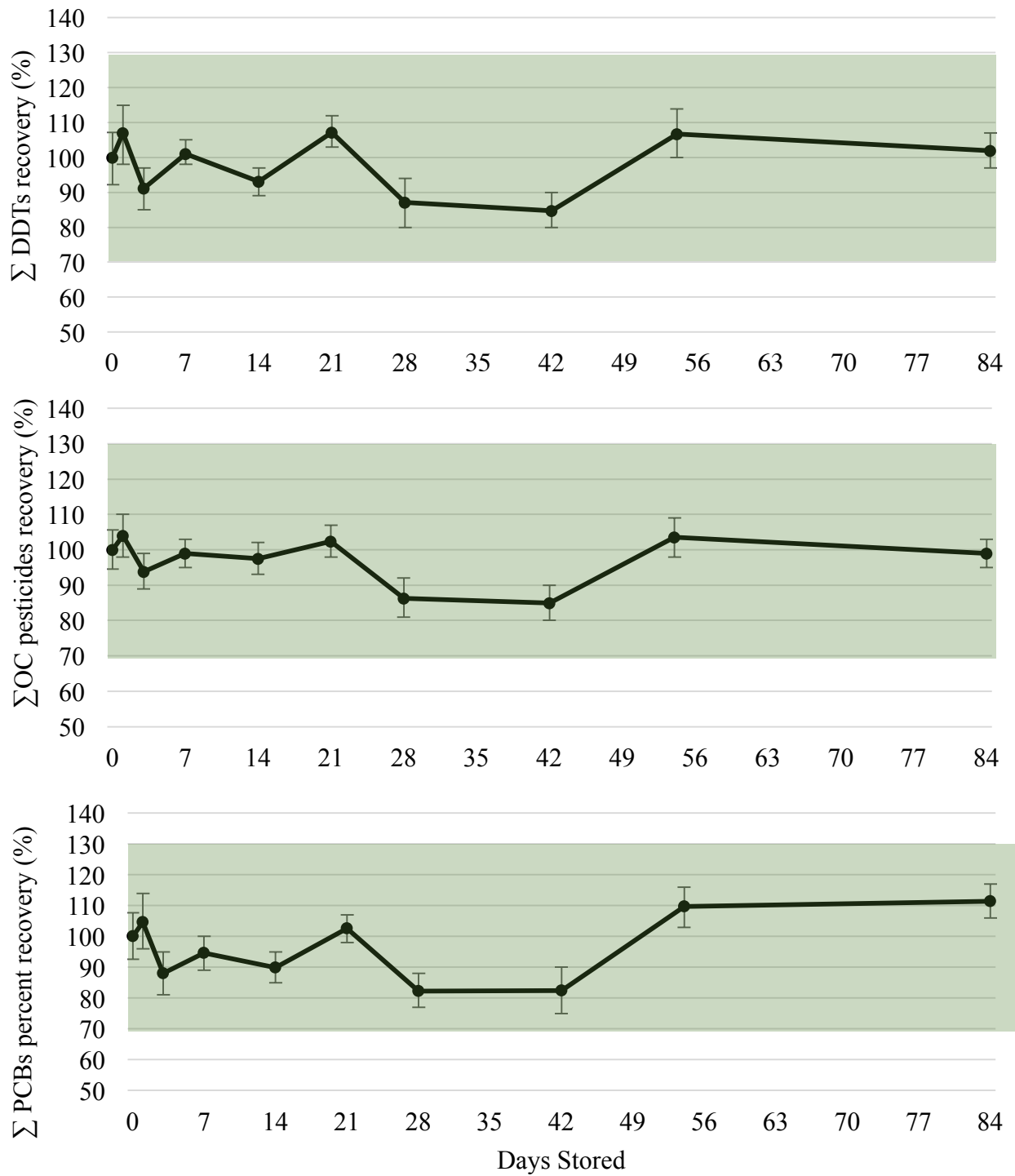
Figure 3: Recovery of total PCBs, OCs, DDTs stored in Oasis® HLB cartridges from 1-84 days. Recovery was calculated based on control concentration of total PCBs, OCs, DDTs found in fortified chicken plasma samples (day 0). Error bars represent the 95% confidence interval of the mean.
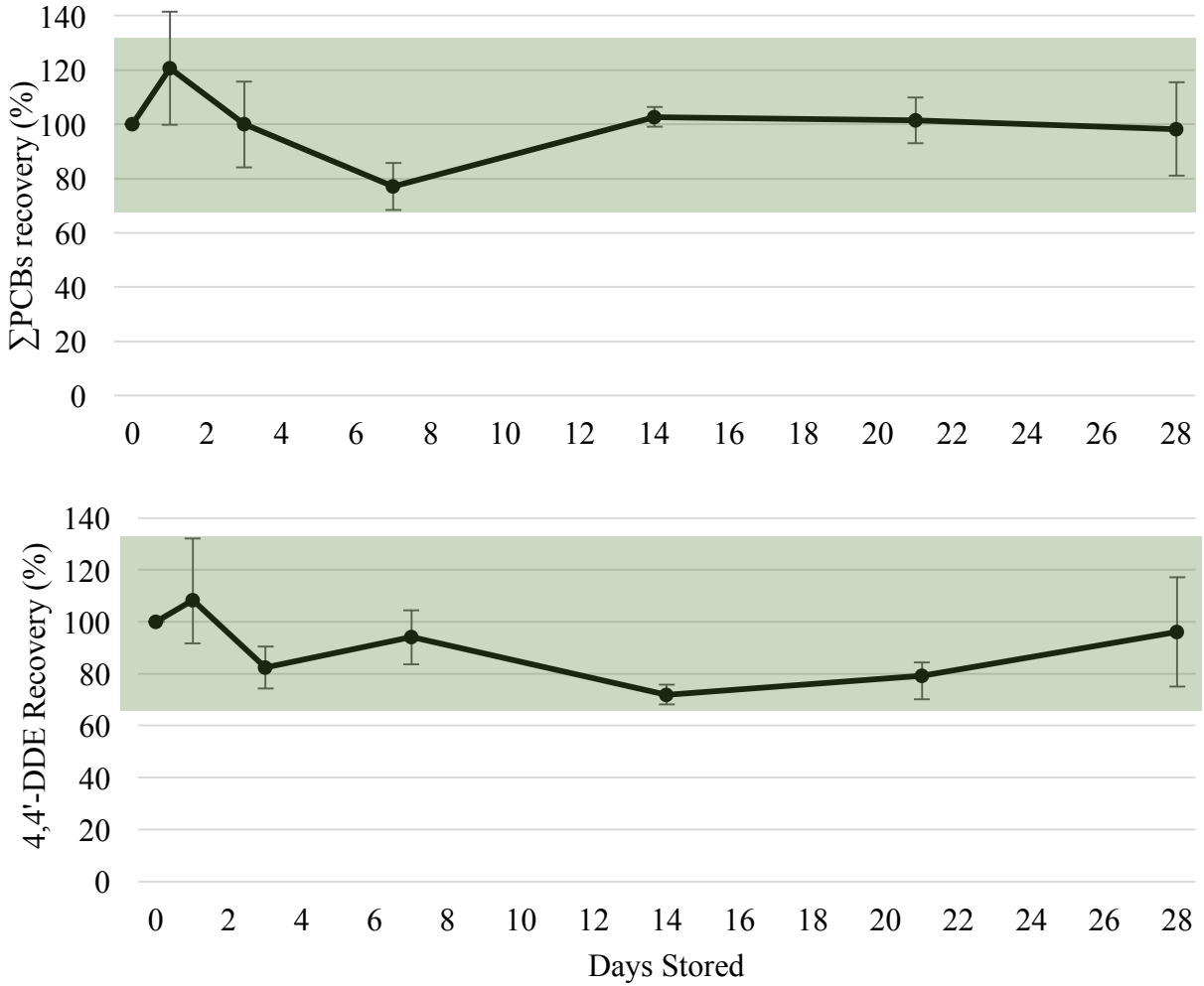

Figure 4: Mean recovery of 4,4'-DDE from field collected bald eagle plasma stored in Oasis® HLB cartridges for 1 to 28 days. Recovery was calculated based on total PCB and 4,4'-DDE concentrations measured in plasma samples with 0 days of storage. Error bars represent the 95% confidence interval of the mean.

Chapter 3: Spatial Temporal Monitoring of Organochlorines in Bald Eagles of Voyageur's National Park 2011-2017

1: Introduction

Bald eagles (*Haliaeetus leucocephalus*) nesting within Voyageur's National Park (VNP) in northern Minnesota have been monitored for legacy organochlorine (OC) compounds since 1989 (Bowerman et al. 1995; Pittman et al. 2015). Organochlorine compounds were used heavily in the mid-twentieth century for electrical stability, insulation, hydraulic lubricants, and insect pest control (Hutzinger et al. 1974; Loganathan and Kannan 1994; Giesy and Kannan 1998). Concern for the human and environmental health impacts of chlorinated compounds emerged when OCs were found to resist degradation and elimination, bioaccumulate and magnify, and cause toxicity to non-target organisms (National Academy of Science 1971; Loganathan and Kannan 1994; Metcalfe and Haffner 1995). The egg-shell thinning effects of the Dichlorodiphenyltrichloroethane (DDT) metabolite Dichlorodiphenyldichloroethylene (DDE) on bald eagles and have been cited as root causes of the bald eagle population decline in the mid-twentieth century (Wiemeyer et al. 1984; Bowerman et al. 1998; Donaldson et al. 1999). Other OC compounds, such as dioxin-like PCBs, elicit toxic effects including endocrine disruption, dermal lesions, weight loss, enzyme induction and inhibition, decreased reproductive health, immune dysfunction and suppression, and teratogenicity (Metcalfe and Haffner 1995; Giesy and Kannan 1998). Bald eagles were identified as an ideal indicator of environmental contamination by OC compounds in the Great Lakes region (International Joint Commission 1991), and the effects of OC contaminants and many other stressors on bald eagles are well documented (Cromartie et al. 1975; Wiemeyer et al 1984; Anthony

et al. 1993; 2007; Elliot et al. 1995; Donaldson et al. 1999; Bowerman et al. 1995; Dykstra et al. 2001; 2005; Cesh et al. 2008).

As an apex predator of the aquatic food web, bald eagles consume a diet comprised primarily of fish (Buehler 2000), which puts individuals at an elevated risk of exposure to persistent organic pollutants (POPs), due to the tendency of these compounds to bioconcentrate in fish (Bowerman et al. 1995). Nestlings eagles are flightless and dependent on adults for food until they are approximately 12 to 14 weeks of age (Gerrard and Bortolotti 1988). Adults forage for food within a home range of approximately 4.9 km^2 to feed the nestlings (Watson 2002); Therefore, OC compounds accumulated within nestling tissues are a reflection contamination in the environment proximate to the nest site (Anthony et al. 1993; Bowerman et al. 2003; MDEQ 1997). By collecting and comparing OC residues in bald eagle nestlings through space and time, we can detect trends and identify areas of concern for OC contamination.

Voyageur's was established as a National Park in 1975 and follows the boundary waters of the United States and Canada for 88.5 kilometers (Voyageur's National Park 2007) (Figure 5). The park is arrayed with over 1,000 islands within three impoundments managed by the International Joint Commission: Rainy, Kabetogama, and Namakan Lakes (Voyageur's National Park 2007). The water-based park provides an ideal nesting habitat and an abundant food source for bald eagles. However, bald eagles in the park are sensitive to disturbance by park visitors and activities such as motor boating (Grim and Kallemeyn 1995; Grubb et al. 2002). The reproductive health and spatial distribution of the bald eagle population in and around VNP has been monitored by the National Park Service since 1973 (Cruz et al. 2017). Prior to

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Voyageur's designation as a national park, nestling success was surveyed by the Superior National Forest from 1962 to 1974 (Grim and Kallemeyn 1995). Thus, the population patterns of bald eagles in VNP are well understood. The dense population of eagles within VNP is unique and allows numerous samples for biomonitoring to be collected in a short period of time.

While VNP was largely secluded from industrial or agricultural point source discharges of OC compounds, the park's bald eagle population was not invulnerable to their effects. Atmospheric deposition of volatilized OC compounds provides a means for the transportation of PCBs and OC pesticides throughout the global environment (Gregor and Gummer 1989; Anthony et al. 2007). Additionally, some historical use of OC pesticides was documented within the park boundaries. Dichlorodiphenyltrichloroethane was heavily sprayed across 5,261 ha between 1958 and 1961 to control spruce budworm (*Choristoneura hebenstreitella*) infestation (Voyageur's National Park 2001). Furthermore, toxic contaminants are cited as a likely source of depressed bald eagle reproduction in the park from 1973-1993 (Grim and Kallemeyn 1995). In 1989, a nestling in VNP had a beak defect and avian pox. Residues of DDE and total PCBs in the plasma of the bird's sibling were 216 and 1600 ppb respectively, suggesting that the malformation may be a result of OC toxicity (Bowerman et al. 1994). The PCB concentrations in this nestling were the greatest among 141 collected bald eagle nestling plasma samples from Michigan, Minnesota, Ohio, Ontario, and Wisconsin between 1987-1989 (Bowerman et al. 1994). Therefore, although OC contamination is comparatively low in VNP, risk of adverse effects by legacy OC compounds should not be ignored.

Recent work demonstrated that DDE and PCB residues were consistently present in bald eagle nestlings and suggested that once-used OC pesticide Dieldrin was increasing in nestlings of VNP from 1995-2010 (Pittman et al. 2015). These data were collected as part of a long-term biomonitoring study of OC trends in bald eagle nestlings of the Great Lakes region. In order to further investigate these findings and continue this longitudinal study, plasma samples were collected from bald eagle nestlings from 2011 to 2017 and residues of legacy OC compounds were quantified. The objectives of this study were to identify the spatial and temporal trends of total PCBs, DDT-related compounds, and Dieldrin in VNP, and to determine if there are new sources of Dieldrin or DDT in the VNP area.

2: Methods

2.1: Field Methods

2.1.1: Occupancy Surveying

Reproductive success was quantified based on nest productivity using calculations and definitions suggested by Postupalsky (1974). Nesting territories, or breeding areas, were defined as areas that contained, or have contained, at least one nest tended by a breeding pair of sexually mature eagles, and where only one pair has bred at a time in a particular year (Postupalsky 1974). Territory occupancy was assumed if at least one adult was perched by, on, or in proximity to, a known nest location within a breeding territory early in the breeding season (Postupalsky 1974). Nest productivity was calculated as the number of young that reach fledging age per occupied nest (Postupalsky 1974). The total productivity of a geographic region was enumerated as the number of successful young per occupied breeding territory per year

(Postupalsky 1974). These data were collected in three stages; an initial aerial survey identifying breeding areas occupied by adults, a second aerial survey confirming nesting success and counting successfully hatched young, and a third survey that was conducted by ground teams that visited a subset of active nests, ascended trees, and confirmed aerially reported nestling counts (Bowerman et al. 2002).

3.1.2: Tissue and Morphometric Data Collection

Ground crews located nest-containing trees using latitude and longitude coordinates taken during aerial observation. United States Forest Service certified climbers ascended the trees to the nests. Eagle nestlings that had reached ages four through nine weeks were safely removed from the nest and delivered by the climber to the team members on the ground in a specially-designed nylon bag. This age range was selected because nestlings are able to properly thermo-regulate (Bortolotti 1984a), adequate blood can be drawn without initiating adverse health effects (Sturkie 1986), and the risk of premature jumping from the nest is low. The initial assessment of age was visually estimated by the climber based on plumage.

Once on the ground, morphometric measurements of each eaglet were taken and used to estimate age and sex as defined by Bortolotti (1984 a, b, c). U.S. Fish and Wildlife Service size 9 identification bands were fitted to each nestling. Three breast feathers were collected from each nestling and placed in coin envelopes until analysis for methyl mercury content (not reported here). Blood was collected from the brachial vein using aseptic techniques into a heparin-lined sterile syringe. Blood volumes that can be collected from avian subjects without detrimental effects are estimated as 10% of the total blood volume which accounts for approximately 7% of the organism's body

weight (Sturkie 1986; Hoysak and Weatherhead 1991). Nestlings sampled ranged from three to seven kilograms and were not harmed by the collection of 10 cubic centimeters (cc) of blood. A portion of the blood collected (approximately one cc) was preserved as whole blood stored in blue-top Vacutainers®. The remaining blood sample was transferred to a labeled, heparin-lined, green-cap glass Vacutainers®. Herperanized blood samples were centrifuged and plasma was removed and placed in another, heparin lined, green-cap glass Vacutainers®, leaving only the red blood cells in the original Vacutainers®. A small blood blot was collected on a gauze pad for DNA analysis (not reported here). The nestlings were then returned to the nest and the climber descended from the tree. All blood cells, plasma, and whole blood were frozen until analysis.

2.2: Laboratory Methods

If multiple birds were sampled from the same nest in one year, plasma from one nestling was randomly selected to represent the territory for that year. It was assumed that nestlings from the same nest in the same year would have similar contaminant loads because they have identical diets (Donaldson et al. 1999). Frozen plasma was thawed and treated with 400 μLs 8M urea to desorb analytes from the matrix and denature and dilute plasma proteins (Sundberg et al. 2006). One hundred μLs of plasma from each nest representative was aliquoted into sterile glass test tubes. Surrogate standard solution with known concentrations was added to each sample and used to confirm extraction efficiency. Positive and negative controls were generated with pooled, mixed-gender domestic chicken (*Gallus domesticus)* plasma in sodium heparin obtained from BioChemed Services (Winchester, VA). Chicken plasma was selected

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because individuals lack exposure to environmental sources of OC contaminants. Thus, any compounds extracted from the chicken plasma are assumed to originate from laboratory manipulation. Negative control samples were spiked with the surrogate standard solution, and positive controls were spiked with known concentrations of PCB and OC pesticides.

Organochlorine compounds were extracted from plasma following solid phase extraction (SPE) described by Sundberg et al. (2006). Oasis® hydrophilic-lipophilic balance (HLB) SPE cartridges were conditioned for extraction using 4 mL aliquots of methanol and equilibrated with 4 mL aliquots of nanopure water (Waters Corporation 2014). Samples were loaded onto SPE cartridges in a vacuum manifold using glass serum pipettes. Glass test tubes were rinsed with 3 mL of nanopure water and the rinse was loaded onto the filter to ensure complete transfer of the sample. Extraction cartridges were rinsed with 3 mL of nanopure water for matrix clean-up after sample loading. Chemicals were eluted from the SPE cartridges into sterile glass test tubes using 2 mL volumes of pesticide grade dichloromethane (DCM). The extracts were dried using a low flow of nitrogen gas through a sample concentrator. An internal standard solution of known concentration was added to each extract prior to analysis to verify analyte concentrations. Extracts were reconstituted in hexane and pipetted into 2-mL amber screw-top gas chromatograph (GC) vials with glass conical inserts. A vehicle blank and a calibration solution (hexane with known volumes of PCB and OC pesticide standard solution, internal standard, and surrogate solution) were analyzed after each batch in to identify potential sample carry over, contamination, and to assure proper calibration.

Chemical standards were obtained for 34 compounds (22 PCBs, 7 OC pesticides, 2 compounds used as internal standards, and 3 compounds used as surrogate standards) from Ultra Scientific (North Kingstown, RI) and Accustandard (New Haven, CT) (Appendix Table 2).

A calibration curve was generated for each analyte and compound-specific method detection limits (MDL) and limits of quantitation (LOQ) were calculated. For all compounds, the MDL is approximately 0.6 and the LOQ is approximately 2.0 (Appendix Table 2). Twenty-two PCB congeners and 7 OC pesticides (Appendix Table 2) were quantitatively analyzed in each sample using an Aligent Technologies 7890A GC with electron capture detection (ECD) with split injection and duel columns, following U. S. Environmental Protection Agency (EPA) Methods 8081 and 8082 (2007a; 2007b). Dual column analysis was used to confirm all results; Extracts were analyzed simultaneously using two different copper columns and the results from each column agreed within 40% for results to be reportable. Surrogate standard recoveries in each individual extracted sample were required to average between 70 and 130% of the nominal concentration of analytes as a quality assurance and quality control (QAQC) acceptance criteria as suggested by EPA method 8000 (2018).

2.3 Data Analysis

Statistical analysis was only performed for OC compounds that were recovered in greater than 50% of samples at quantities greater than the MDL. Samples were categorized based on nest location into one of the three VNP impoundments: Namakan, Rainy, and Kabetogama lakes. Statistical analyses were conducted in Statistical Analysis System (SAS) software, version 9.4 (SAS Institute Inc., Cary, NC). Due to

the left-censored and right-skewed nature of environmental contaminant data, normality was not achieved for parametric analysis, so non-parametric statistical analyses were computed. The non-parametric Kaplan-Meier (K-M) method was used to compute summary statistics for individual compounds with <50% censoring (Helsel 2005). This is the standard method for estimating summary statistics for right-censored survival data and can be modified for estimating mean and standard error of left-censored environmental data without needing to substitute values for censored data (Helsel 2005). In addition to non-parametric K-M means for individual compounds, geometric means were computed by assigning censored values half of the compound-specific MDL in order to compare findings with previously reported bald eagle biomonitoring data in VNP. Summed values for describing groups of compounds (total PCBs and total DDTs) were calculated by assigning each censored constituent half of the compound-specific MDL (Leith et al. 2010). Due to the fact that the summed values can vary widely depending on how censored data is handled and substituted, minimum and maximum range values were calculated to illustrate the interval range of possible true total PCBs and total DDTs. Maximum estimates were approximated by assigning censored measurements the compound-specific MDL and minimum estimates were computed by assigning all censored measurements a value of nearly 0 (0.0001 for the purpose of statistical computations).

Total PCBs and 4,4'-DDE were compared to the no observable adverse effect concentration (NOAEC) for bald eagle reproduction (36.4 and 11.4 μg kg^{-1} respectively) (Bowerman et al. 2003).

The non-parametric Kruskal-Wallis test was used to assess if residue values varied in rank scores among the impoundments by year. This analysis was repeated for each compound or compound group detected in >50% of samples at values greater than the MDL. When rank scores were significantly different by year, regression analysis was completed to determine if statistically significant trends existed. Previously reported data by Pittman et al. (2015) was included in regression analyses in order to determine comprehensive trends of this longitudinal study. When rank scores differed significantly by lake, multiple comparisons tests were conducted for pair-wise comparison of groups using the Bonferroni correction. Individual comparisons level was calculated by dividing the number of comparisons by the desired family-wise alpha-level (Helsel 2005). Five-year geometric means were calculated to compare with previously reported data and to determine percent change from 1995 to 2017.

A ratio describing the relationship between metabolite-compound DDE and its parent compound DDT ("DDE:∑DDT") was calculated for each sample to estimate the age of DDT (Harner et al. 1999; Tavares and Costa 1999; Qiu et al. 2004). Various methods have been used to describe the relationship between DDT and metabolites in the environment such as calculating [DDE + DDD]/DDT (Muñoz-Arnanz and Jiménez 2011), DDD/∑DDTs (Macgregor 1974; Aguilar 1984), the ratio of isomers (2,4'-DDT:4,4'-DDT) (Qiu et al. 2010), and the ratio of DDE:DDT (Tavares and Costa 1999; Qiu et al. 2004). The comparison of 4,4'-DDE to total 4,4'-DDT-derived compounds was selected over other comparison methods because of the degree of censoring in the data; the computation required the use of only three compounds and there was therefore less uncertainty in the calculated value. Historic sources of DDT can be identified by

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high ratios of DDE:∑DDT, while lower or decreasing DDE:∑DDT ratios indicate more recent exposure or release of DDT into the environment (Hitch and Day 1992; Tavares et al. 1999; Qio and Zhu 2010). Technical grade mixtures of DDT were approximately comprised of 80% 4,4'-DDT (also known as p,p'-DDT) isomer and 20% 2,4'-DDT (also known as o,p'-DDT) isomer (Lundholm 1997; Kamrin 1997). Therefore, the ratio of 4,4'-DDE to ∑4,4'-DDT-derived compounds was selected because they are more likely to be detected in samples.

3: Results

Plasma samples were collected and analyzed from 121 nestlings from 2011 to 2017 with at least one sample from each impoundment (Table 5). These samples represent 59 unique nests of 42 territories in VNP. The residue data was highly censored with a large proportion of non-detected (ND) values. Statistical analyses were completed for total PCBs, total DDTs, and 4,4'-DDE. Dieldrin detections are also reported in order to compare with previously reported findings by Pittman et al. (2015). However, Dieldrin was below the MDL >50% of samples.

3.1: Total PCBs

At least one PCB congener was detected in 95% of samples at levels greater than the MDL. On average, 20 of 22 PCBs analyzed in each sample were below the LOQ (Table 6). Total PCBs ranged from ND to 158.5 µg kg^{-1} and annual geometric means of total PCBs ranged from 10.7 to 24.1 µg kg^{-1} (Table 7 and Figure 6). Rank scores of total PCBs did not vary significantly among lakes (p=0.0755) but rank scores did vary significantly by year (p<0.0001). Geometric mean total PCBs decreased 17.93% from the 1995-2001 five-year mean to the 2013-2017 five-year mean.

However, no statistically significant decreasing regression trends were found. The maximum estimates (using the MDL of each censored constituent) of annual geometric mean of total PCBs were below the NOAEC for bald eagle productivity (Figure 7), and minimum estimates (using 0 for each censored constituent) were <2 in 2014 (Figure 7).

3.2: Total DDTs

At least one DDT-derived compound was detected in 99% of samples at levels greater than the MDL. However, in 61% of samples 4,4'-DDE was the only DDT-derived compound detected above the MDL. Total DDTs ranged from ND to 149.7 µg kg^{-1} (Table 7). Mean rank scores of total DDTs did not vary significantly among lakes (p= 0.8952), but rank scores did vary significantly by year (p<0.0001). Geometric mean total DDTs decreased 50.47% from the 1995-2001 five-year mean to the 2013-2017 five-year mean and regression analysis indicated that total DDTs decreased significantly from 1995 to 2017 (p<0.0001) (Figure 8). The minimum estimates of annual geometric mean of total DDTs were below the LOQ in five of the seven years of this study (Figure 9).

3.3: 4,4'-DDE

Residues of 4,4'-DDE were detected in 99.2% of samples. Of these detections, 33% were below the QL for 4,4'-DDE (Table 7). Mean rank scores of 4,4'-DDE did not vary significantly among lakes (p=0.1474) but did vary significantly by year (p<0.0001). Geometric mean 4,4'-DDE decreased 48.47% from the 1995-2001 five-year mean to the 2012-2017 five-year mean and regression analysis indicated that 4,4'-DDE decreased significantly from 1995 to 2017 (p<0.0001) (Figure 10). Kaplan-Meier annual mean estimates were higher than geometric means for all years except 2017

where the geometric mean was greater (Table 7). Geometric mean 4,4'-DDE was consistently below the NOAEC for bald eagle productivity in (Figure 10).

Study wide DDE:∑DDT ratio in VNP was 0.919 (Table 8) and values ranged 0.557 to 1.00. Annual mean DDE:∑DDT ratio was greater than 0.86 for every year of this study (Table 8).

3.4: Dieldrin

Dieldrin was detected in 62.8% of samples, however 91% of these detections were censored. Therefore, statistical analyses were not conducted (Figure 11).

4: Discussion

Today, VNP is largely secluded from industrial and agricultural influence. Legacy OC contamination in bald eagle nestling plasma was very low in VNP from 2011-2017, with few of the analytically targeted compounds detected at levels greater than the MDL in more than 50% of samples. Total PCBs, total DDTs, and 4,4'-DDE did not vary significantly among the impoundments during this study, indicating that contaminant trends are consistent within the park boundaries. However, residue concentrations of Dieldrin, total PCBs, total DDTs, and 4,4'-DDE fluctuated annually in VNP from 1995 to 2017 (Figures 6, 8, 10, 11). Due to the lack of point sources of contamination in the park, these annual differences are likely a result of micro-environmental changes such as water-level fluctuation, food availability, food web dynamics, or atmospheric deposition which may cause legacy OC contaminants to vary slightly in bioavailability on a year-to-year basis.

The climate in northern Minnesota is highly variable and temperature and precipitation fluctuate annually and throughout the year (Weeks et al. 1998). The Rainy

Lake basin and downstream Namakan Chain of Lakes (Rainy-Namakan basin) are highly susceptible to extreme rain and drought conditions, responding rapidly to changes in water supply (International Joint Commission, 2017). In 2000, VNP water managers began adhering to a new water level management regime implemented by the IJC (International Rainy Lake Board of Control, 2004). The lake levels in VNP are controlled by dams in two different areas: one large dam at the northeastern corner of Rainy Lake and two smaller dams in Namakan Lake (Holmberg et al. 2005). Biomonitoring in the park prior to and after the implementation of this water plan suggested that the regulation may contribute to fluctuations of OC compounds accumulated in bald eagles (Pittman et al. 2015). Extreme and persistent rain in 2001 resulted in the highest water levels measured in the Rainy-Namakan basin since 1968, and two extreme weather events in 2002 led to the highest water levels in Rainy lake since 1950 (International Rainy Lake Board of Control, 2002). Effects of flash flooding and persistent rain in VNP may be exasperated by the thin soils that allow for rapid surface runoff (Voyageur's National Park 2007). In response to these weather events, a performance evaluation of the 2000 prescribed water level regime was conducted from 2015-2017, and ultimately resulted in a new supplementary order for emergency weather responses in the Rainy-Namakan basin that was implemented in 2018 (International Joint Commission, 2017). This new ruling allows water managers to better respond to drought or extreme rain conditions. The drastic year-to-year differences in water level from impoundment implementation can have impacts on relative fish abundance (Erman 1973), aquatic ecology (International Rainy Lake Board of Control, 2002; Holmberg et al. 2005), and therefore the prey base for bald

eagles. Additionally, lake level in VNP is significantly and positively correlated with young of the year walleye (*Sander vitreus*) and yellow perch (*Perca flavescens*) abundance (Kallemeyn 1987). Thus, food availability and trophic structure in the park may not be consistent from year to year due to water level fluctuations, and changes in food web structure can change the rate at which organochlorine compounds bioaccumulate (Hebert et al. 2000). Bioavailability and bioaccumulation of legacy OC compounds may follow abnormal patterns within the park due to the inconsistent water level and management.

Spikes of Dieldrin residue concentrations in bald eagle plasma were of concern in VNP because of the compound's persistence in the environment and tendency to bioaccumulate (Jorgenson 2001; Pittman et al. 2015). Additionally, Dieldrin has been related to embryo lethality and sub-lethal effects in adult bald eagles (Wiemeyer et al. 1984; Coon et al. 1970). However, from 1995-2017 the park-wide annual geometric mean concentration of Dieldrin in nestling plasma was greater than the LOQ on only 6 occasions (1995, 1998, 2000, 2003, 2009, 2010) (Figure 11). The elevated concentrations in back-to-back years from 2009-2010 suggested that there may be a new source of Dieldrin in the VNP area (Pittman et al. 2015). However, further investigation of this possibility revealed that Dieldrin levels subsided in VNP from 2011-2017 (Figure 11), and were similar to those reported in other parts of the mid-western United States in the 1990s (3.0-5.1 µg kg^{-1}) (Donaldson et al. 1999). Dieldrin was only detected at concentrations greater than the LOQ in 3% of samples and the geometric means for all years from 2011-2017 were below the MDL for Dieldrin (Figure 11). Although there were too few Dieldrin detections for formal statistical

analyses, it appears that the spikes in 2009 and 2010 were natural fluctuations, rather than the result of new inputs, and these fluctuations may be a result of extreme rainfall events releasing either sediment or soil bound legacy Dieldrin.

Total PCBs and 4,4'-DDE followed similar patterns during 2011-2017. Both total PCBs and 4,4'-DDE were consistently below the NOAECs for bald eagle productivity established by Bowerman et al. (2003). Therefore, despite the persistence of these compounds in the park, they are not likely generating population level effects. This is supported by the steady growth of the Voyageur's eagle population from less than 10 mating pairs in the late 1970s to over 48 mating pairs in 2016 (Cruz et al. 2017). Total PCBs and 4,4'-DDE residue concentrations in eagle nestlings were lower than those reported in eagles by others on Great Lakes shorelines in Wisconsin and Michigan, and on the upper Mississippi River (Dykstra et al. 2010; Weirda et al. 2016). Eagle nestlings on the St. Croix River had similar residue concentrations of 4,4'-DDE and total PCBs to nestlings in VNP (Dykstra et al. 2010). It is noteworthy that 4,4'-DDE and total PCBs have decreased appreciably from levels in VNP at the initiation of this biomonitoring program. From 1989-1992, the geometric mean of total PCBs in VNP was 47.36 µg kg^{-1} (Bowerman 1993), and has decreased 64% to 16.91 µg kg^{-1} (2011-2017 geometric mean) (Figure 6). Similarly, the geometric mean 4,4'-DDE concentration in VNP from 1989-1992 was 20.28 µg kg^{-1} (Bowerman 1993) and has since deceased 84% to 3.45 µg kg^{-1} (2011-2017 geometric mean) (Figure 10). However, although sizeable changes are detectible over the 28 years of monitoring, short term changes from 2010 to 2017 (during this study) in 4,4'-DDE (6.36-6.25 µg kg^{-1} respectively) and total PCBs (13.84-19.29 µg kg^{-1} respectively) are not easily

discernable. This emphasizes the importance of long-term monitoring of OC compounds; while minor fluctuations over time can be detected, long-term data sets are required to detect gradual changes due to the characteristically slow degradation rates of POPs.

Point sources of DDT in the developed world were largely eliminated in the 1970s by new legislation (Mowbray 1988; Goldberg 1991). Although levels fluctuated throughout the course of this study, total DDTs, and 4,4'-DDE exhibited significant downward trends, which is common to previous analyses. It is not surprising that total DDTs and 4,4'-DDE follow the same trend because 4,4'-DDE is the dominant constituent of total DDTs in VNP (Table 8). Significant decreasing trends have been recorded in the Great Lakes as well. 4,4'-DDE declined significantly in bald eagle nestlings from 1987 to 2008 (Weirda et al. 2016) and in lake trout from 1970-2003 in Michigan (Carlson et al. 2010). The significant decreasing trend of the already low total DDTs in VNP emphasizes the issue of data censoring and NDs. If DDTs continue to decrease, it will be increasingly difficult to quantify less-prevalent DDT-derived compounds and calculate reliable estimates of total DDTs. Additionally, the consistently high ratio of 4,4'-DDE to total 4,4'-DDT-derived compounds suggests that DDT compounds in VNP are a relic of historic, rather than recent, use (Table 8). The 4,4'-DDE metabolite contributed over 90% of the DDT-originating compounds from 2011-2017. When DDT is metabolized, it is converted into many degradation products, but the DDE metabolite is the most stable and not often excreted by biota (Aguilar 1984). It has been demonstrated that the proportion of the parent-compound DDT decreases with time, while the proportion of the metabolite DDE increases (Aguilar

1984). However, without new inputs or sources of DDT, the concentration of DDE cannot continue to increase beyond the historic contributions. Instances where DDT levels, relative to DDE, increased have been attributed to new inputs such as atmospheric deposition, illegal use, or DDT-impurities in the pesticide dicofol (Hitch and Day 1992; Tavares and Costa 1999; Qiu et al. 2004; 2010; Turgut et al. 2009; Muñoz-Arnanz and Jiménez 2011). The ratio of DDE to total DDTs in marine mammals in 1984 ranged from 0.47-0.62 and significant increasing trends over time were observed (Aguilar 1984). Soils with only legacy exposure to DDT in California had DDE:DDT ratios of up to 0.83 (Hitch and Day 1992). Thus, it is likely that historic use is the primary source of DDT-derived compounds in VNP.

Contrary to DDT compounds, there was not a significant decreasing trend of total PCBs in VNP from 1995 to 2017. Nonetheless, levels remained below the NOAEC (36.4 µg/ kg^{-1}) for bald eagle productivity for the entirety of this longitudinal biomonitoring program. Bayesian assessments of PCB residue concentrations in a variety of fish species in the Great Lakes indicated that PCB levels declined gradually from the 1970s to the 1990s after point sources were eliminated (Visha et al. 2018). After this initial decline, the rate of PCB removal slowed and concentrations reached steady state within species with sizeable year-to-year variation (Visha et al. 2018). This model is consistent with our findings in fish-consuming bald eagles and could partially explain the annual variations in PCB residues.

Low concentrations of PCBs and DDTs can cause sublethal and adverse effects on wildlife at the individual and population levels (Best et al. 1994; Bowerman et al. 1995; 2003; Dykstra et al. 2001; 2005; Elliot et al. 1995; Elliott and Norstrom 1998).

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Despite the decreasing trends of legacy OC compounds in VNP, consistent monitoring of legacy OC compounds is invaluable for characterizing and identifying areas of concern and the potential risk of sublethal effects.

5: Conclusions

Legacy DDT and PCB contamination in VNP has decreased since 1989 and the DDT in the park appears to be a consequence of historic use. Moreover, there does not appear to be novel sources of Dieldrin in the Rainy-Namakan basin influencing the VNP food web. Concentrations are approaching undetectable levels and may be reaching a steady state within the environment of VNP. The frequent changes in water resource management of the Rainy-Namakan basin could be contributing to the temporal fluctuations of contaminant residues detected in bald eagle nestlings.

Table 5: Annual bald eagle plasma samples from each impoundment

Year	Rainy Lake	Kabetogama Lake	Namakan Lake	Total
2011	6	7	6	19
2012	7	10	4	21
2013	4	6	6	16
2014	5	10	4	19
2015	5	9	6	20
2016	5	7	4	16
2017	2	4	4	10

Table 6: Number bald eagle nestling samples collected from 2011-2017 with residues detected below the method detection limit (MDL), between the MDL and the limit of quantitation (LOQ) and above the LOQ for each PCB congener.

PCB Congener	Number of Detections			>MDL (%)
	<MDL	MDL<x<LOQ	>LOQ	
8	42	67	12	65.3%
18	83	37	1	31.4%
28	1	6	114	99.2%
44	34	73	14	71.9%
52	37	65	19	69.4%
66	46	71	4	62.0%
77*	68	53	0	43.8%
101	34	84	3	71.9%
105*	1	103	17	99.2%
110	97	24	0	19.8%
118*	48	64	9	60.3%
126*	36	83	2	70.2%
128	74	44	3	38.8%
138	53	59	9	56.2%
153	27	83	11	77.7%
156*	41	77	3	66.1%
170	47	70	4	61.2%
180	32	79	10	73.6%
187	103	18	0	14.9%
195	88	31	2	27.3%
206	60	58	3	50.4%
209	65	54	2	46.3%

* Dioxin-like PCB congeners

Table 7: Annual geometric mean of total PCBs, total DDTs and 4,4'-DDE in plasma samples (calculated by assigning non-detects a value of half of the method detection limit), the range of means (non-detects assigned 0 – non-detects assigned MDL), and the range of the values measured. Kaplan-Meier mean± standard error is reported for 4,4'-DDE (because it is not a summed value) and the proportion of censored 4,4'-DDE measurements in the Kaplan-Meier assessment. When minimum value was below the method detection limit, they are noted as "ND."

Year	n	PCBs ug kg^{-1}	DDTs ug kg-1	4,4'-DDE ug kg-1	4,4'-DDE ug kg-1	
		Geometric mean ± sd (range of means) range of values			K-M mean	censored measurements (%)
2011	19	17.8±1.26 (11.2-24.3) 18.7-31.7	3.1 (0.1-5.1)* ND-11.4	1.0±3.72* (0.3-1.5)* ND-9.8	3.2±0.51	52.6%
2012	21	24.1±1.49 (17.3-30.8) 22.0-98.0	9.4 (7.5-11.2) 3.7-72.7	7.5±2.07 (7.5-7.5) 2.1-64.7	10.4±2.83	0%
2013	16	14.0±2.05 (2.8-21.3) 13.8-152.0	7.0 (0.79-9.4)* ND-150.8	3.8±5.64 (0.7-4.5)* ND-149.2	15.2±9.09	25.0%
2014	19	10.7±1.91 (0.8-18.0)* 13.8-124.3	2.8 (0.1-5.0)* ND-57.4	0.7±4.57* (0.0-1.3)* ND-55.8	5.5±3.07	73.7%
2015	20	15.3±1.48 (8.2-22.2) 16.3-61.4	3.7 (0.8-5.8)* ND-62.5	1.2±4.68* (0.0-1.7)* ND-60.9	6.0±2.98	50.0%
2016	16	17.0±1.39 (10.1-23.6) 16.9-40.4	6.0 (ND-8.0) ND-19.03	3.7±3.07 (1.3-4.0)* ND-17.5	5.8±1.03	87.5%
2017	10	19.3±1.29 (12.8-25.6) 20.0-35.3	9.5 (7.9-11.1) 3.9-17.5	6.3±1.72 (6.3-6.3) 2.3-15.9	7.2±1.32	0%

*calculated mean fell below the method detection limit

Table 8: The mean ratio of 4,4'-DDE to 4,4'-DDT in bald eagle nestling samples for each year and collectively from 2011-2017.

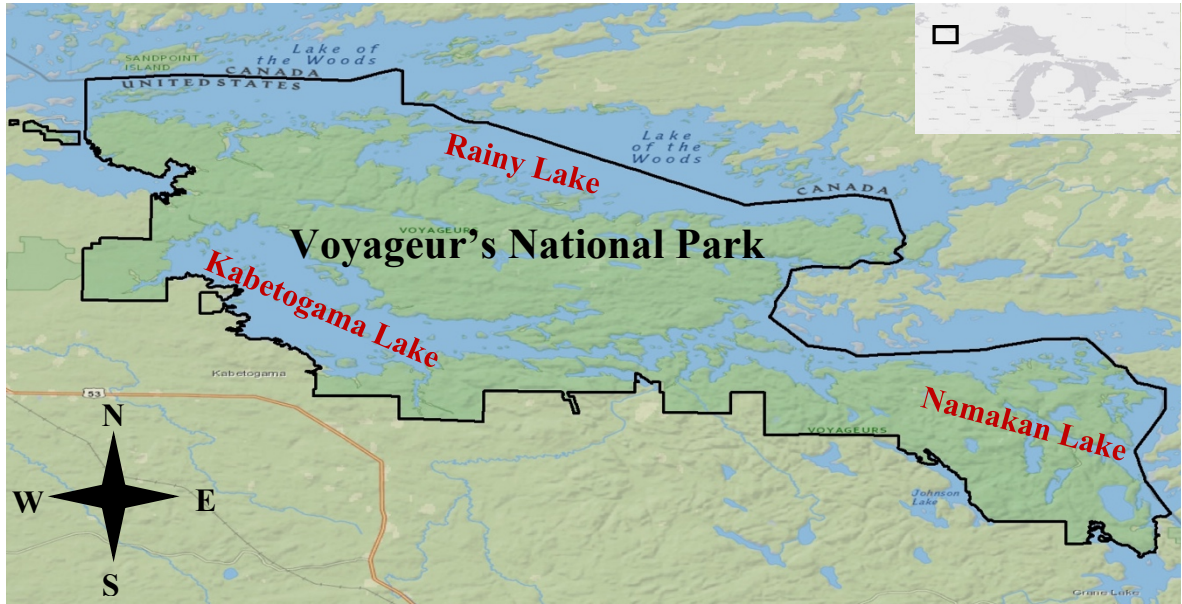| Year	4,4'-DDE/∑4,4'-DDTs
2011	91.2±7.8
2012	94.2±3.54
2013	94±12.33
2014	98.9±2.73
2015	89.2±12.2
2016	86.1±10.41
2017	86.4±4.74
2011-2017 Average	91.9±9.43

Figure 5: Map of Voyageur's National Park generated using the National Geographic base map in Arc Maps 13 and boundary data retrieved from the National Parks Service.
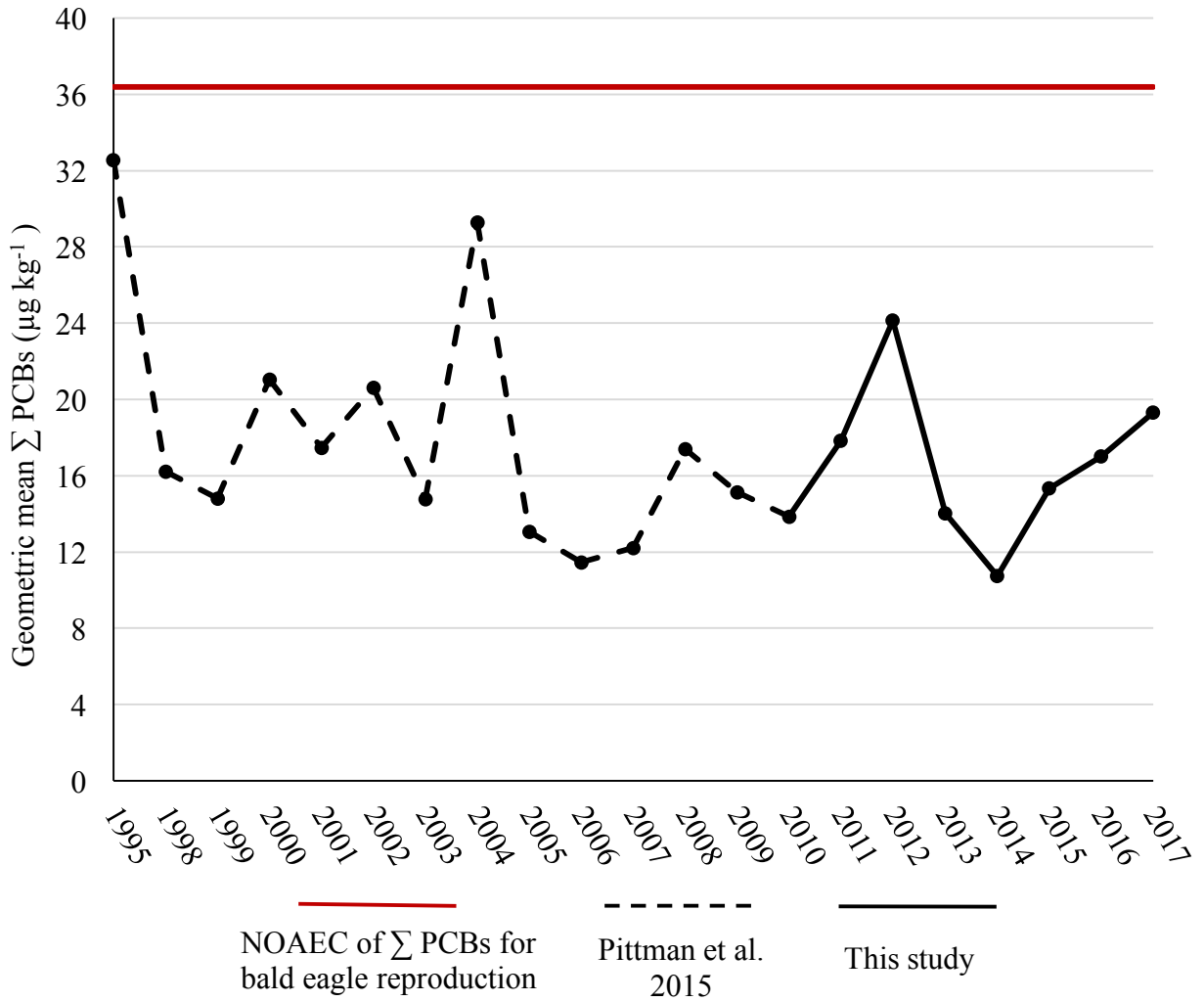
Figure 6: Annual geometric mean total PCBs from 1995-2010 (data from Pittman et. al 2015) and 2011-2017 calculated by assigning non-detects half of the method detection limit. The NOAEC of total PCBs for bald eagle productivity (36.4 µg kg^{-1}; Bowerman et al. 2003) is included.
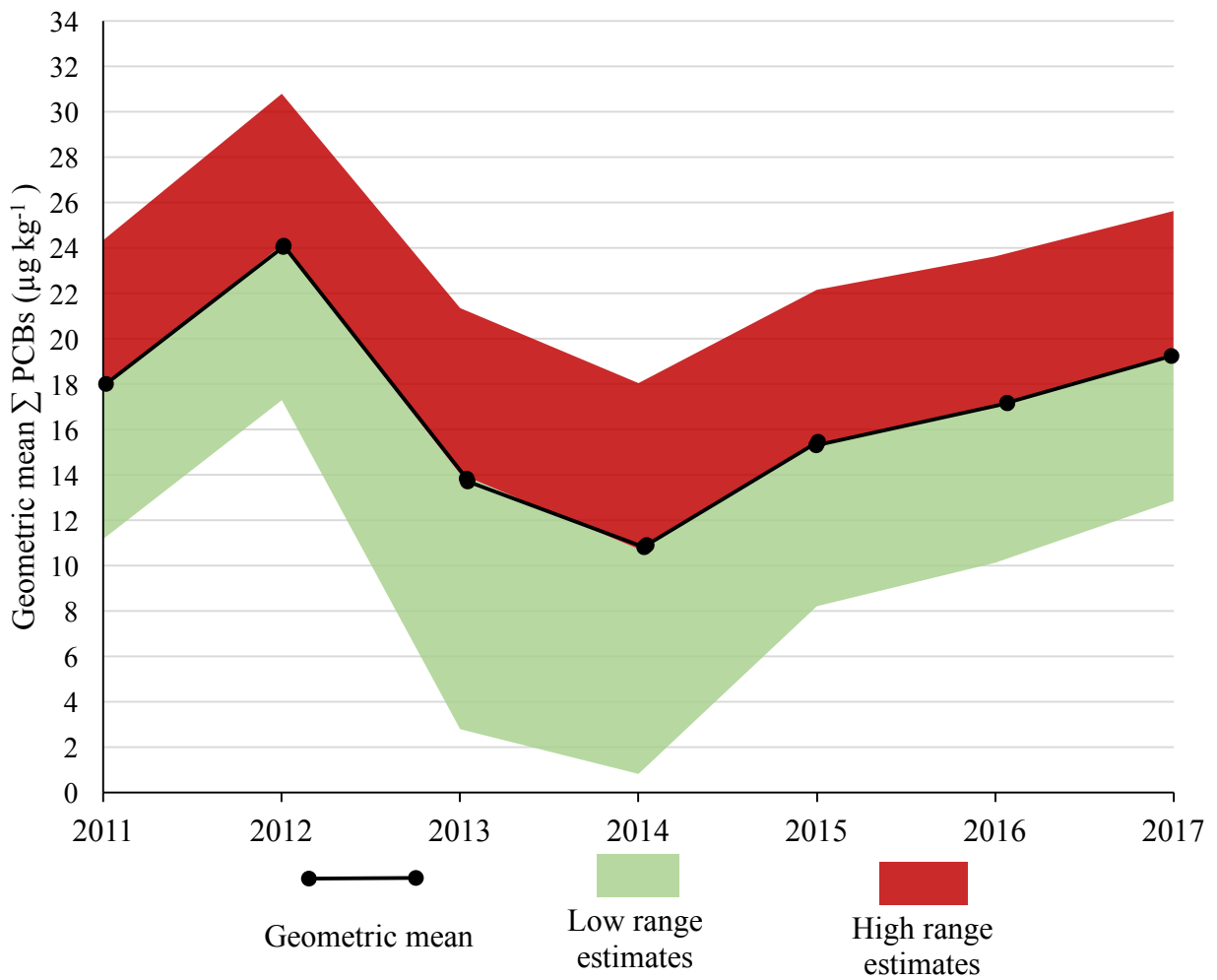
Figure 7: Annual geometric mean total PCBs calculated by assigning non-detects half of the method detection limit. High range (maximum) estimate calculated by assigning non-detects the method detection limit. Low range minimum estimate calculated by assigning non-detects a value of nearly 0 (0.0001).
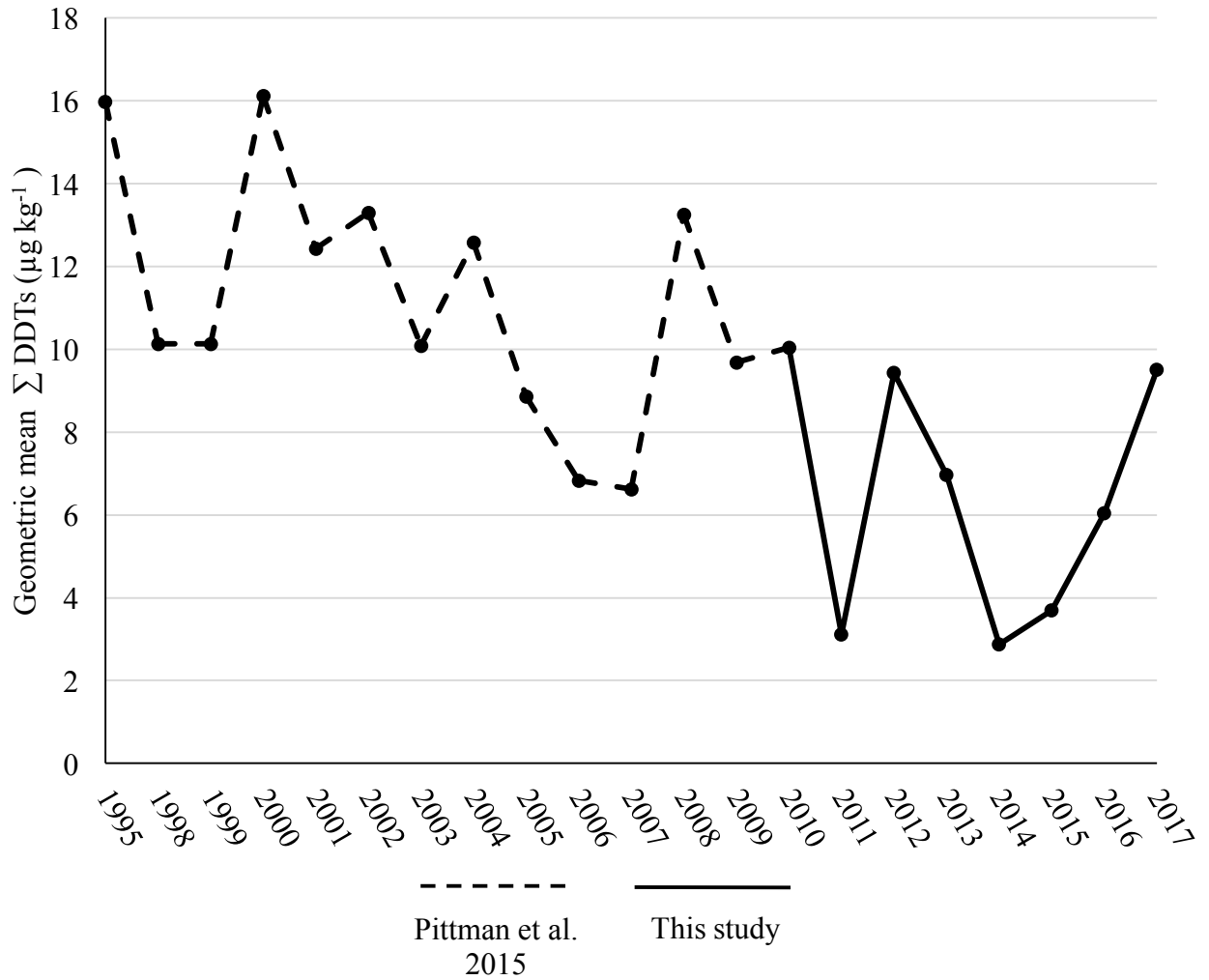
Figure 8: Annual geometric mean total DDTs from 1995-2010 (data from Pittman et. al 2015) and 2011-2017 calculated by assigning non-detects half of the method detection limit.
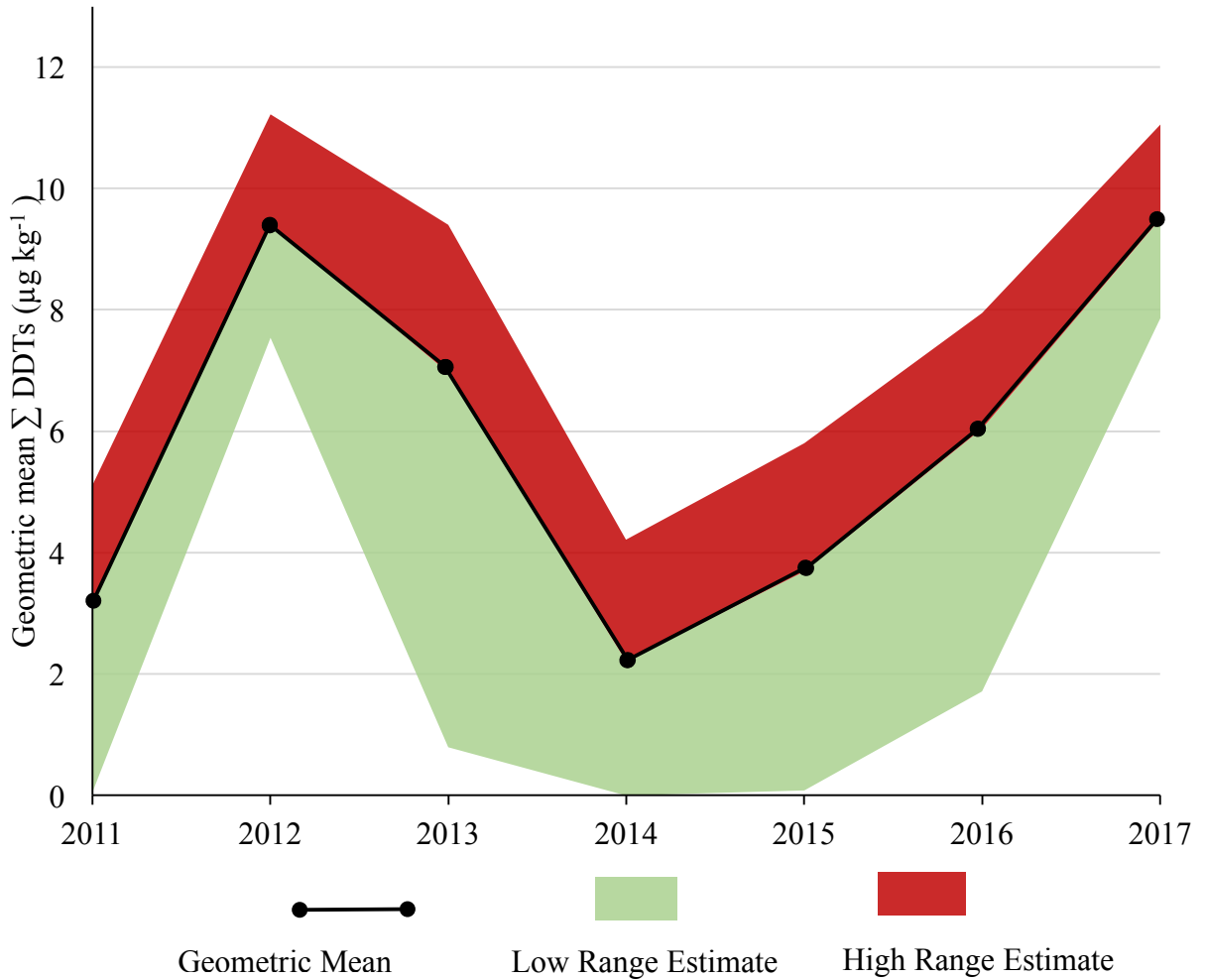
Figure 9: Annual geometric mean total DDTs calculated by assigning non-detects half of the method detection limit. High range (maximum) estimate calculated by assigning non-detects the method detection limit. Low range minimum estimate calculated by assigning non-detects a value of nearly 0 (0.0001).
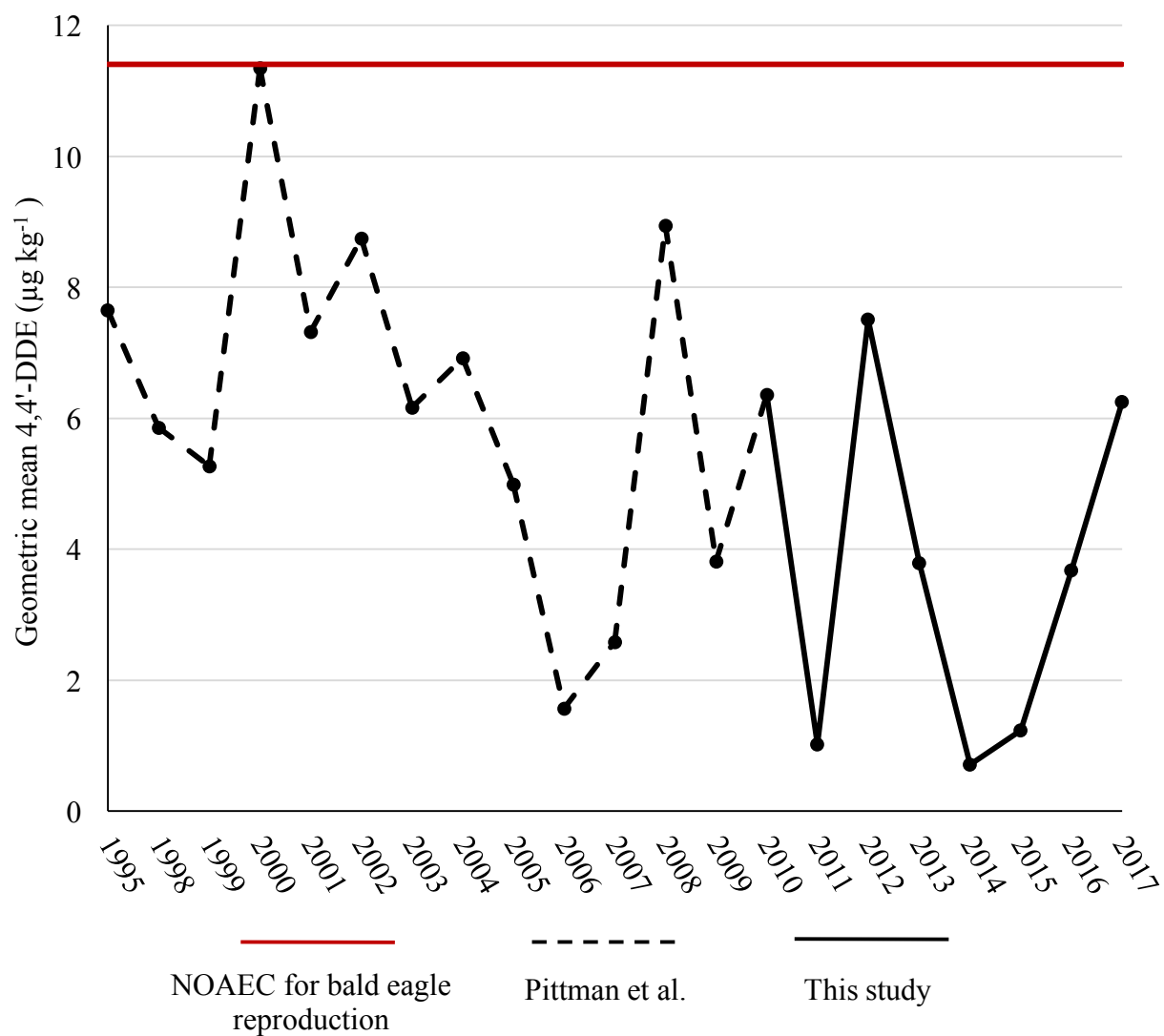
Figure 10: Annual geometric mean 4,4'-DDE from 1995-2010 (data from Pittman et. al 2015) and 2011-2017 calculated by assigning non-detects half of the method detection limit. The NOAEC of 4,4'-DDE for bald eagle productivity (11.4 µg kg^{-1}; Bowerman et al. 2003) is included.
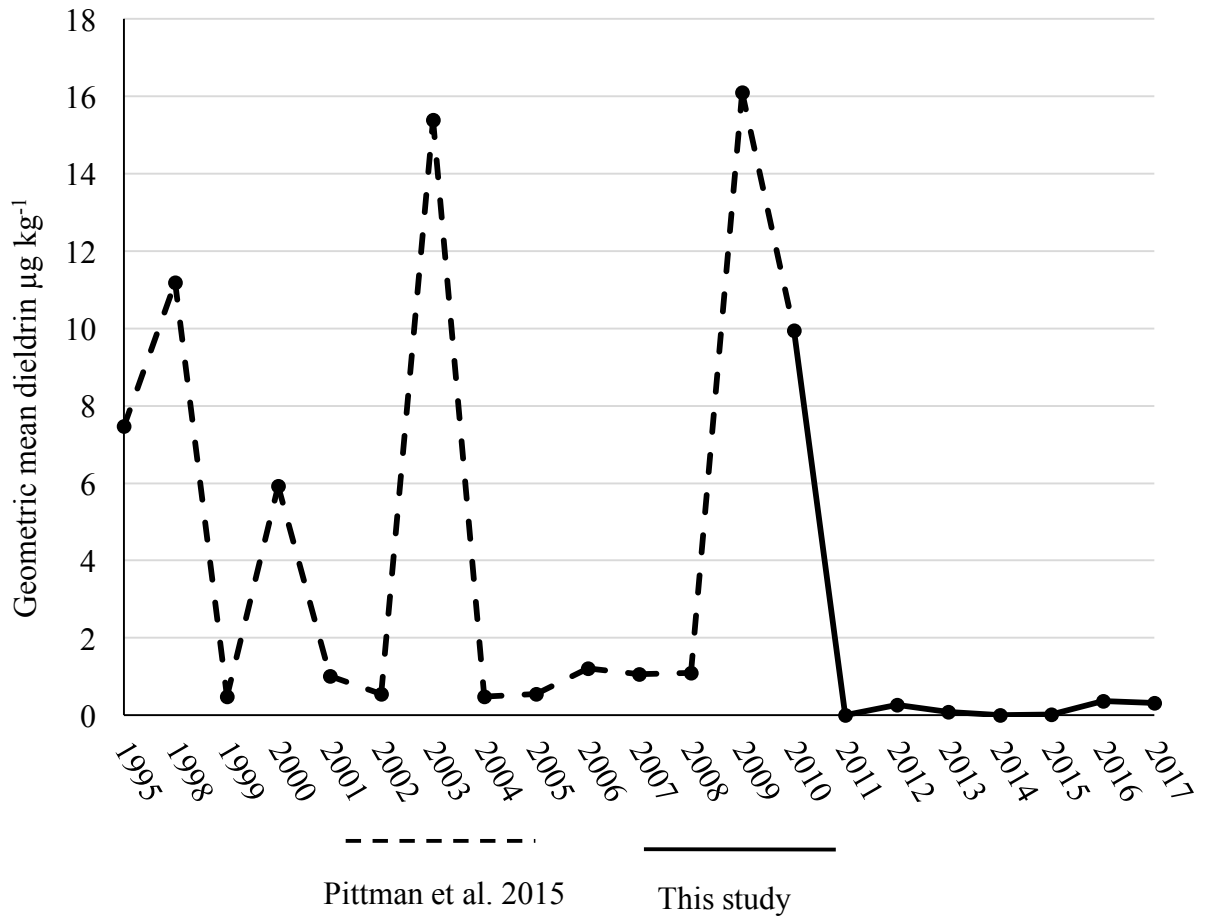
Figure 11: Annual geometric mean Dieldrin from 1995-2010 (data from Pittman et. al 2015) and 2011-2017 calculated by assigning non-detects half of the method detection limit.

Chapter 4: Summary

Environmental levels of organochlorine compounds have slowly decreased since the 1970s. Organochlorine compounds used historically are still present in the Voyageur's National Park (VNP) ecosystem, biomagnifying in the food web, and accumulating in bald eagles. However, we demonstrated that the concentrations are not great enough to impact bald eagles at a population level and do not vary among the impoundments in the National Park. Evidence of gradual declines of environmental organochlorine concentrations in VNP and elsewhere are encouraging. The newly implemented water management regime by the International Joint Commission will help dam managers better respond to extreme weather events and control water level, which may decrease the bioavailability of legacy organochlorine compounds in soils and sediments to the aquatic food web in VNP. Nevertheless, the continued use of some organochlorine compounds in the developing world and provides an ongoing source of these contaminants globally. Therefore, continued investigation of the distribution, persistence, and effects of organochlorines should be conducted world-wide.

The stability of organochlorine analytes in commercially available Oasis ® HLB solid phase extraction cartridges offers an opportunity for optimizing international biomonitoring of organochlorine compounds. We determined that the analytical accuracy and precision of measurements after storing extracts within the cartridges for 84 days does not change, which suggest that analytes can be extracted and stored within extraction cartridges for 3 months while being transported internationally and prepared for analysis. Using this method for extraction eliminates the need to transport biological samples across international borders which could

accelerate contaminant biomonitoring. Future research should be conducted to validate the storage capabilities of Oasis ® HLB cartridges using field collected plasma samples with different chemical makeup than the samples used here. Additionally, extracts from stored Oasis ® HLB cartridges should be assessed for the presence of avian influenza virus in order to confirm that the transport of these cartridges is epidemiologically safe.

The long-term monitoring of organochlorine compounds in the bald eagle population of VNP demonstrates the long-lasting effects of these compounds, and the potential for gradual environmental recovery after their removal from use. Continued use of organochlorine compounds for vector management may be negatively impacting non-target species in the developing world. The stability of organochlorine analytes in commercially available solid phase extraction cartridges offers an opportunity to monitor organochlorine compounds globally and more efficiently.

Appendix
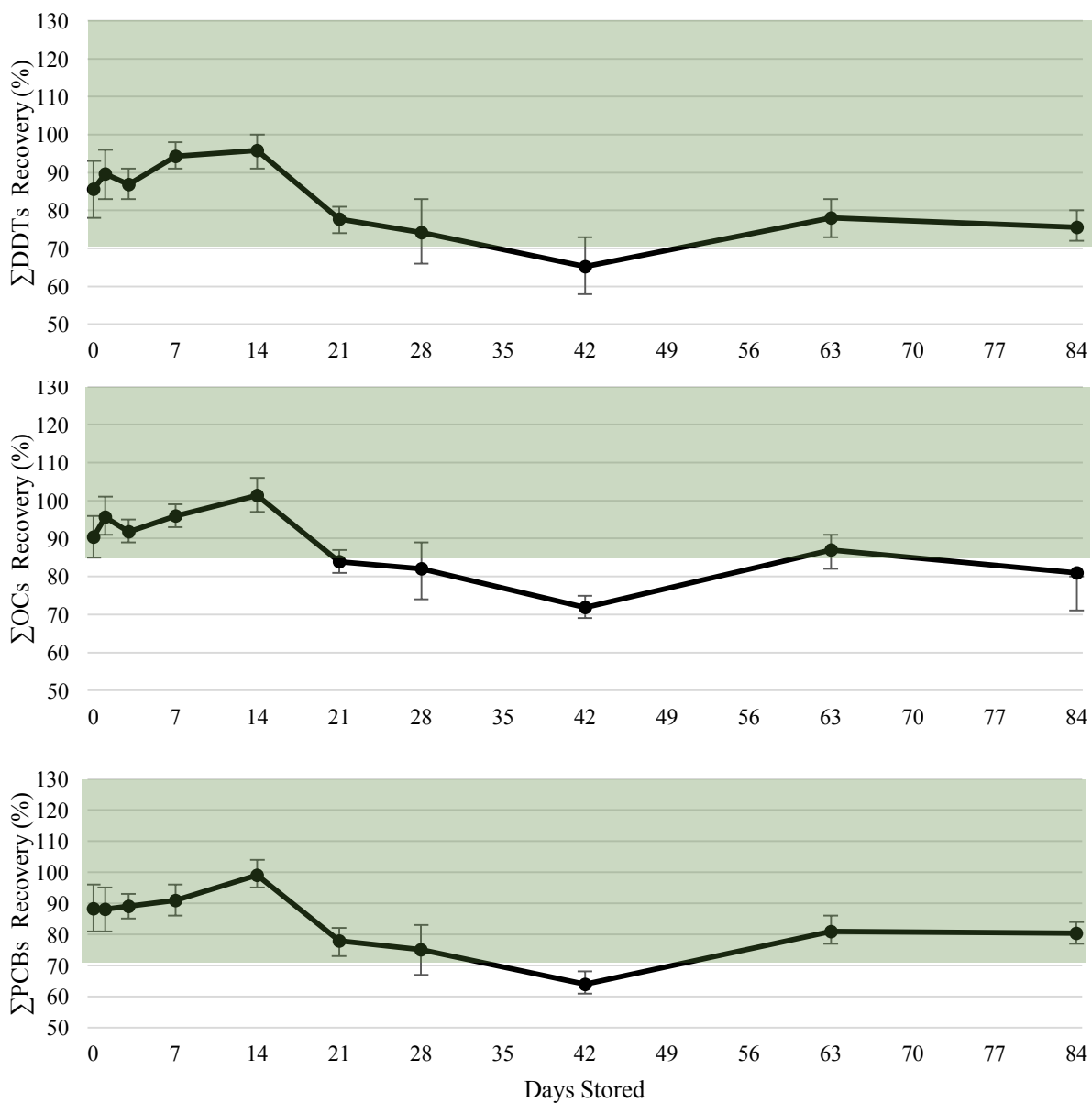
Appendix Figure 1: Nominal recovery of compounds stored in Oasis® HLB cartridges from 1-84 days. Recovery is calculated based on the concentration of total PCBs, OCs, DDTs in the spike solution added to chicken plasma. Error bars represent the 95% confidence interval of the mean.

Appendix Table 1: Mean nominal percent recovery and 95% confidence interval for the mean of total DDTs, OCs, and PCBs at each storage time point.

Treatment (days)	Mean nominal percent recovery (95% confidence interval of the mean)		
	DDTs	OCs	PCBs
0	86% (78-93%)	90% (85-96%)	88% (81-96%)
1	90% (83-96%)	96% (91-101%)	88% (81-95%)
3	87% (83-91%)	92% (89-95%)	89% (85-93%)
7	94% (91-98%)	96% (93-99%)	91% (86-96%)
14	96% (91-100%)	101% (97-106%)	99% (95-104%)
21	78% (74-81%) [B]	84% (81-87%) [B]	78% (73-82%) [B]
28	74% (66-83%) [AB]	82% (74-89%) [B]	75% (67-83%) [AB]
42	65%* (58-73%) [AB]	72%* (69-75%) [AB]	64%* (61-68%) [AB]
63	78% (73-83%) [B]	87% (82-91%) [B]	81% (77-86%) [B]
84	76% (72-80%) [B]	81% (71-80%) [B]	80% (77-84%) [B]

*indicates that the treatment mean was significantly different than that of the untreated controls
[A] indicates lower confidence limit falls outside of the QAQC acceptable range
[B] indicates that the lower confidence limit falls below the confidence limit of the untreated controls

Appendix Table 1: Organochlorine analytes and limits of detection and quantitation

Organochlorine Analyte	Detection Limit	Quantitation Limit
2,4'-Dichlorodiphenyldichloroethylene (DDE)	0.63	2.01
4,4'-DDE	0.63	2.01
2,4'- Dichlorodiphenyldichloroethane (DDD)	0.63	2.01
4,4'-DDD	0.63	2.00
2,4'-Dichlorodiphenyltrichloroethane (DDT)	0.63	2.01
4,4'-DDT	0.63	2.01
Dieldrin	0.63	2.01
PCB 8	0.62	1.98
PCB 18	0.62	1.98
PCB 28	0.63	1.99
PCB 44	0.62	1.98
PCB 52	0.62	1.98
PCB 66	0.63	2.00
PCB 77	0.63	2.00
PCB 101	0.63	2.00
PCB 105	0.62	1.98
PCB 110	0.63	2.01
PCB 118	0.63	1.99
PCB 126	0.63	2.00
PCB 128	0.63	1.99
PCB 138	0.63	2.00
PCB 153	0.63	1.99
PCB 156	0.63	2.01
PCB 170	0.62	1.98
PCB 180	0.63	2.00
PCB 187	0.62	1.98
PCB 195	0.63	2.00
PCB 206	0.62	1.98
PCB 209	0.63	1.99

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