ABSTRACT

Approaches to Understanding the Cumulative
Effects of Stressors on Marine Mammals

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In 2017, The National Academy of Sciences reported on the approaches to understanding the cumulative effects of stressors on marine mammals and suggested the use of baleen whale earplugs to evaluate both stress and stressors. The objective of this dissertation is to investigate historic and current organic and inorganic contaminants within the marine ecosystem to better understand the cumulative effects of stressors on marine mammals. Simultaneously, this dissertation also demonstrate links between.

The long-term health impact of organic contaminants was assessed in harbor porpoises off the coast of Washington State. This study focused on the POP burden within the blubber of three females (two adults and one juvenile), one of which had a B-cell lymphoma. POP blubber concentrations from different life stages were used to assess the lifetime POPs burden. The juvenile porpoise had the highest contaminant burden followed by the adult female with lymphoma and the nonlymphoma adult.

POP life history exposure profiles were reconstructed using baleen whale earplugs. This earplug study expands upon previous earplug studies of both spatial and

temporal trends. POPs analysis using earplugs provided contaminant data 30 years prior to the first reports in marine species. Chemical exposure profiles and bioaccumulation rates were reconstructed for a total of six earplugs from the North Pacific and Atlantic Ocean basins. Bioaccumulation rates were found to be 56 times higher in the North Pacific compared to the North Atlantic, suggesting a higher risk of exposure in the North Pacific.

Lastly, a single earplug was used to investigate temporal profiles of inorganic elements. The final study used a single fin whale earplug to produce more than 1,600 data points, which were used to reconstruct 48 distinct profiles of toxic, essential, nonessential, rare earth, and other non-biologically relevant elements. This research, in conjunction with concurrent studies, aimed to examine stress profiles and other stressors (e.g., reproduction and whaling; see appendix). Earplug data from this study provides insight into biological and biogeochemical processes as well as preliminary data for further elemental analysis of more earplugs.

Approaches to Understanding the Cumulative
Effects of Stressors on Marine Mammals

by

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ATTRIBUTIONS

Drs. Sascha Usenko and Stephen J. Trumble assisted with conceptualization, funding acquisition, and mentorship. Zach C. Winfield assisted in the sample preparation, extraction, analysis, and writing of Chapters Two, and Three, as well as the data interpretation and writing of Chapter Four. Dr. Stephanie Norman assisted with conceptualization, sample and funding acquisition, and the writing of Chapter Two. Additionally, Dr. Barry H Rickman, Matthew Klope, Susan Berta, Sandra Dubpernell, Howard Garrett, Mary Jo Adams, Dyanna Lambourn, Jessica L Huggins, Dr. Nadine Lysiak, Dr. Adelaide E Clark, and Rebel Sanders assisted with sample analysis and interpretation. For Chapter Two, only the abstract, introduction, and results and discussion related to contaminants in marine mammals were included. Farzaneh Mansouri assisted with sample analysis of Chapter Three and assisted in the interpretation of Chapter Four. Danielle D. Crain assisted with the delamination of earplugs used for Chapters Three and Four. Dr. Martin Shafer and his team performed elemental analysis and data interpretation for Chapter Four. Richard Sabin and Charles Potter provided museum samples for Chapters Three and Four.

To the memory of my grandfathers, Darwin Eugene Winfield, and Lindon Edmond Hodges, who always encouraged me to ask questions.

CHAPTER ONE

Introduction

Marine mammals are considered both ocean engineers[1] and sentinels[2] within their ecosystem. Marine mammals can face a multitude of stressors due to the anthropogenic activity, from chemical and noise pollution, loss of habitat, entanglement, whaling, and ship collision over their entire lifetime. Scientists must consider the full impact of these anthropogenic stressors on populations or ecosystems, and whether the additional activity could pose a threat that would result in population decline. There have been many efforts made to understand the response of marine mammals to stressors. However, it is difficult to determine the contribution to stress over time from multiple stressors that vary in both magnitude and duration. This is especially true for long-lived mammals, such as baleen whales, which can live for decades to centuries. For example, an 80-year-old blue whale living in the Atlantic Ocean could experience periods of industrial whaling, an increase in ocean noise, (i.e., acute and chronic exposure), and global pesticide use (i.e., long-term organic contaminant exposure), and climate change.

Typically, studies focus on cortisol (and related stress hormones) to study stress in marine mammals. A stress response from either an anthropogenic or natural stressor will impact the hypothalamic-pituitary-adrenal (HPA) axis and result in elevated glucocorticoid levels within wildlife.[3] The HPA axis is responsible for the release of glucocorticoids into the bloodstream and moderating levels through negative feedback.[4] Once released, glucocorticoids bind to tissue receptors and alter the expressions of genes

that affect multiple physiological processes, including metabolism. Animals that are constantly exposed to stressors are expected to have higher baseline levels of glucocorticoids, which increases the glucocorticoid response to additional stressors, and lengthens the time for levels to return to baseline.[5] Measurements of glucocorticoids represent a possible proxy for cumulative stress and health in marine mammals as the glucocorticoids are produced as the results of a variety of stressors.

In 2017, The National Academy of Sciences (NAS) reported on the approaches to understanding the cumulative effects of stressors on marine mammals.[6] NAS distinguished between two kinds of stressors: intrinsic, which is an internal factor or stimulus that results in a significant change to an animal's homeostatic condition, and extrinsic, which is a factor originating from the external environment that produces a stress response within an animal, see Table 1.1. NAS also described ecological drivers, which is an abiotic or biotic feature of the environment that affects multiple components of the ecosystem which either directly or indirectly introduce multiple extrinsic stressors.

Extrinsic stressors, described by NAS include contaminants, ship strikes, entanglement, noise, and psychological factors. The above-mentioned ecological drivers are also considered extrinsic factors and include the loss of keystone or foundation species (i.e., a species that is largely depended on by its ecosystem), recurring climate patterns (i.e., El Niño), and climate change. Intrinsic stressors include pregnancy, lactation, migration, molting, fasting (e.g., during capital breeding season). NAS's list was not all-inclusive, however, the above stressors were included as they have a likelihood to interact with other stressors.

Table 1.1 Partial recreation of examples of types of stressors from the National Academy of Sciences Report.

Stressor Type	Examples
Intrinsic Stressor	Pregnancy, lactation, migration, molting, fasting (e.g., during the breeding season in capital breeders)
Extrinsic Stressor	Anthropogenic: Pollutants, ship strike, entanglement, noise, psychological factors (e.g., perceived threat)
	Natural, but potentially influenced by anthropogenic activity: Harmful algal blooms, resource limitation, predator pressure, pathogens, temperature, salinity, naturally occurring chemicals, intra- or interspecific competition
Ecological Drivers	Loss of keystone or foundation species, recurring climate patterns such as El Niño, climate change

To evaluate stress and stressors within marine mammals is no simple task, however, in recent years, research at Baylor University, as well as external collaborators, has developed into a multiple stressors and stress platform. Currently, research is being performed to investigate ocean productivity (using carbon and nitrogen stable isotopes), diet (using fatty and amino acids), pregnancy (particularly rates using progesterone), and stress (using cortisol). The goal of this dissertation is to investigate the contributions of contaminants within the marine environment, specifically persistent organic pollutants, and inorganic elements (many of which are known toxins), by reconstructing life-time profiles using age estimates and chemical analysis of baleen whale earplugs. Using reconstructed chemical and elemental profiles, this study investigates maternal offloading, spatiotemporal trends, and bioaccumulation rates of pollutants. The results of this study will then be paired with stress measurements in future studies.

Persistent Organic Pollutants

Persistent organic pollutants (POPs) are chemical contaminants, described as an extrinsic stressor, that have a long half-life in the marine environment (i.e., persistent), bioaccumulative (i.e., increase in concentration over the entire lifetime of an individual due to limited excretion pathways or a lack of degradation) and are known to cause reproductive impairment or developmental effects[7-9], immune dysfunction, disease susceptibility[10-12], as well as limited endocrine disruption.[13-16] POPs include historic pesticides (e.g., dichlorodiphenyltrichloroethane[17] (DDT) and chlordane), industrial-use chemicals (e.g., polychlorinated biphenyls[18] (PCBs)), and emerging contaminants of concern (e.g., polybrominated biphenyl ethers (PBDEs)[19], previously used as a flame retardant). These compounds are bioaccumulative due to their lipophilicity and their affinity to the lipid-rich blubber of marine mammals.[20] PCBs, DDT, and its metabolites dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD), are frequently found in the tissues of marine mammals all over the world. They are particularly higher in concentrations in coastal regions near heavy development or industry.[21-24] DDT and PCBs have become a health concern for marine mammals as they are related to a variety of adverse health effects. PBDEs have also been shown to have similar toxic effects on the endocrine system as PCBs and have also been reported to cause development issues related to learning and memory impairment in rodent studies.

The effect of stressors depends on the location of the individual, their vulnerability to stressors at their given life-stage, as well as the relative contributions of other stressors within their environment. For marine mammals living during the 1930s – present, the exposure to POPs can potentially lead to both development and long-term

health concerns. Typically, POP concentrations in blubber increase over the animal's lifetime, though there is some evidence of variation due to parturition followed by lactation and seasonal changes in blubber thickness. Parturition and lactation both result in the transfer of POPs to the mother's offspring, also known as maternal offloading, which may be particularly harmful during periods of development (i.e. pups or juvenile calves).[27, 28] Seasonal blubber changes result in the mobilization of POPs from blubber to bloodstream where POPs can reach target organs that trigger adverse health effects[6]. Seasonal blubber changes and maternal offloading highlight the temporal variations that contribute to stress over an individual's life. Additionally, marine mammals, such as baleen whales, have long lifespans and are highly migratory, process hundreds of tons of food a year, which all could contribute to a variety of chemical and elemental exposure scenarios. For example, individual whales with lifespans spanning multiple decades may be exposed to a variety of POPs and POP concentrations resulting in an array of exposure scenarios. Spatially, POP concentrations may also vary between geographic regions of heavy production and use, and remote locations via long-range atmospheric transport.[29-31] Some marine mammals have wide ranges within their resident ocean basin and these long-distance movements can introduce different chemical stressors as they transition from one location to the next. Alternatively, populations with limited ranges are likely to be continuously exposed to specific stressors found within their specific region.[32]

Long-term exposure to POPs is associated with a wide range of health effects, briefly described above. Nonlethal stressor exposure can affect an animal's ability to maintain baseline cortisol levels and reduce their ability to respond to stressors. POPs have been suggested to disrupt the HPA axis and interfere with glucocorticoid receptors,

or the synthesis of adrenal steroids[33-35], however further studies are needed as multiple biochemical pathways can be impacted by POPs and other contaminants (e.g., toxic elements such as cadmium, lead, and mercury).

In humans, POPs are known to have immunosuppressive effects as there is evidence of a greater chance of infection in relation to POP exposure.[36, 37] Maternal offloading of POPs from mother to offspring during developmental periods is a cause of concerns as POPs have been found to affect the brain and central nervous system. PCBs have been linked to developmental effects in human offspring which resulted in a reduction of cognitive function.[38-41] This same decrease in cognitive function in marine mammal offspring would likely reduce their chance of survival in their early life stages as their survival is dependent on their response to predators and their foraging efficiency following weaning. In addition to maternal offloading, individuals that lived during the active usage of mass production of POPs could also offload contaminants to their offspring that are now found in lower concentrations than those present in the current environment, thus contaminating current generations with historic chemicals.

Inorganic Contaminants and Elements

Elements occur naturally within the Earth's crust and enter the marine ecosystem by atmospheric deposition, run-off, shelf sediments, hydrothermal vents, and ocean crust.[42] Though ubiquitous within the marine ecosystem, elements are not spatially homogeneous and small anthropogenic contributions can cause increased concentrations within local biota due to biomagnification.[43, 44] Similar to POPs, there are two major routes of elemental intake for marine mammals including, maternal offloading (i.e., across the placenta and during lactation) and their food.[45] Following weaning, the uptake

of metals is predominantly from food, and specific areas of the world may show higher concentration within marine mammals as a result of anthropogenic and natural sources. For example, studies have found that mercury concentrations were higher in dolphins originated from the Mediterranean Sea when compared to dolphins originating from Japan or the north-east Atlantic.[46-48] This difference was attributed to the larger concentration occurring naturally in the Mediterranean Sea.[47] For the purpose of this dissertation, metals/elements studied have been divided into five classes: toxic, essential, nonessential, rare earth elements, and other non-biologically relevant elements. Essential and nonessential elements are related to biochemical processes[49] (chemical processes within living organisms) and or biogeochemical cycles[50] (flow of elements and compounds between the biological and physical environment).

Many studies have reported elemental burdens, particularly elements that are considered toxic (e.g. immunosuppressive) and have known adverse health effects.[51],[52] The majority of toxic metal studies focus on mercury and cadmium in liver, muscle, or kidneys[53] and often report susceptibility to disease[54-56] and neurotoxicity[57]. The following elements have a chemical species with a potential to be neurotoxic including aluminum (Al), arsenic (As), bismuth (Bi), cadmium (Cd), lead (Pb), lithium (Li), manganese (Mn), mercury (Hg), selenium (Se), tellurium (Te), and thallium (Tl)[58, 59]. Many of these trace elements use the same mechanism to impair the nervous system. For example, Hg, Cd, and Cu can alter the electron transport chain which leads to cell death.[60]

In most cases, metals and metalloids are more neurotoxic in their organic form as the more fat-soluble compounds can better cross the blood-brain barrier easier.[61] Most

notable, methylmercury (MeHg), an organic form of Hg (and one of the most heavily studied environmental pollutants), crosses this barrier and damages the central nervous system, which can result in sensory and motor decline and behavioral changes.[62] The severity of the neurotoxicity is dependent on multiple factors, including age (early developmental periods), sex, and the mixture of pollutants. Early exposure to neurotoxins has long-lasting health impacts on mammalian systems. Juvenile marine mammals are more vulnerable to neurotoxic chemicals as the blood-brain barrier is not fully formed and the developing structures are more sensitive.[58] For example, Cd is capable of passing through the blood-brain barrier during gestation and post-partum but is unable to cross an adult's blood-brain barrier.[63] The sex of the individual alters the development of the brain through genomic and endocrine mechanisms which also influences the neurotoxicity of elements[64, 65] as well as the behavioral and metabolic differences between females and males.[57] There is evidence to support that seabirds and marine mammals have means to convert MeHg to its less toxic inorganic form. When investigating the health impacts of contaminants, it is important to consider the adverse health effects of POPs and metals as both contaminant classes have been reported to increase the susceptibility to disease in cetaceans.[54-56]

Chemical Extraction and Analysis

Selective Pressurized Liquid Extraction

Sample preparation can contain many steps, including sample pretreatment, extraction, and clean-up. Due to the multitude of steps, sample preparation typically requires more time and is labor-intensive, especially for the analysis of organic

contaminants in biological tissues. For example, Soxhlet extraction can take multiple hours just to extract the analytes from the matrix. Unfortunately, the more steps prior to the analysis of a sample often result in an increase in error associated with the measurement.[66] Pressurized liquid extraction (PLE) has been around for over 20 years and has specific advantages over more traditional extraction techniques such as Soxhlet including automation, extraction time per sample, and typically less solvent. PLE uses both high temperature and pressure to extract analytes from a given matrix. PLE is a three-part extraction process often performed with an Accelerated Solvent Extractor (ASE).[67] The first involves desorption of the analytes from the matrix. The second involves the diffusion of the analyte through the solvent inside the matrix. The third process involves the bulk transfer of the analyte to the flowing liquid. This is done through automation by loading a stainless-steel extraction cell into an oven. The cell is heated and filled with solvent with applied pressure to keep the added solvent in the liquid state. The solvent volume and pressure are maintained for a specified static time. Finally, the extraction solvent is removed using compressed nitrogen and collected in a bottle below. The sample can be extracted multiple times using the same or different combinations of extraction solvents. Once the specified number of cycles is finished the cell is flush with solvent and purged for a specified time with compressed nitrogen.

PLE is considered an exhaustive extraction method and often extracts target analytes along with the sample matrix, requiring a post-extraction clean-up, such as silica packed column chromatography or gel permeation chromatography. Selective pressurized liquid extraction (SPLE) was developed for the simultaneous extraction of target analytes and the retainment of matrix compounds. This is accomplished by adding adsorbent(s) to

the extraction cell below the sample homogenate.[66] Adsorbents serve to retain or destroy potential interferences. By using SPLE, post-extraction clean-up can be avoided, thereby reducing error propagation while maintaining automation. In addition, the elimination of post-extraction clean-up steps also serves to help reduce the opportunity for sample contamination.

SPLE is a preferred method when extracting organic contaminants from baleen whale earplugs, which are mainly composed of lipids and proteins. Earplugs form as the earwax is secreted from the glove finger into the ear canal. As the whale migrates, the secretion color alternates between light and dark laminae. Over the entire lifetime of the whale, the earwax forms a plug that remains preserved until removed post-mortem. Historically, researchers have counted the alternating light and dark laminae to determine the age of the individual whale. The combination of one light and dark layer is equivalent to approximately 1 y. Once aged, the earplug is delaminated, and the laminae are used for multiple chemical analyses. Using SPLE, earwax, with as little as 150 mg, can be extracted quickly and used to analyze multiple organic contaminants, including, pesticides, PCBs, and PBDEs. Following extraction and analysis, lifetime history profiles can be reconstructed using age estimates.

Using earplugs, it is possible to reconstruct contaminant profiles spanning multiple decades as a function of time and age. For other matrices, such as blubber, repeated sampling comes with many logistical hurdles as well as limitations in age estimates. When the age of a marine mammal is unknown, the body length or age group (i.e., calf/pup, juvenile, or adult) is used instead. While common practice, it is impossible to accurately determine the age of exposure of contaminants or the contributions of

location or time (e.g., usage period of POPs). Earplugs provide a unique opportunity to capture multiple age classes over many decades to reconstruct and correct for contributions of the age and lifespan of each individual. Historic earplugs have been archived within museum collections and more recent plugs have been collected from stranded whales. Earplugs from museums can be used to investigate more historic stressors and more recent plugs from individuals, some living 80 years or more, can be used to investigate both historic and current stressors.

Chemical and Elemental Analysis

Analytical techniques used to measure POPs and metals varied in accuracy over time and between laboratories.[53] Due to these differences, it is difficult to compare datasets between studies over multiple decades and between laboratories, however significant improvements in procedures have made comparing data easier over the past decades. Quality assurance, quality control, and proper sample storage and preparation were necessary to compare data and prevent contamination.

For this study, contaminant analysis was performed using a variety of analytical techniques and quality assurance and control measures to ensure consistency across analyses. POPs extraction was performed on blubber and earplugs using SPLE. Robinson et al. previously developed both extraction and quantitation methods of POPs for blubber[68] and earwax[69]. Quantification was performed using gas chromatography-mass spectrometry (GC-MS) in two different ionization modes, electron, and chemical ionization. However, due to chemical interference produced during the extraction of POPs in earwax, an additional method was developed using gas chromatography-tandem mass spectrometry (GC-MS/MS) in electron ionization mode and can be found in the

supporting information of Chapter 3. Elemental extraction was performed using mixed acid microwave-aided digestion and quantification was performed using sector field – inductively coupled plasma – mass spectrometry (SF-ICP-MS, Thermo-Finnigan, Element 2) and was performed at the University of Wisconsin-Madison, Madison, WI. Additional analysis was performed to quantify Hg and MeHg using cold vapor atomic absorption (CVAA). Details can be found in Chapter 4.

Evaluating stress and stressors in the marine ecosystem is difficult but using marine mammal tissues and secretions is possible. By analyzing contaminants in blubber, a link was established to adverse health effects (i.e., carcinogenesis)[70]. Baleen earplug analysis allows for the reconstruction of lifetime chemical profiles spanning multiple decades[71] or even centuries[72]. Further analysis of these unique samples will only expand our understanding of stress and stressors within the marine environment.

CHAPTER TWO

Persistent Organic Pollutant and Hormone Levels in Harbor Porpoise with B Cell Lymphoma

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Abstract

B-cell lymphoma, a common morphologic variant of non-Hodgkin lymphoma, has been associated with persistent pollutants in humans, but this association is not well-characterized in top-level predators sharing marine resources with humans. We characterized and compared blubber contaminants and hormones of a pregnant harbor porpoise (*Phocoena phocoena*) with B-cell lymphoma, with those in two presumed healthy fishery, bycaught porpoises with no lymphoma: a pregnant adult and female juvenile. Common historic use compounds, including polychlorinated biphenyls, polybrominated diphenyl ethers, and pesticides, were evaluated in blubber samples from three porpoises. In addition, blubber cortisol, and progesterone levels (ng/g) were determined in all three animals. Total pollutant concentrations were highest in the juvenile porpoise, followed by the lymphoma porpoise and the non-lymphoma adult. Blubber cortisol concentrations were 191% greater in the pregnant with lymphoma porpoise compared with the pregnant no lymphoma porpoise, and 89% greater in the juvenile female compared with the pregnant no lymphoma porpoise. Although both

adults were pregnant, progesterone levels were substantially greater (90%) in the healthy compared with the lymphoma adult. Health monitoring of top-level marine predators, such as porpoise, provides a sentinel measure of contaminants that serve as indicators of potential environmental exposure to humans.

Introduction

Lymphomas are malignant tumors of the lymphoid system reported in humans[73] and higher vertebrate animals.[74, 75] Two major types currently are recognized in humans: non-Hodgkins (NHL) and Hodgkins.[73] B-cell lymphoma (BCL) is a common morphologic variant of NHL (80–85% of all cases). B-cell lymphoma is documented in humans[76] and other mammals, including horses[77] and orangutans[78]. However, BCL is relatively uncommon in cetaceans[79] and has not been described in porpoises.

The incidence or diagnosis of NHLs, such as BCLs, has been increasing in humans during the latter twentieth century.[80, 81] Coincident with this time period was increased use of and environmental contamination by polychlorinated biphenyls (PCBs), before their manufacturing was banned in the United States in 1979.[82] This convergence prompted several epidemiological studies to determine that the link between PCBs and NHL resulted from several important factors: temporal correspondence between exposure to PCBs and increasing incidence of NHL; toxicological and epidemiological evidence for the immunotoxicity and carcinogenicity of PCBs; and the structural similarity between PCBs and dioxins, which are known human carcinogens that also are associated with NHL.[83] Polybrominated diphenyl ethers (PBDEs), organobromine compounds first put into commercial production as flame retardants in the mid-1960s, have raised environmental and health concerns due to their widespread use and increased production

since the 1990s.[84] The use and persistence of PCBs and PBDEs also represents a significant toxicological concern for higher trophic level organisms, such as marine mammals.[85] PCBs are associated with deleterious health effects in marine mammals, including increased susceptibility to infectious diseases, immunotoxicity, neoplasia, and endocrine disruption.[86-89]

Published evidence from human studies suggests the more highly chlorinated PCB and PBDE congeners may contribute to the risk of NHL.[90-93] In marine mammals, similar potential associations are reported in belugas (Delphinapterus leucas)[94] and California sea lions (Zalophus californianus)[87]. Although PCBs are not considered directly genotoxic, they may encourage lymphomagenesis through immune dysregulation and immunotoxicity, resulting in genetic mutations.[83, 95] In a rodent study, individual PCB congeners caused DNA adduct formation and cellular mutations. The PCBs were hypothesized to be metabolized into genotoxic metabolites in vitro and in vivo, acting as initiating agents, and inducing cancer.[96] Those PCB congeners most associated with increased NHL risk include coplanar PCBs 156, 180, and 194[92] and 118, 138, 153, and 170.[97, 98] Studies suggest PBDE-47 in adipose tissues is associated with increased risk of NHL in humans.[91, 99]

An association between pesticide exposure and incidence of lymphoma has been observed, most notably in dogs exposed to lawn pesticide treatments and humans involved in agricultural occupations[100, 101]. Within Puget Sound, recent levels of DDTs in marine fish and sediments are generally lower than concentrations of PCBs[102, 103], with ∑DDT levels measured in whole bodies of out-migrating juvenile Puget Sound Chinook

salmon (Oncorhynchus tshawytscha) more than two times lower than ∑PCB concentrations.[104]

The inland transboundary waters of Washington State have historically received large amounts of endogenous and exogenous pollutants from agricultural, industrial, and household sources.[105, 106] These included wastewater and agricultural runoff, wood and paper pulp mills, and industrial activities, such as manufacturing and gravel mining. Numerous sites within Puget Sound, Washington were heavily contaminated by PCBs from industrial activities.[103, 107] In addition to these localized sources of pollutants, more global-type sources transported these chemicals to the Salish Sea through atmospheric transport[108] and via food webs from prey fish species, such as Pacific herring (Clupea pallasii)[103] and salmon (Oncorhynchus spp.)[107].

Harbor porpoise (Phocoena phocoena) are among the top predators of the coastal trophic system in Washington's inland waters, exposing them to contaminant accumulation from the local food web. Moreover, this species and other regional marine mammals have accumulated persistent organic pollutants (POPs), such as PCBs and PBDEs.[109, 110] Prey species, such as herring, may play an important role in transferring POPs to predators at higher trophic levels due to the former's relatively high abundance, geographic distribution and lipophilic content.[103] Given their coastal inland water home range and prey preferences, harbor porpoise within the Salish Sea are a good model to investigate pollutants and potential links to disease. Therefore, the purpose of this initial study was to characterize and compare the blubber POPs, along with stress and sex hormone concentrations, of an adult pregnant harbor porpoise with B-cell lymphoma, to those in presumed healthy fishery by-caught porpoises with no lymphoma: an adult,

pregnant harbor porpoise and a young female yearling, as well as discuss the potential association between POPs and lymphoma.

Material and Methods

Harbor Porpoise Samples

Detailed pathologic examinations were conducted, and causes of death determined, for the case porpoise with lymphoma (abbreviated as adult lymphoma positive, AL+) and two comparison presumed healthy, fishery by-caught animals: an adult pregnant female with no lymphoma (adult lymphoma negative, AL-) and a female yearling with no lymphoma (YL-), all from Washington State's central inland waters and collected under stranding response permits. Full-thickness blubber samples, extending down to the underlying muscle, for the AL+ were collected from a 167 cm, female, adult harbor porpoise at a standardized site (approximately midway on the dorsum between the blowhole and just anterior to the insertion of the dorsal fin), placed in clean aluminum foil, and frozen at -20 C. This porpoise was found fresh dead, in poor nutritional condition (dorsal blubber thickness [DBT] = 1.1 cm), on 1 November 2013 on Whidbey Island and was subsequently diagnosed with B-cell lymphoma in the lungs, mediastinal lymph nodes, and spleen. She was pregnant with a 5.5 cm long, early gestational fetus in the left uterine horn. Standard blubber samples also were collected from two apparently healthy fishery bycaught porpoises (excellent nutritional condition) and frozen at -20 C. The AL- was an approximately 165-cm, adult lactating harbor porpoise (DBT = 1.6 cm) with a 7 cm fetus found 31 October 2012 in Kingston, and YL was a 110.5 cm female yearling (DBT = 2.1 cm) with blunt force trauma and hemorrhage of the skull base soft

tissues and first two cervical vertebrae from fishing gear entanglement, collected 21 September 2014 from Whidbey Island.

Contaminant Analysis

The initial analyte list screened by gas chromatography– mass spectrometry (GC–MS) consisted of 65 compounds. The final target analytes, detected in at least one of the study animals, are reported in ng/g and include six PBDE congeners (47, 49, 66, 99, 100, 153), 16 PCB congeners (101, 105, 110, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 180, 187, 189), and 15 organochlorine pesticides (alpha-, beta-, and gamma-hexachlorocyclohexane, heptachlor, heptachlor-epoxide, mirex, hexachlorobenzene, trans-chlordane, cis-chlordane, transnonachlor, cis-nonachlor, endrin ketone, o, p' -DDD, o, p' - DDE, o,p' -DDT, p, p' -DDD, p, p' -DDE, and p, p'-DDT).

Blubber aliquots were extracted using accelerated solvent extraction (ASE 350, Thermo, Sunnyvale, CA) as previously described.[68] Briefly, 100 mL cells were loaded with 5 g of silica gel and 55 g of acidic silica gel. The cells were conditioned using a 1:1 v/v mixture of dichloromethane and hexane. The full-thickness blubber punches (0.50–1 g) were homogenized in sodium sulfate using a mortar and pestle and loaded into the cell. Samples were spiked with isotopically labeled surrogate standards and left on the bench for 1 h for quasi-equilibration. Samples were then extracted using hexane. Once extracted, samples were blown down to ≈ 200 μL and spiked with isotopically labeled internal standards. Samples were analyzed using the instrumentation described below.

DDT, DDE, and DDD related compounds were detected and quantified using a Varian CP-3900 GC system coupled to a Saturn 2100T ion trap mass spectrometer with electron impact ionization using a previously described method.[69] The remainder of the

target analytes was detected and quantified using an Agilent 7890 GC coupled to an Agilent 5974 MS in electron capture negative ionization mode as previously described in Trumble et al. (2012).[29] Combined, the average surrogate recovery was 55% for GC–MS analysis.

Results

Of the 76 target pollutants, 38 were detected in at least one of the three porpoises, with 34 recovered from both the AL+ and AL and 35 from the YL- (Table 2.1). Total pollutant concentrations (in ng/g lipid) were slightly higher in the YL porpoise (8300) than the AL+ (7900). Total blubber PBDE (Figure 2.1) and ∑PCB (Figure 2.2) concentrations were highest in the YL- with 1700 and 4700, respectively, compared with AL+ and AL-, whereas total pesticide concentration (Figure 2.3) was greatest in the AL+ (4500). Additionally, of the 34 analytes recovered from the AL+ and AL-, 25 (74%) were higher in the AL+. With the greatest total number of contaminants detected in the YL-, individual analyte concentrations were correspondingly greatest in this animal, compared among all three porpoises. DDTs were detected in the AL+ and YL-, but not in the AL-; however, quantification was limited due to unknown chemical interference. PBDEs 28 and 33 were only detected in the YL- (12.7) as was PCB congener 169 (27.3). ∑DDE levels in the AL+ porpoise was 3700 but could not be precisely quantified due to the value being beyond the linearity of the calibration curve.

Table 2.1 Persistent pollutants in the blubber of harbor porpoises in the Salish Sea, Washington State. Each value represents the mean of three replicate extractions, unless otherwise noted.

	Contaminant concentrations (ng/g lipid) in the blubber of:		
Analyte	AL+	AL-	YL-
PBDEs			
PBDE 28 + 33	ND	ND	12.7
PBDE 47	525.5	299.8	1177.3
PBDE 49	97.8[a]	37.7[a]	105.8
PBDE 66	9.8	2.4[a]	20.8[a]
PBDE 99	150.1	130.0	110.3
PBDE 100	143.0	93.8	150.1[a]
PBDE 153	40.4	32.9[a]	2.4[b]
PBDE 154	58.3	67.9	33.3
Total PBDEs	1024.9	708.9	1702.3
PCBs			
PCB 101	188.7	106.9	385.9
PCB 105	44.8	25.4	90.8
PCB 110	69.1	38.7	103.0
PCB 114	2.4	3.1	ND
PCB 118	140.8	77.0	377.6
PCB 123	4.1[a]	5.7	ND
PCB 126	5.6[a]	6.1	9.5
PCB 138	692.2	424.7	1520.1
PCB 153	623.4	393.4	1429.9
PCB 156	4.8[a]	2.9[a]	8.3
PCB 157	3.2[a]	2.7	8.4
PCB 167	11.3	ND	24.4
PCB 169	ND	ND	27.3[a]
PCB 180	267.8	202.4	362.1
PCB 187	306.8	225.4	375.3
PCB 189	4.8	3.6	4.1
Total PCBs	2369.8	1518.0	4726.7
Pesticides			
Alpha-HCH	31.2	37.4	50.2
Beta/Gamma-HCH	46.8	39.9	59.7
Heptachlor	ND	2.9	1.0[a]
Heptachlor-epoxide	ND	5.3	8.1
Mirex	14.1	12.0	ND
HCB	20.2	15.7	86.7
Trans-chlordane	6.1	6.9	8.2
Cis-chlordane	82.0	62.7	181.3
Trans-nonachlor	67.3	48.8	155.5
Cis-nonachlor	40.6	27.4	85.4
Endrine ketone	6.8	5.5	9.8
ΣDDTs	96.7	ND	55.6
ΣDDEs	3697.0	469.7	1085.5
ΣDDDs	402.7	54.2	137.1
Total Pesticides	4511.5	812.3	1924.1
Total Pollutants	7906.2	3039.2	8353.1

Abbreviations: AL+, adult pregnant harbor porpoise with lymphoma; AL-, adult pregnant harbor porpoise with no lymphoma; YL-, young female porpoise no lymphoma; HCH, hexachlorocyclohexane; HCB, hexachlorobenzene; ND, not detected.
[a]Two replicate extractions. [b]Single extraction.

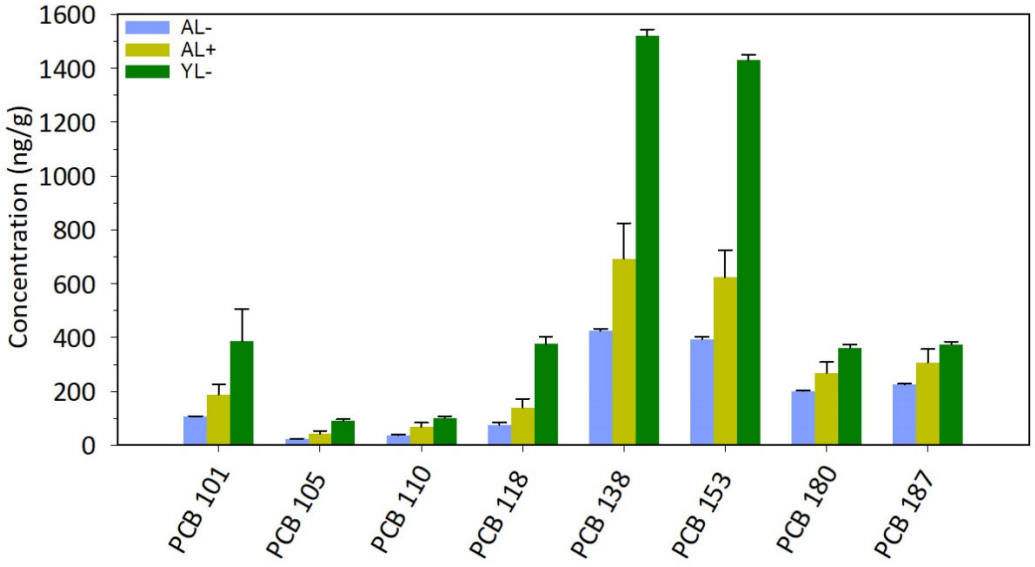

Figure 2.1 Mean blubber PCB levels and means (ng/g lipid) in pregnant adult harbor porpoises with (AL+) and without (AL-) lymphoma, and a female yearling with no lymphoma (YL-). Nondetected and analytes making up less than 5% of the total burden were omitted from graphs.

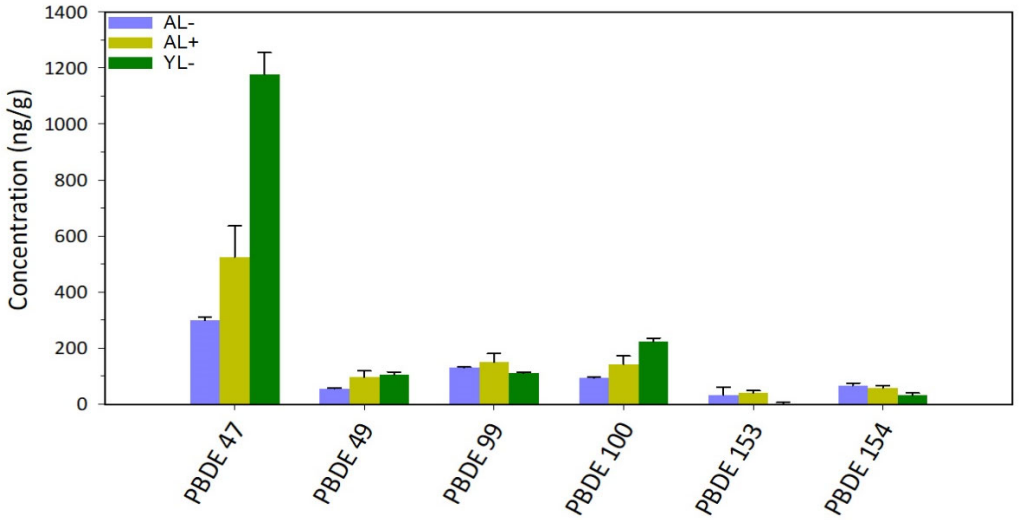

Figure 2.2 Mean blubber PBDE levels and means (ng/g lipid) in pregnant adult harbor porpoises with (AL+) and without (AL-) lymphoma, and a female yearling with no lymphoma (YL-). Nondetected and analytes making up less than 5% of the total burden were omitted from graphs.

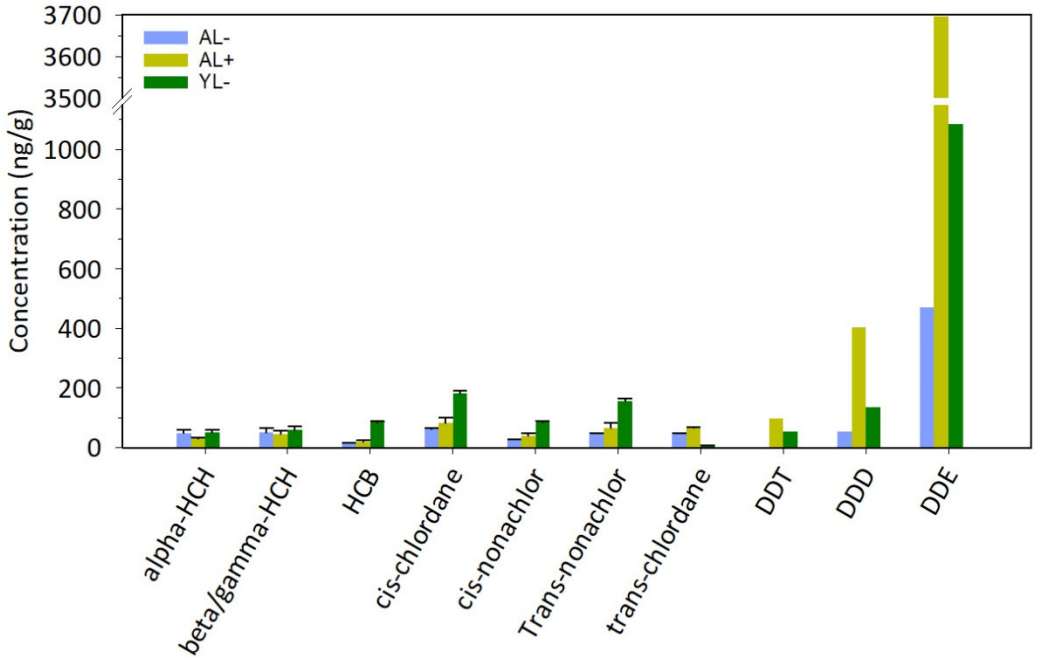

Figure 2.3 Mean pesticide levels and means (ng/g lipid) in pregnant adult harbor porpoises with (AL+) and without (AL-) lymphoma and a female yearling with no lymphoma (YL-). Nondetected and analytes making up less than 5% of the total burden were omitted from graph.

Discussion

Many variables may affect measured pollutant loads in blubber and include season sampled, condition of the blubber sampled, and the animal's age, health, and nutritive condition, gender, reproductive status, prior maternal offloading, and diet.[12, 111] In general, contaminant levels were highest in the YL-, likely reflecting receipt of maternal transfer of pollutants during gestation and lactation.[112] Between the two pregnant adults, contaminants were consistently higher in the AL+. However, the concentrations noted in the lymphoma case may have been partly due to mobilization of pollutants from the blubber, as often is observed as an animal loses its nutritional condition due to chronic illness or poor diet.[12] Differences between the two individuals may be reflective of age or number of pregnancies and therefore additional maternal

offload. Transfer rates to offspring tend to decrease with mother's age; therefore, contaminant levels are higher in primiparous compared with multiparous females, because they have not yet offloaded their contaminants to their offspring.[113] Transfer of contaminants to offspring follows birth order, diminishing with the dam's age, with the highest load transferred to the first-born compared with subsequent calves.[114] The age of the AL+ was estimated to be between 8 and 12 years based on tooth growth layer group counts[115]; however, the age of the AL- was not estimated.

Although not directly comparable to immunotoxic thresholds observed in other studies and marine mammal species, ∑PCB ranges in this study were near or less than those determined to cause dose-related alterations in gene expression, immune, and endocrine function (vitamin A and thyroid hormones) (1300 ng/kg lipid)[116] or to alter or reduce natural-killer T-cell function in harbor seals (Phoca vitulina) (17,000 ng/kg lipid).[86] Lack of inferred or demonstrated data on immune function effects of PCBs and PBDEs in harbor porpoises precludes further comparisons beyond those to other regional porpoise populations. Blubber PCBs in porpoises from Washington, Oregon, and California were 2000–129,000 ng/g lipid[109], whereas in British Columbia measured 5000–17,000 ng/g lipid.[117] These harbor porpoise populations were thus likely to include individuals with PCB levels near the immunotoxic range observed in harbor seals.

Although the US sales and distribution of PCBs ended in the 1970s, PCBs are commonly reported in animal tissues worldwide.[29, 118] The likely dominant pathway of acquiring contaminants in the adult porpoises was through bioaccumulation upon ingestion of mature prey items, such as Pacific herring, known to be significantly contaminated with PCBs.[103] Recent work in Salish Sea harbor seals demonstrated a

decline in PCBs (81%) and PBDEs (71%) in sampled tissues from 1984 to 2003.[110] The decline was attributed to marked reduction of source input of these chemicals and increased regulatory implementations.[110] Ongoing efforts continue to reduce source inputs of contaminants into the Salish Sea and promote environmental remediation of existing sites of pollution.[119] Although a definitive causal relationship between contaminants, cortisol, and BCL is yet to be demonstrated, this research adds to the weight of evidence of a possible link; further samples are needed to elucidate these associations.

PBDEs levels were greatest in the YL- porpoise, likely reflecting the animal's young age and therefore, not yet having offloaded contaminants through transplacental transfer and lactation. Nonetheless, the levels of PBDE-47 recorded in the present study were at least 30 times greater than the mean observed in human patients with NHL.[120] While evidence linking PBDEs with lymphoma is less-defined than that for PCBs, findings to date indicate that these modern additives to plastics may similarly effect human health (and possibly other mammals) as DDT and PCBs, by inducing genetic recombination that are linked to a number of diseases, including cancer.[121] DDTs were lower compared with concentrations reported in a bottlenose dolphin (Tursiops truncatus) with immunoblastic lymphoma[74], in dolphins in the Indian River Lagoon, and Charleston, South Carolina[122], and were below toxicological significant levels[123]. The high proportion of DDEs, metabolites of DDT, suggests the DDT exposure was not recent.

CHAPTER THREE

Eighty Years of Chemical Exposure Profiles of Persistent Organic Pollutants Reconstructed Through Baleen Whale Earplugs

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Abstract

Despite decades of effort, significant knowledge gaps still exist regarding the global transport and distribution of persistent organic pollutants (POPs) in marine ecosystems, especially for periods prior to the 1970s. Furthermore, for long-lived marine mammals such as baleen whales, POPs impacts on early developmental (first years of life), as well as lifetime exposure profiles for periods of use and phase-out, are not well characterized. Recently, analytical techniques capable of reconstructing lifetime (i.e., birth to death; ~6 mos. resolution) chemical exposure profiles in baleen whale earplugs have been developed. Earplugs represent a unique opportunity to examine the spatiotemporal trends of POPs in the marine ecosystem. Baleen whale earplugs were collected from six whales (one blue whale (*Balaenoptera musculus*) and five fin whales (*Balaenoptera physalus*)), including four from archived collections and two from recent strandings. Lifespans for some of these individuals date back to the 1930s and provide insight into early periods of POP use. POP concentrations (reported in ng g^{-1} dry wt.) were determined in laminae (n = 35) and were combined with age estimates and calendar year to reconstruct lifetime POP exposure profiles and lifetime bioaccumulation

rates. Dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) were found to be the most dominant POPs (spanning the past 80 y), were detected as early as the 1930s and were ubiquitous in the North Pacific and Atlantic Oceans. Lifetime bioaccumulation rates determined using baleen whale earplugs were 56 times higher in the North Pacific as compared to the North Atlantic. This may suggest that baleen whales in the North Pacific may be more likely to be exposed to POPs.

Introduction

Select persistent organic pollutants (POPs) were initially measured in marine mammal tissues in the mid-1960s.[124] Over the next half-century, studies continued to measure POPs in various marine mammal tissues (i.e., blubber, skin, whiskers, and hair), providing insight into POPs physiochemical properties (*e.g.*, persistent, toxic, and bioaccumulative). Many of the POPs identified from marine mammal tissues are considered semi-volatile organic compounds (SVOCs), capable of undergoing long-range atmospheric transport and global distillation[30, 31] and reported to negatively affect the health, specifically with regards to hormone and immunological dysfunction, reproductive disorders, and endocrine disruption.[70, 125-127] Marine mammals have been used to measure POPS in various tissues but assessing longitudinal trends within individuals has had limited success.[128]

The most commonly measured POPs recovered from whale tissues include heavy use pesticides, dichlorodiphenyltrichloroethane (DDT)[17], industrial multi-use chemicals polychlorinated biphenyls (PCBs)[129], and polybrominated diphenyl ethers (PBDEs, flame retardants)[19]. It is estimated that 2.8 million metric tons[17] and more than 1 million metric tons[130] were produced globally of DDT and PCBs, respectively. DDT and PCBs were

used heavily in the Northern Hemisphere from the 1930s to 1970s until restricted or phased out.[17, 129] PBDE usage began in the 1960s until replaced in the 2000s and annual global production was estimated to be more than 67,000 metric tons.[131] While considered a legacy contaminant, DDT is still being applied to control mosquito-borne pathogens[17] and it was estimated that about 3,500 metric tons were produced annually on average from 2008 to 2014.[132] Similarly to DDT, with the decline of use over time, the amount of PCBs and PBDE to the environment has been steadily decreasing. However, despite the phase-out and the passage of time, many POPs can still be measured throughout the environment.[17, 18] Despite their recalcitrant nature, select POPs can also undergo biotransformation (*e.g.*, DDT to DDE).[17]

The opportunistic sampling of subcutaneous blubber samples is routinely used to assess the lifetime accumulation of POPs in marine mammals. However, without age/time-corrected POP concentrations, it is difficult to assess the spatiotemporal variations in POPs across individuals, populations, and generations. This may be especially difficult in free-ranging marine mammals that migrate thousands of miles every year with lifespans that range through periods of heavy POP use and phase-out. Furthermore, spatial and temporal differences in foraging behavior and food availability may influence their exposure, while sex, body size and condition could influence lifetime burden.[133, 134] Sample collection for temporal studies of wild cetaceans is difficult as the collection process is invasive and heavily regulated through permits.[128] While POP sampling has been limited temporally and spatially, datasets have provided evidence on the ubiquity of POPs both at the animal and environment level. However, with very few POP samples collected from marine mammals prior to the 1970s, understanding

environmental transport, trophic exposure and accumulation or excretion patterns in marine mammals is unknown.

The baleen whale earplug was initially described in the early 20th century[135] with subsequent histological and aging studies ensuing.[136, 137] From these detailed studies, it was determined that the whale earplug is excreted continuously from the tympanic membrane into the auditory canal over the whale's entire lifetime. The lipid-based earplug accumulates as light and dark layers, plausibly associated with foraging differences, with each light/dark grouping representing approximately a year.[138-141] Recently, whale earplugs have provided researchers the ability to access both labile (*e.g.*, hormones) and inert (*e.g.,* POPs) compounds as a function of time and whale age.[142] While the earwax is excreted, the kinetically labile and inert POPs within the body are archived within the earplug.[142] By combining chemical analysis with age/time estimates, earwax acts as a surrogate for repeat sampling and provides a unique opportunity to correct for natural biological variability when comparing across individuals, as well as to reconstruct POP exposure profiles and bioaccumulation rates.

The goal of this research was to characterize POP exposure scenarios within fin whales (*Balaenoptera physalus*) as a function of time/age from baleen whale earplug laminae spanning 1929-2015 using methods previously developed for blue whale (*Balaenoptera musculus)* earplugs.[69, 142] In addition, relative changes in POP concentrations will be assessed to determine extrinsic and intrinsic factors, such as maternal offloading, bioaccumulation rates, as well as long-term spatiotemporal trends of POPs within the marine ecosystem. Specifically, this study sought to 1) reconstruct lifetime POP profiles and bioaccumulation rates using whale earplugs, (2) assess the

spatiotemporal heterogeneity of POPs in the Northern Hemisphere (i.e., Atlantic and Pacific Oceans) and (3) combine reconstructed POP profiles from multiple baleen whales to assess the ecosystem exposure history in the North Pacific from 1930 to the present.

Materials and Methods

Chemicals, supplies, extraction, and quantification were performed as previously described by [69]). The complete target list contains 53 analytes from three major POP groups including, pesticides, PCBs, and PBDEs (Supporting Information). Due to matrix interference, the DDX analysis (including both isomers, o,p', and p,p', of DDT, DDE, and DDD was performed using gas chromatography-tandem mass spectrometry in electron ionization mode (GC-MS/MS EI; Supporting Information).

Baleen whale earplugs (N = 6) for this study, including fin whales (N = 5), were delaminated, aged, and stored as previously described as well as one (N = 1) blue whale.[72, 142] Of the five fin whales sampled, three were from archived museum collections whereas two from strandings. The lifespans (estimated at 1.5 to 31 y) of the fin whales sampled spanned 87 years (1928-2016). One previous sampled male blue whale earplug from the North Pacific with an estimated lifespan from 1995 – 2007 was included in this study.[142] Individual whale/earplugs were identified by species as well as ocean basin of origin (*e.g.,* NA or NP for North Atlantic or North Pacific, respectively; Table 3.1)**.** The estimated age of each whale was based on individual lamina counts[29] and along with a year of death, provided each lamina group with an associated calendar year. Single fin whale laminae from each earplug were analyzed for POPs providing a minimum aliquot was achieved (i.e., 150 mg dry wt.), however, when per lamina sample mass was insufficient, adjacent laminae were combined which also resulted in a mean age or date

sampled (*e.g.*, combining laminae 8, 9 and 10 with an estimated age of 4, 4.5, and 5 respectively, would result in an estimate age of 4.5 y).

A total of 35 earwax sample extractions (N= 35, including samples of individual lamina and combined laminae) were used to reconstruct lifetime POP profiles in five fin whales (Table 3.1). All POP concentrations were reported as ng g^{-1} dry wt. and were corrected for the mass of earwax (g) used in the extraction. POP contaminant profiles were produced by plotting the summation of contaminants within each lamina against the age or year determined through the aging process. Bioaccumulation rates were determined by combining the sum of contaminant burden of the current and previous lamina versus the age and applying linear regression.[142] For each fin whale earplug, sex, lifespan in years, estimated age, ocean of origin, lifetime POP burden (ng $g^{-1)}$, and bioaccumulation rate (ng·g^{-1} $y^{-1)}$ was determined (Table 3.1). Fixed effect multiple regression analysis of the bioaccumulation rates (summation of contaminant burden vs age) analysis was performed using JMP 14 (SAS Institute, Cary, NC) to determine if the ocean of origin, sex, lifespan, and species affect the POP burden.

Results and Discussion

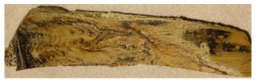
POPs were detected and quantified in all fin whale earplug laminae sampled from historical collections and more recent strandings. Using this technique, the historical earplugs sampled allowed for the reconstruction of POPs data an estimated 40 years (i.e., the 1930s – 1960s) before the first published POPs study of marine mammals.[124] It should be noted that the chronological nature of this dataset spans the introduction and heavy use of POPs within the northern hemisphere. By combining the POP concentrations from each lamina with an age/time estimate, lifetime POP profiles can be

reconstructed for the five fin whales (Section 3.1) as well as calculate the lifetime bioaccumulation rates (Section 3.2). These results were also compared to similar contaminant data from a previously analyzed blue whale earplug.[142] The estimated lifespan based on the three fin whale earplugs (NA1, NA2, and NP1) spanned from the 1920s to the early 1960s (Table 3.1) and were used to assess periods of POP usage in the North Pacific and Atlantic Ocean. The remaining three earplugs, including two fin whales (NP3 and NP4) and one blue whale (NP2)[142], provide estimated lifespans covering 1995 to 2016 were used to assess contaminant profiles decades after phase-out.

Reconstructed Lifetime POP Profiles

Reconstructed estimated lifetime POP profiles (i.e., birth to death) from the six baleen whale earplugs reveal the relative contribution of select POP classes over the past 80 years as ΣDDXs >> ΣPCBs > ΣPBDEs, chlordanes (ΣCHLRs), nonachlors (ΣNCHLRs), hexachlorobenzene (HCB), pentachloroanisole (PCA), and gamma-hexachlorohexane (γ-HCH) (Figure 3.1). It is important to note that DDT isomers and metabolites (DDD and DDE) were detected within the lamina dating to the 1930s (Figure 3.2). The presence of DDT (and metabolites) detected in lamina dating back to the 1930s suggest that DDT was rapidly transported from source regions after its introduction (e.g., agricultural activities) to the marine ecosystems where it entered the food web and accumulated in marine mammals.

Table 3.1 Individual baleen whale images and data including ID, species, sex, lifespan, estimated age, ocean of origin, lifetime burden, and lifetime bioaccumulation rates (slope and r^2). All concentrations are reported in ng g^{-1}.

Earplug Images	Sample ID	Species	Sex	Ocean of Origin	Estimated Lifespan	Estimated Age (y)	Lifetime Burden (ng g^{-1})	Bioaccumulation Rate (ng·g^{-1} y^{-1})	r^2
	NA1	*Balaenoptera physalus* (fin)	♀	Atlantic	1937-1955	18	126	5.88	0.93
	NA2	*Balaenoptera physalus* (fin)	♂	Atlantic	1937-1956	19	52.4	2.66	0.86
	NP1	*Balaenoptera physalus* (fin)	♂	Pacific	1928-1961	33	16,100	474	0.99
	NP2	*Balaenoptera musculus* (blue)	♂	Pacific	1995-2007	12	5,170	360	0.99
	NP3	*Balaenoptera physalus* (fin)	♀	Pacific	2013-2015	2	241	95.4	0.90
	NP4	*Balaenoptera physalus* (fin)	♂	Pacific	2014-2015	1.5	60.1	43.4	0.99

Similarly to DDT, the presents of other POPs in laminae (from the 1930s and 1940) suggest this phenomenon was not unique to DDT as many POPs entered the marine food web shortly after widespread use.[17] The POP profiles of historic fins also showed an increase in concentration within the laminae from the 1930s to 1940s (Figure 3.1), which may be corresponding to the widespread scale-up of POP use in 1930 through 1950. Specifically, NA1 and NA2 had increasing POP levels from 1938 to 1944, and NP1 had increasing POP concentrations in laminae from 1929 to 1943. Concentrations of POPs within the laminae of historic plugs plateaued from the mid-1940s through the 1950s in all three historic earplugs (Figure 3.1). However, plateaued concentrations were notably different among whales during this period with the average concentration of 18.8, 6.61, and 1270 ng g^{-1} for NA1, NA2, and NP1 respectively. The lifetime contaminant burdens can be found in Table 3.1.

Reconstructed earplugs profiles of whales NP1, NA1, and NA2 were relatively similar in overall lifetime exposure profiles (Figure 3.1). However, some differences in the overall POP profiles among individuals revealed individual disparities in DDX, PCBs, PBDEs, and PCA detections. The reconstructed POP profile of NP1, which had an estimated lifespan from 1928 – 1961, had a profile including, DDX, PCBs, PCA, γ-HCH, HCB, chlordanes, and nonachlors. Interestingly, ΣDDX measurements within the lamina of NP1 dated back to 1935 was 1,200 ng g^{-1}, which was 63 times that of DDX measured in NA1 (19 ng g^{-1}, 1937) and 210 times that of NA2 (5.6 ng g^{-1}, 1938). The male blue whale's (NP2; 1995-2007), contaminant profile was also dominated by DDX, consisting mainly of metabolites (i.e., DDD and DDE). This contaminant profile is similar to more recent samples that correspond to decades after widespread DDT use and restriction.[17]

The presence of DDT and or it's metabolites in laminae from the 1930s to the 2000s highlights the widespread and persistent nature of DDT contamination in our world's oceans.

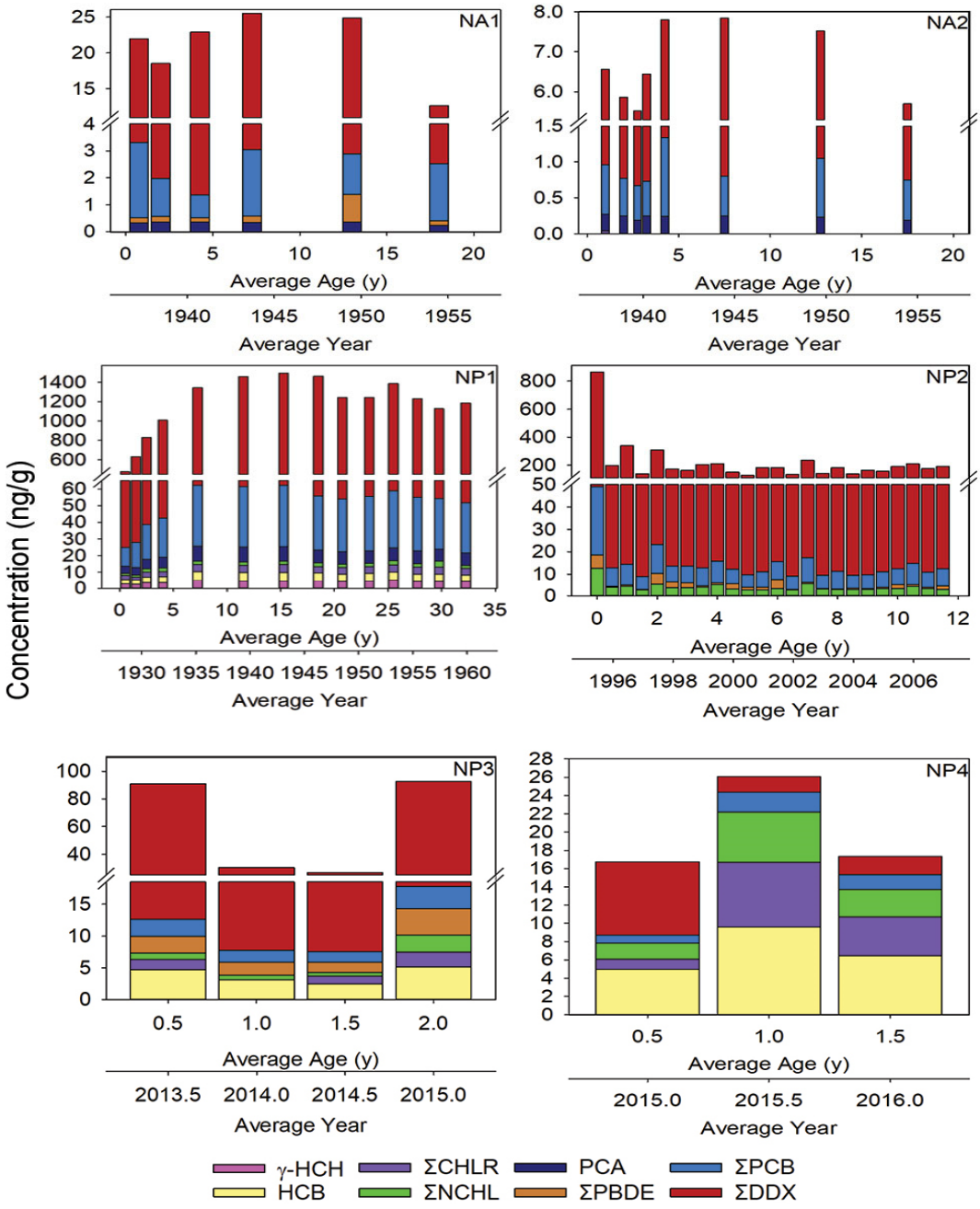


Figure 3.1 Reconstructed contaminant profiles of baleen whales spanning 1928 – 2015. γ-HCH (gamma-hexachlorohexane), ΣCHLR (sum of cis- and trans-chlordanes), PCA (pentachloroanisole), ΣPCBs (sum of analyzed PCB congeners), HCB (hexachlorobenzene), ΣNCHL (sum of cis- and trans-nonaclor), ΣPBDEs (sum of analyzed PCB congeners, ΣDDXs (includes total of measured isomers (i.e., p,p' and o,p') of dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyldichloroethane (DDD).

When comparing lifetime POP exposure profiles and overall burdens across individuals, populations, and species, it is important to consider differences prey.[143] For example, fin whales consume krill as well as small schooling fish[144], while blue whales are known to exclusively feed on krill.[145] In addition to diet and trophic position, contaminant burden may also be influenced by several factors including lifespan and human activity (e.g., pesticide use; which changes as a function of time and space), sex, proximity to sources (*e.g.*, nearshore), the location (i.e., regional or ocean basin).[133, 134]

Decades after use, earplugs from recent strandings (i.e., NP3 and NP4) have similar contaminant profiles (*e.g.*, DDX, PCBs, and PBDEs), however, contaminant concentrations levels were lower than those measured in NP1 and NP2. Interestingly, these two young fin whales had a higher POP burden than NA1 and NA2, which lived for nearly ~18 and 19 years respectively during the heavy usage period of POPs. The contaminant profile of NP4 was unique compared to the other five individuals, as it was dominated by HCB and not DDX. NP3 and NP4 had similar lifespans and originated from the North Pacific however, the first lamina of their profiles contained different isomers of DDX. For example, NP3 Additionally, the first lamina of the fin whale NP3 contained isomers of DDX (p,p'- and o,p'- DDT, DDE, and DDD) whereas NP4 contained only isomers of DDT and DDD (Figure 3.2). These differences in POP profiles between NP3 and NP4 could be due to regional (distance from the source of local or long-range contamination).

POP exposure in the North Pacific was modeled for DDX and PCBs as a function of time by combining exposure profiles from three individuals. The lifetime contaminant burden profiles plotted as a function of the year (Figure 3.3), which spanned the 1930s –

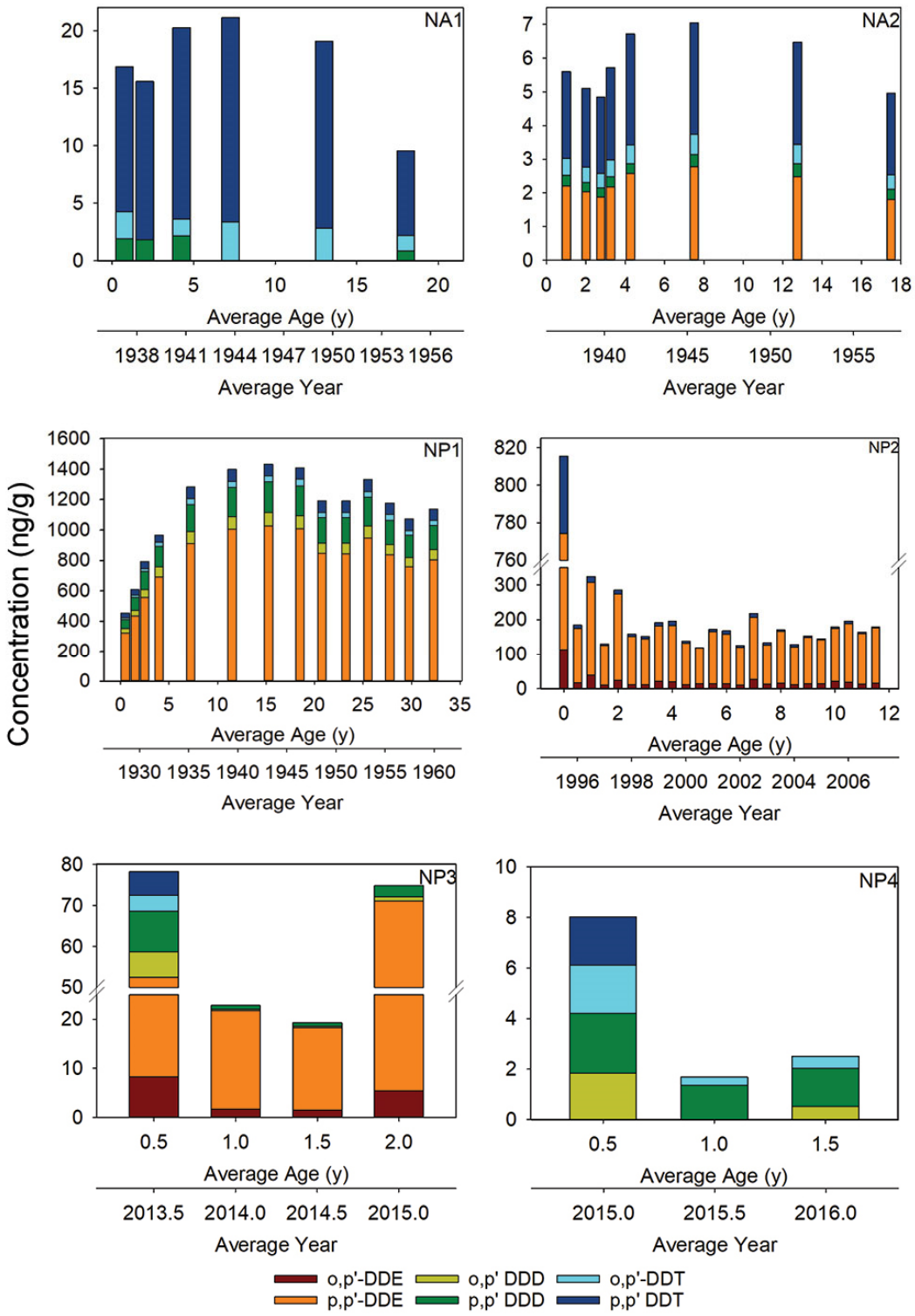


Figure 3.2 Reconstructed ΣDDTs profiles of baleen whales spanning 1928 – 2015 of p,p'- and o,p'-isomers of dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyldichloroethane (DDD).

1960s and 1995 to 2016, revealed increases in DDX and PCB concentrations from the 1930s to the early 1950s and were a relatively stable period in the 1950s and 1960s (i.e., NP1). This increase in concentration in the earplug corresponding to the 1930s likely coincides with the mass production and usage of DDT and PCBs (1930s – 1970s). After the 1960s, DDX and PCB concentrations appear to decrease through the 1990s (i.e., NP2) and into the 2010s (i.e., NP3 and NP4). The decreasing trend over the past few decades suggests that restrictive actions have reduced concentrations in the North Pacific.

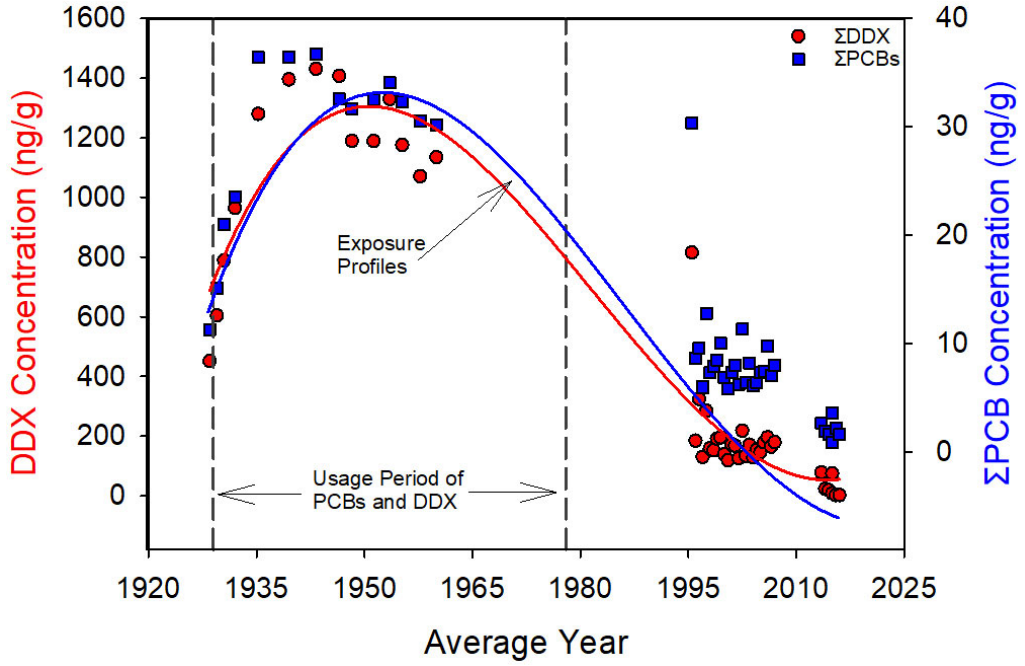


Figure 3.3 Reconstructed POP exposure profiles of DDX and PCBs in the North Pacific Ocean from multiple individual whales. Modeled environmental exposure profiles for ΣDDX and ΣPCB spanning the past 90 years. Modeled exposure profiles were developed from chemical observations reconstructed from baleen whale earplugs (solid red and blue lines). Dashed black lines indicate periods of use for PCBs (1929 - 1977) and DDT (1930s to 1972).

Lifetime POP profiles reconstructed from whale earplugs also reveal differences in POP exposure profiles during early life. For example, NP2's contaminant profile

contained PCBs, nonachlors, PBDEs, and the highest contaminant concentrations were found in the first lamina (contaminant sum of 864 ng g^{-1}), while the subsequent lamina contaminant concentration was 197 ng g^{-1}. Similar to the blue whale's (NP2) contaminant profile, the first lamina from NP3 also had the highest contaminant burden than the proceeding lamina. Whereas the second lamina of NP4 was higher than the first (see Figure 2.1). However, NP2 – NP4 have the highest DDX contaminant burden within the first lamina (see Figure 3.2). Isomers of DDT were not detected in NP3's following laminae and p,p'- DDT was also not detected in the remaining laminae of NP4 (Figure 3.2). The profile difference between the first and subsequent laminae could be a result of the transfer of POPs during lactation and a subsequent change in foraging or feeding behaviors after weaning. A similar conclusion was drawn in a previous study of humpback whales where POPs recovered from blubber varied by geographic feeding areas of the North Pacific and North Atlantic.[146] Another study by Metcalfe and colleagues (2004) reported maternal offloading of POPs in humpback and blue whales, with their young reaching POP equilibrium during gestation and lactation. Assuming that POP concentrations reach equilibrium between mother and calf during lactation may indicate the first earplug lamina as the contaminant profile transferred from the mother with subsequent layers representing environmental exposure. NP4 revealed a POP profile that included relatively high concentrations of HCB and ΣCHLRs, followed by ΣDDXs and ΣPCBs. NP4's contaminant burden within the first lamina was not higher than the proceeding laminae and could indicate higher exposure from the environment in comparison to maternal offloading or a difference in birth order (i.e., the mother of NP4 had transferred the majority of her contaminants to prior progeny).[113, 147]

It is important to note that fin and blue whales fast[144, 148], however we detected POPs in every lamina. It is important to note that POPs are lipophilic and that in cetaceans, lipids can be hydrolyzed, depending on fasting state, and subsequently release POPs back into the bloodstream and therefore throughout the body.[20] The degree to which POPs are released is not only dependant on the fasting state, but also other factors such as the octanol-water partition coefficient of the POP.[149] Contaminants with higher chlorination are generally more lipophilic (larger octanol-water partition efficient) and would likely remain in the blubber and those that are less lipophilic (less chlorination, smaller octanol-water partition coefficient) will likely be released back into the bloodstream during lipolysis.[149] Due to a potential flux of POPs between blubber and bloodstream, the possibility exists for contaminants to partition back into the bloodstream after initial exposure and recirculate over the individual's entire life. We acknowledge that the complexities of POPs partitioning within the tissue and organs of marine mammals is difficult as partition coefficients vary between compartments within the body [150], however, due to the lack of external exposure, earplug lamina appears to maintain a 6-month record of POPs circulating within the blood. In other words, earplugs can offer a record of recirculating POPs during known periods of fasting in large baleen whales.[20] In the initial study including the male blue whale (NP2), the earplug used to estimate that the lifetime burden was approximately 10% different than the total POP burden determined from the blubber of the same individual.[142] It is also important to note that female mammals are capable of offloading their contaminant burden in their fat-rich milk reserves to their offspring during lactation.[151]

Bioaccumulation Rates Determined using Baleen Whale Earplugs

In addition to the lifetime contaminant profile, age-corrected lifetime bioaccumulation rates (burden as a function of age; $ng \cdot g^{-1}\ y^{-1}$) were calculated. A relationship between the sum of contaminant burden ($ng\ g^{-1}$) was calculated as a function of time for each whale (i.e., burden from the current and previous laminae against the age (y) of an individual). Bioaccumulation rates can be used to investigate changes in exposure within and among individuals, populations, generations through time and space. Lifetime bioaccumulation rates (Figure 3.4) are extremely rare, especially in free-ranging long-lived marine mammals. To the authors' knowledge, bioaccumulation rates of POPs in baleen whales have only previously been modeled in a single blue whale[142] or modeled in captive odontocetes.[150]

POP bioaccumulation rates in the whales sampled ranged from 2.6 to 470 $ng \cdot g^{-1}\ y^{-1}$, with coefficients of determination (r^2) from 0.86 to 0.99 (Table 3.1 and Figure 3.4). The analysis of POP bioaccumulation rates of baleen whale earplugs revealed that whales sampled from the North Pacific (n = 4) rates were, on average, 56 times higher than those sampled in the North Atlantic (n = 2). The fin whale, NP1, had the highest bioaccumulation rate of 470 $ng \cdot g^{-1}\ y^{-1}$, followed by the blue whale, NP2 at 360 $ng \cdot g^{-1}\ y^{-1}$, fin whale, NP3 at 95 $ng \cdot g^{-1}\ y^{-1}$, and fin whale NP4 at 43 $ng \cdot g^{-1}\ y^{-1}$. The POP bioaccumulation rates measured in the North Atlantic were 5.9 and 2.7 $ng \cdot g^{-1}\ y^{-1}$ for whales NA1 and NA2, respectively. This is further supported, when comparing the mean contaminant burden of earplugs with similar lifetimes (*e.g.*, NP1, 1928-1961; NA1, 1937-1955; NA2 1937-1956). NP1 ($1150 \pm 313\ ng\ g^{-1}$, n = 14) had a statistically significant (*t*

test, $p < 0.0001$) mean contaminant burden than individuals originating in the North Atlantic (12.8 ± 8.01 ng g^{-1}, n = 14). The POP bioaccumulation rate difference among

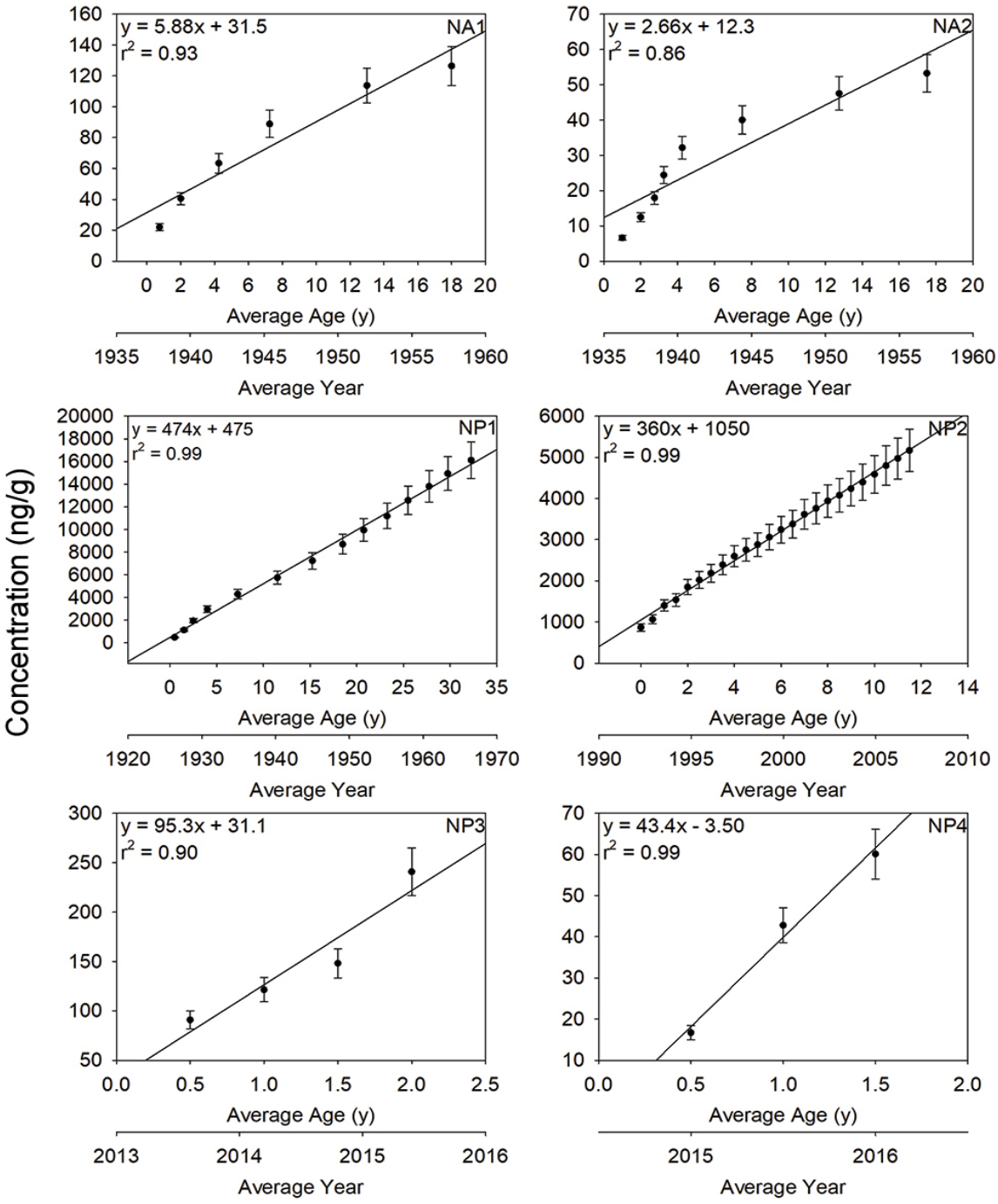


Figure 3.4 Reconstructed bioaccumulation rates of baleen whales spanning 1928 – 2015. Bars indicate a 10% error.

individuals suggest factors other than age may impact individual burden. The authors acknowledge the number of samples within this study is limited and multiple factors including, physiology (*e.g.*, sex), foraging behavior (*e.g.*, prey type), characteristics of contaminant source (*e.g.*, regional usage), and lifespan are known to impact burden.[133, 134] However, using a fixed-effect model we determined ocean basin ($p < 0.0001$) and lifespan ($p = 0.0035$) are significant factors when examining POP burden in comparison to species ($p = 0.20$) and sex ($p = 0.62$). This suggests contaminant exposure is influenced by location of whale as well as when the individual whale lived.

The usefulness of an animal as an ecosystem model or sentinel is dependent on their ecological diversity and variability inherent in their ecosystem. Baleen whales act as both sentinels[152-154] and marine ecosystem engineers[1], that rely on healthy marine ecosystems for their survival.[152-154] By recording decades of longitudinal data, whale earplugs can provide long-term profiles and allow opportunities to investigate a wide range of scientific topics such as legacy and emerging contaminants (using bioaccumulation models), global and regional differences as well as spatial and temporal differences (using contaminant profiles). While assessing the contaminant impact of an entire ecosystem remains difficult, earplugs do provide a unique opportunity, though not without limitations (*e.g.*, 6 mos. resolution, sex and species differences, excretion pathways). However, long-term longitudinal data collected jointly with samples of increased resolution (blubber/feces) can provide POP variability and possibly the health of the marine ecosystem.

Conclusion

This is the first study to recreate multiple lifetime contaminant profiles and bioaccumulation rates of individual fin whale using a previously developed method for blue whale earplugs. These profiles were then used to investigate maternal offloading, long-term spatiotemporal trends, and bioaccumulation rates within the marine ecosystem. Baleen whale earplugs used in this study provided contaminant data from the 1930s onward and provided a unique opportunity to investigate the transport of the first mass-produced usage of DDT and PCBs in the marine environment. Longitudinal contaminant data from individuals inhabiting the North Pacific revealed the initial introduction of DDT and PCBs, their rise in concentration, and subsequent decrease in this ecosystem following remediate action within the northern hemisphere. Additionally, using longitudinal data profiles, bioaccumulation rates in the North Pacific region were shown to be magnitudes of difference greater than those originating in the North Atlantic. The higher rates of those sampled in the North Pacific suggest a higher rate of POP exposure compared to the North Atlantic or more contaminants in the pacific versus the Atlantic. This study highlights the variability between regions and suggests baleen whale earplugs can be used to further assess differences between species, region, foraging behavior, and prey availability.

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Supporting Information

Separation and Quantitation of Total DDXs using GC-MS/MS EI

Chemical analysis of both o,p' and p,p' isomers of dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyldichloroethane (DDD) was performed using gas chromatography-tandem mass spectrometry in electron ionization mode (GC-MS/MS EI). An established temperature program using a Thermo Scientific Trace 1310 gas chromatograph with DB-5MS capillary column and ultra-high-purity helium.[69] Samples were injected using a TriPlus RSH Autosampler (Thermo Fisher Scientific, MA USA). Ionization and identification were performed using a Thermo Scientific TSQ 8000 Evo tandem mass spectrometer in selective reaction monitoring mode (SRM) using EI. SRM method development was performed using the Thermo Scientific AutoSRM software. Each

analyte and isotopically labeled surrogate ($^{13}C_{12}$ - DDT) and internal standard (d_{12}-Benzo(e)pyrene) were identified by retention time and initial precursor fragments. The selected fragments were evaluated using the product search function within AutoSRM. Multiple injections were performed to determine the most abundant product ions through collision-induced dissociation with argon gas. The product ion collision energy was optimized at 2 eV intervals and used for the final SRM method. Target analytes were identified by retention time as well and quantitative and qualitative ions. Target analytes were further confirmed using ion response ratios, ± 20% from known standards. Retention time, precursor ions, production ions, and collision energies can be found in the supporting information.

Calibration and quantitation were performed using Chromeleon Chromatography Data System 7.2 software (Thermo Fisher Scientific, MA USA). Calibration curves spanned four orders of magnitude. Continuing calibration verification (CCV) solutions were ran following three extracts. All samples were blank corrected, and concentrations were normalized to earwax mass.

Table 3.2 Complete target analyte list.

Pesticides	Polychlorinated Biphenyls (PCBs)	Polybrominated Diphenyl Ethers (PBDEs)
Aldrin	PCB 77	PBDE 15
Alpha-hexachlorocyclohexane	PCB 81	PBDE 17
Beta-hexachlorocyclohexane	PCB 101	PBDE 28
Bifenthrin	PCB 105	PBDE 33
Chlorothanolil	PCB 110	PBDE 49
Dacthal	PCB 114	PBDE 66
Delta-hexachlorocyclohexane	PCB 118	PBDE 75
Deltamethrin	PCB 123	PBDE 85
Gamma-hexachlorocyclohexane	PCB 126	PBDE 99
Heptachlor	PCB 138	PBDE 100
Hexachlorobenzene	PCB 153	PBDE 119
Lambda-cyhalothrin	PCB 156	PBDE 153
o,p' Dichlorodiphenyldichloroethane	PCB 167	PBDE 154
o,p' Dichlorodiphenyldichloroethylene	PCB 169	PBDE 155
o,p' Dichlorodiphenyltrichloroethane	PCB 180	PBDE 183
p,p' Dichlorodiphenyldichloroethane	PCB 187	
p,p' Dichlorodiphenyldichloroethylene		
p,p' Dichlorodiphenyltrichloroethane		
Pentachloroanisole		
Pentachlorobenzene		
Pentachloronitrobenzene		
Trifluralin		

Polychlorinated Biphenyl (PCB) Congener Percent Contribution

The most frequently detected PCB were tetra- to hepta-chlorinated and included PCBs 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 180, and 187 consistent with previous studies.[143, 155, 156] The most dominant PCB congeners were 153 and 118 in both the North Atlantic and North Pacific during and post usage, see Figure 3.5. In the North Atlantic, NA1 had the largest variety of congeners detected, 12. NA2 had a total of 7 congeners, and except for PCB 105, all congeners detected in NA2 were also detected in NA1. In the North Pacific, congeners (PCBs 118,153,180, 187) were similar between the fin whales (NP1, NP3, NP4), except for PCBs 157 and 167. However, the percent contribution varied over time and indicated an increase in PCB 118 and 153 and a decrease in PCBs 180 and 187. PCBs 153, 118, and 138 were the largest contributors to NP2. The PCB profile is influenced by a variety of factors (i.e., the source[18], and the chemical structure[149]). PCBs were produced in mixture of assorted congeners and were

widely used and over time PCBs can undergo long-range atmospheric transport and global distillation[18], movement from warmer locations of application to colder remote locations. It is important to note that the lipophilicity of PCBs also plays a role in contaminant profiles and the recirculation within the bloodstream. The more chlorinated the PCB the more lipophilic and the less likely it is to remain in the bloodstream or return during lipolysis.[149] Within cetaceans, PCB profiles predominantly contained higher chlorinated congeners.[21] Due to the physiological need for blood for form the earplug it is possible that less lipophilic PCBs and other contaminants will reach the cerumen and be deposited in lipid instead. Further studies are needed to determine the difference between contaminant burdens in blubber and earwax from the same individual.

Jensen et al. were the first to report PCBs in marine species, including, common seals, ringed seals, and grey seals in 1969. Using baleen earplugs, we are able to report PCB profiles 30 years earlier.[157] The analysis included the total measurement of PCBs, as individual congeners were not identified by mass spectrometry until 1971.[158] The earliest baleen whale study was conducted in the early 1970s and included gray, fin, blue, humpback, and sei whales. The analysis was conducted using blubber and oil samples analyzed on GC-ECD and included PCBs, DDT, and its metabolites, dieldrin, and α-chlordane.[159] Similar to our data, ΣDDX concentrations were higher than ΣPCBs in the 1960s and 1970s. The ubiquitous presence in the 1930s (similar to DDT) also indicates a rapid transition from one environmental compartment to another. However, following the restriction of PCBs their contaminant concentrations have declined to below initial usage period, see Figure 3.3, but similar patterns of congener percent contribution between individuals from the North Pacific indicate the persistence of PCBs over multiple

decades. In a previous study of Pacific killer whales, Ross et al. found PCB 153 and 138 were the most dominants within three different communities (northern and southern residents, and transients) off the coast of British Columbia, Canada. Their data suggested that PCB congener accumulation is strongly related to age, sex, and trophic level and would coincide with increased PCB contaminant burden, see Figure 3.1.[160] The profiles of NP1, NP3, and NP4 suggest a similar diet or regional overlap. NP1 and NP3 had the same congeners (118, 153, 167), but at different percent contributions and could possibly indicate a similar region of feeding and diet.[21, 161, 162]

Table 3.3 DDX internal, surrogate, and target analyte GC-MS/MS EI SRM compound retention time, precursor and product ions (m/z) and optimized collision energy generated using Chromeleon AutoSRM.

Compound	Retention Time (min)	Precursor,Product Ions (m/z)	Collision Energy (eV)
Benzo(e)pyrene-D12	29.02	132,118	12
		264,260	36
Dichlorodiphenyltrichloroethane-13C12	22.77	247,176	38
		247,177	22
o,p' Dichlorodiphenyldichloroethylene	19.30	176,150	22
		246,176	32
p,p' Dichlorodiphenyldichloroethylene	20.32	176,150	22
		246,176	30
o,p' Dichlorodiphenyldichloroethane	20.52	235,165	22
		235,200	8
p,p' Dichlorodiphenyldichloroethane	21.62	235,165	22
		235,200	8
o,p' Dichlorodiphenyltrichloroethane	21.69	165,115	32
		235,165	22
p,p' Dichlorodiphenyltrichloroethane	22.77	165,115	32
		235,165	22

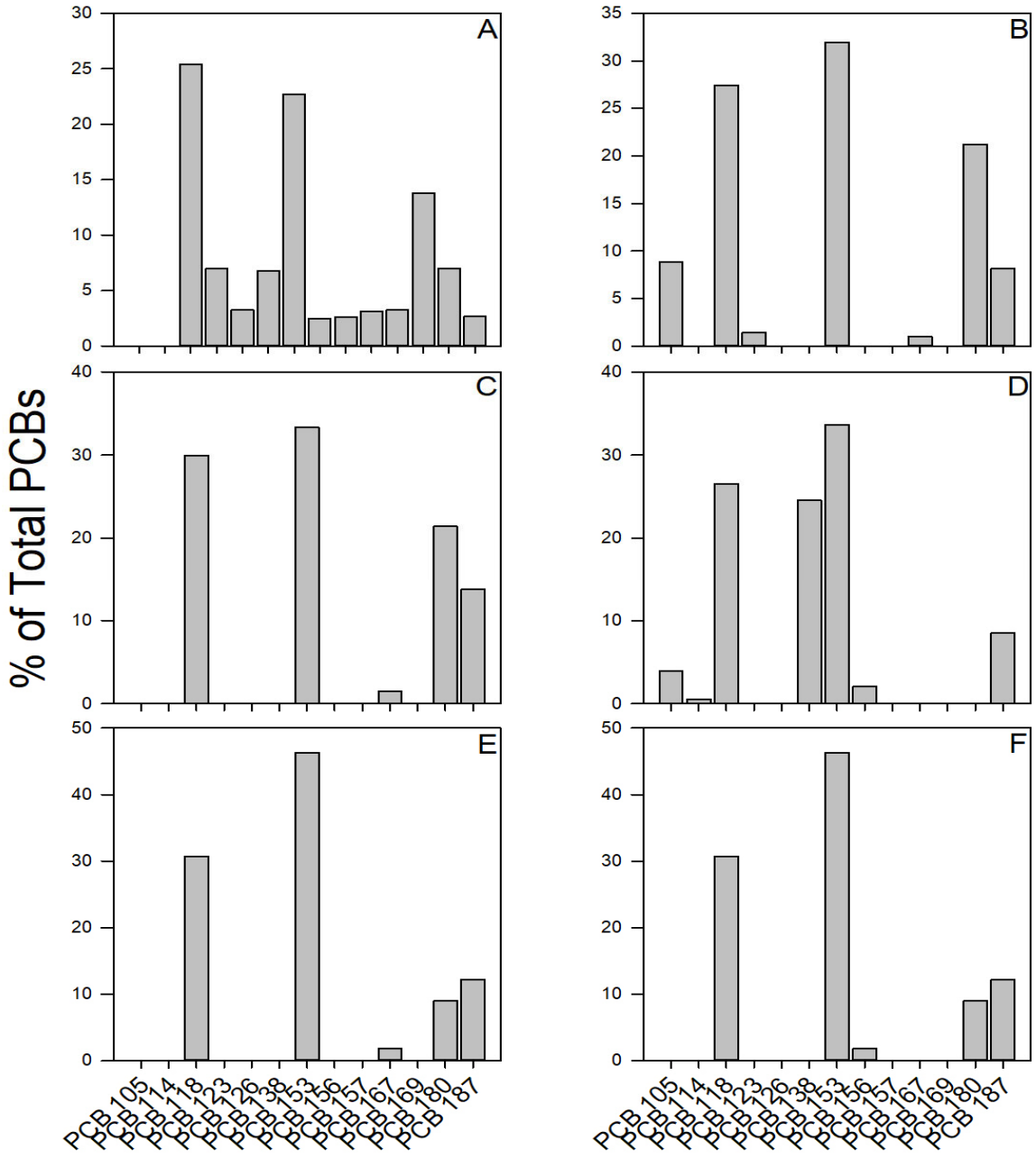

Figure 3.5 Percent contribution of detected PCB congeners of the total sum for individual whales from 1928 – 2015. A) NA1, B) NA2, C), NP1, D) NP2, E) NP3, F) NP4. Ocean basin congeners were similar for fin whales however percent contribution varied over time. All profiles were dominated by PCB 153 and 118.

CHAPTER FOUR

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Temporal Elemental Analysis of a Fin Whale (*Balaenoptera physalus*) Earplug

Abstract

Lifetime element profiles have been reconstructed using an earplug for an individual female fin whale (*Balaenoptera physalus*). These lifetime profiles (i.e. birth to death; 1913-1954) were reconstructed with ≈ six months resolution (n = 34 laminae) by combining previously established aging techniques with elemental analysis. Elemental analysis focused on 48 elements including toxic, essential, nonessential, rare earth elements, and other non-biologically relevant elements. Early periods of the individual's life history highlight a transition period for select metals, including, calcium, cadmium, cobalt, manganese, phosphorus, sodium, selenium, and zinc, which seem to correspond with reaching sexual maturity. Toxic metals showed evidence of bioaccumulation (i.e. increase in metal concentration over the individual lifespan) and their corresponding lifetime bioaccumulation rates were calculated and range from 7.38 to 3.1 x 10^5 $ng \cdot g^{-1}$ y^{-1}. Methylmercury and inorganic mercury profiles suggest variability in the contribution or source as a function of time. The analysis of 49 elements in 34 laminae resulted in over 1,600 data points describing the life history of different biochemical processes and biogeochemical cycles. The utility of earplugs and earwax has been significantly expanded as a result of this research and will provide significant insight into the spatial and temporal variability of metals in the world's oceans.

Introduction

Elements occur naturally in the Earth's crust and typically enter the marine ecosystem through atmospheric deposition, run-off, shelf sediments, hydrothermal vents, and ocean crust.[163] Sources and subsequent release of many elements have increased at alarming rates due to human activity (e.g., increasing human populations, urbanization, rapid economic development, and bad management of coastal areas).[164] Currently, there are still significant knowledge gaps in our understanding of the availability, trends, and profiles of many elements in the world's oceans and higher trophic marine mammals. This knowledge gap is further exacerbated for periods from the past century and in the southern hemisphere.

Marine mammals, such as cetaceans (i.e., whales, dolphins, and porpoises), are long-lived species with low reproductive rates, late maturity[165] and are susceptible to the negative impact of anthropogenic activity.[72] Tissues, such as blubber, liver, and skin, have been collected from marine mammals and are frequently analyzed for essential, nonessential, and toxic elements.[166] In more recent studies, biological tissues that grow incrementally over an individual organism's life (e.g. coral skeleton, fish otoliths, mammalian teeth, scales, baleen, and claws), have been used to evaluate the exposure and experiences of an organism.[167] By measuring elements within these incremental growth tissues, researchers can reconstruct environmental conditions and physiological responses over the lifetime of marine organisms.

Baleen whale earplugs are a biologic secretion that is deposited in incremental layers or laminae over the individual's life and as such provides a unique opportunity to "repeatedly sample" a single individual over its entire lifetime. Elemental data can be

assigned age estimates using the incremental layers to normalize concentrations to both a function of time (date) and age. Previously, a blue whale earplug has been used to reconstruct lifetime profiles of a single element, (i.e. total mercury; Hg).[142] This blue whale study also reported reconstructed lifetime profiles of select persistent organic pollutants and hormones. Earplugs are secreted within the whale's ear canal over its entire lifetime in alternating light and dark layers. The ear canal is never exposed to the external environment and represents a lifetime archive of chemical and elemental exposure. Once the earplug is aged, inorganic Hg lifetime profiles were reconstructed, and lifetime burdens and bioaccumulation rates were calculated. Mean Hg concentrations were 14.1 ± 2.6 ng g^{-1} and the profile included two distinct peaks possibly related to regional and/or anthropogenic increases of mercury. The bioaccumulation rate was calculated to be 28.2 ng·g^{-1} y^{-1} with an r^2 of 0.99. Though limited to just total Hg, this study was the first to report a life history profile of a baleen whale.

Temporal data is critical for understanding different contaminant exposure scenarios. For instance, the emissions of contaminants into the environment often change as a function of production and or use, and the time of exposure (e.g., early development or adulthood) impacts the severity of the health outcome. When incremental growth tissue samples are unavailable, marine mammals are often described by their body length, or by age groups such as calves, juveniles, or adults. With limited age data to accompany or correct contaminant data, quantifying and examining different exposure scenarios is difficult. This is further complicated when the factors contributing to the contaminant burden are considered. For example, individuals with long lifespans (e.g., baleen whales), often lived through usage periods of POPs as well as their subsequent ban or restriction.[71]

Without both an age and year, it is difficult to determine which is contributing directly to the exposure at the time of sampling. By normalizing measurements by age or year it is possible to evaluate contaminant exposure from the same individual over a lifespan and extended periods.

The goal of this research is to provide a proof of concept and increase the environmental data that can be derived from a whale earplug. Specifically, this paper helps characterize elemental profiles of 48 elements, and both inorganic and organic forms of mercury, in a fin whale from the southern hemisphere with a lifespan from the early 20th century. In addition, relative changes in select elements were compared to known changes in biochemistry, specifically reaching sexual maturity. Lifetime bioaccumulation rates were calculated for non-essential toxic elements with profiles that showed evidence of bioaccumulation. Profiles of both inorganic and organic mercury were also produced.

Methods

Earplug Delamination

A single preserved fin whale earplug originating from the Southern Atlantic Ocean Basin that died in 1954 was delaminated as previously described[72], however, precautions were taken to eliminate metals contamination. Briefly, the earplug was aged and then bisected lengthwise with a ceramic blade by a researcher wearing protective equipment, including a metal-free coat, pants, shoe covers, face mask, and face shield within a Biosafety Level 3 clean space equipped with a Class 2 type A2 laminal flow hood (NUAIRE). Delamination was performed with a ceramic scalpel. Elemental

analysis required a minimum sample mass of 100 mg. Due to this limitation, individual laminae were combined to meet requirements. Laminae 1 - 12 representing approximately birth to 6 y were separated individually. Laminae 13 – 21 were combined in samples of 1 y (2 laminae per sample) and included the age ranges of 7 – 15 y. Lastly, laminae 22 – 34 were combined in samples of 2 y (4 laminae per samples) and represented approximately 17 – 41 y of age. Once delaminated, samples were homogenized within their vials and stored in nitrogen. The earplug was delaminated at Baylor University, Waco, TX, and was then shipped to the University of Wisconsin-Madison, Madison, WI for elemental analysis.

Analyte List

A total of 48 elements (including multiple measures of Hg and MeHg) were analyzed in digested samples of earwax. Toxic elements are those that are potentially harmful at low concentrations and include cadmium (Cd), lead (Pb), and Hg.[44] Essential elements are required for biological function and can be toxic at high concentrations, however, deficiencies of essential elements can also cause adverse health effects (e.g., developmental disorders and abnormal biological functions).[49] In some reports of toxic elements, arsenic (As) and chromium (Cr) are considered toxic. Arsenic is considered an essential element in low concentrations and for this study, it is considered nonessential. Chromium is also considered an essential element and will be classed as such within this study. For example, Cr deficiency often presents as glucose intolerance and abnormalities in glucose and lipid metabolism and nerve disorders.[168] For this study, essential elements included in alphabetical order are calcium (Ca), chromium (Cr), cobalt (Co), copper (Cu), iodine (I), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo),

phosphorous (P), potassium (K), selenium (Se), sodium (Na), and zinc (Zn). The overall presence of elements within the body is dependent on the needs of biological functions as well as the distribution of elements across the oceans.[45] Essential elements are generally split into two categories, macro- and micro-elements.[169] Macro-elements are required in higher concentrations[170] greater than 100 mg dL^{-1} and include Ca, P, and Na within this study. Micro-elements are required in lower concentrations less than 100 mg dL^{-1} and include Cr, Co, Cu, I, Fe, Mg, Mn, Mo, K, Se, and Zn. Some elements are needed in ultra-trace concentrations and are believed to be essential, in this study, ultra-trace elements were labeled as nonessential and include arsenic (As), boron (B), lithium (Li), nickel (Ni), and vanadium (V).

Sample Preparation

Whale cerumen was digesting using a mixed acid microwave-aided Teflon bomb with Pro 24 Microwave Rotor. Briefly, 100 mg of cerumen was dissolved in 4 mL of 16 M nitric acid, 4 mL of 32 M sulfuric acid, 0.8 mL of hydrofluoric acid, and 2 mL of hydrogen peroxide and then loaded into the microwave digestor at room temperature. Samples were then heated to 210 ºC at a rate of 15 ºC per minute and then held at 210 ºC for 20 minutes. Samples were then diluted to 30 mL using ultrapure deionized water. Samples were then filtered and diluted to a 1:3 ratio for sector field - inductively coupled plasma – mass spectrometry (SF-ICP-MS, Thermo-Finnigan, Element 2).

Sample Analysis

SF-ICP-MS was performed for all elements within this study and included a variety of quality assurance and quality control (QA/QC) parameters. For all samples

analyzed using SF-ICP-MS, a mean batch-specific method blank, with outlier detection, was applied to all sample data. Additionally, uncertainly estimates for each sample/element included the standard deviation of triplicate analysis of each sample, blank subtraction from 4-5 method blanks from each batch (5 batches total), and digestion recovery corrections using a long-term standard deviation of National Institute of Standards and Technology Standard Reference Materials (SRMs) recoveries. QC of each batch included the SRM of marine sediment and mussel tissues. Aqueous mixed-metal digestion bomb spikes, bottle blanks, sample analysis and digestion duplicates, sample spikes and recoveries, reagent (method) blanks, initial calibration blanks, initial calibration verifications, continuing calibration blank, and continuing calibration verifications were used throughout the analysis. The averages of element concentrations were taken when multiple measurements were made for a single lamina (e.g., analysis and digestion duplicates). Only a single isotope of mercury with a mass number of 202 was analyzed via SF-ICP-MS and will be denoted as ^{202}Hg. Total mercury analysis was also performed using cold-vapor atomic fluorescence spectrometry (CVAF) using Brooks-Rand instruments and will be denoted as THg. Analysis including Hg and MeHg followed the Environmental Protection Agency method 1631 and 1630, respectively.

Statistics and Modeling

Elemental concentrations were reported as ng g^{-1} dry weight (dw) and were corrected for the mass of cerumen (g) used in the digestion. Elemental profiles were produced by plotting the summation of elements within each lamina versus the estimated age determined through the aging process. Bioaccumulation rates of toxic elements were determined similarly to previously published rates of persistent organic pollutants.[142, 171]

Briefly, rates were determined by combining the elemental concentration of the current and previous laminae versus the age and applying linear regression ($ng \cdot g^{-1}\ y^{-1}$). Percent MeHg (%MeHg) was calculated by determining the percent of MeHg within inorganic Hg (which is calculated by subtracting the concentration of MeHg determined by CVAF from ^{202}Hg determined via SF-ICP-MS) which is denoted as Hg_i. Pearson Correlations including all elements, age, %MeHg, and the molar ratio of Se and Hg_i (Se:Hg_i) (df = N – 2 = 32, $\alpha = 0.05$) were determined and plotted using Tableau Desktop 2020 (Salesforce, Seattle, WA).

Results

Forty-eight elements (including MeHg and THg) were analyzed within 34 laminae (n = 45 total samples including laminae and digestion duplicates) of a historic female fin whale originating from the Southern Atlantic Ocean basin that had an approximate lifespan from 1913 to 1954. This single earplug produced a dataset of over 1,600 total data points. Of the elemental classes analyzed, toxic, essential, and nonessential, are discussed in more detail. Rare earth elements and other non-biologically active elemental measurements and profiles are included within the Supporting Information.

Elemental profiles were reconstructed by normalizing elemental data to the age and year estimates associated with each lamina. These profiles were then used to investigate lifetime trends of toxic, essential, and nonessential elements. Using earplugs, it is possible to investigate the burden of toxic elements as well as their bioaccumulation rates over the entire lifetime of an individual whale's life. Additionally, SF-ICP-MS and CVAF can be used to quantify Hg and MeHg. This additional data of both the inorganic

and organic forms of Hg can be used to evaluate exposure at different life stages of the individual whale's life.

Table 4.1 Sum, mean, standard deviation, and range of toxic, essential, and nonessential elements.

Elemental Class	Element	Sum (ng g^{-1})	Mean ± s (ng g^{-1})	Range (ng g^{-1})
Toxic	$^{111}Cd^{e}$	8,900	260 ± 87	140 - 460
	Pb*[e]	1.5×10^{6}	$4.3 \times 10^{4} \pm 2.8 \times 10^{4}$	2.3×10^{4} - 1.5×10^{5}
	^{202}Hg	2,200	64 ± 26	26 - 140
	Hg**	3,000	88 ± 37	45 - 220
	MeHg**	300	8.9 ± 1.8	5.3 - 13
Essential	^{44}Ca	4.2×10^{8}	$1.2 \times 10^{7} \pm 4.5 \times 10^{6}$	6.8×10^{6} - 2.4×10^{7}
	$^{52}Cr^{t}$	4,700	140 ± 110	36 - 590
	^{59}Co	5,000	150 ± 28	110 - 220
	^{63}Cu	2.8×10^{6}	$8.1 \times 10^{4} \pm 9{,}400$	6.5×10^{4} - 1.0×10^{5}
	^{127}I	2,500	74 ± 75	20 - 370
	^{56}Fe	2.3×10^{6}	$6.7 \times 10^{4} \pm 1.2 \times 10^{4}$	2.5×10^{4} - 1.6×10^{5}
	^{25}Mg	1.8×10^{6}	$5.3 \times 10^{4} \pm 7{,}000$	4.3×10^{4} - 7.1×10^{4}
	^{55}Mn	2.2×10^{5}	6,600 ± 5,100	1,200 - 2.0×10^{4}
	^{95}Mo	1,500	43 ± 10	29 - 68
	^{31}P	1.1×10^{8}	$3.2 \times 10^{6} \pm 2.3 \times 10^{6}$	4.6×10^{5} - 8.6×10^{6}
	^{39}K	4.3×10^{5}	$1.3 \times 10^{4} \pm 4{,}600$	9.2×10^{3} - 3.0×10^{4}
	^{77}Se	1.4×10^{6}	$4.1 \times 10^{4} \pm 6{,}900$	3.1×10^{4} - 5.7×10^{4}
	^{23}Na	2.1×10^{6}	$6.2 \times 10^{4} \pm 2.9 \times 10^{4}$	3.0×10^{4} - 1.4×10^{5}
	^{66}Zn	2.3×10^{6}	$6.8 \times 10^{4} \pm 3.9 \times 10^{4}$	2.5×10^{4} - 1.6×10^{5}
Nonessential	$^{75}As^{t}$	4,600	140 ± 39	59 - 200
	^{11}B	6.9×10^{4}	2,000 ± 370	1,500 - 3,300
	$^{7}Li^{e}$	500	15 ± 9.4	4.1 - 40
	^{60}Ni	3.6×10^{5}	$1.1 \times 10^{4} \pm 1{,}700$	7,500 - 1.5×10^{4}
	$^{51}V^{e}$	7,000	210 ± 30	170 - 300

[e] Evidence of essentialness
[t] Also considered toxic
* Sum of Pb
** Quantified via CVAF

Toxic Elements

Toxic element concentrations spanned multiple orders of magnitude. The sum, mean, standard deviation, and range of toxic, essential, and nonessential elements can be found in Table 4.1. Lead was detected in the highest average concentrations followed by Cd > THg > ^{202}Hg > MeHg. Lead concentrations were also higher than some essential and non-essential elements including (K > Mn > Co > I > Mo > Se and Ni > B > V, respectively). MeHg, on average, was four magnitudes lower in concentration than Pb with a mean and standard deviation of 8.9 ± 1.8 and 4.3 x10^4 ± 2.8 x10^4 ng g^{-1}, respectively. MeHg was detected in concentrations lower than all toxic elements as well as both essential and nonessential elements.

Essential and Nonessential Elements

Essential elemental means spanned a total of six orders of magnitude with Ca had the highest concentration on average followed by P > Cu > Zn > Fe > Na > Mg > Se > K > Mn > Co > Cr > I > Mo. Though necessary in higher concentrations, some essential elements were found in lower concentrations than other toxic and nonessential elements. Nonessential element concentrations spanned three orders of magnitude and were also found in higher concentrations than toxic elements. Of the nonessential elements, Nickel was detected in the highest concentration, on average, followed by B > Cr > As > Li.

Reconstructed Lifetime Elemental Profiles

Reconstructed lifetime toxic, essential, and nonessential profiles spanning 41 y were produced for each element within their respective class using the corresponding ages. Many of these elemental profiles exhibited periods of relative stability, increasing or decreasing trends, or both. Periods with no visible increasing or decreasing trend were reported as a mean ± standard deviation. Increasing or decreasing concentrations over

multiple years are described by the slope and r^2. Elemental trends were not distinct within each class. For example, Cd, Ca, and Li (toxic, essential, and nonessential elements, respectively) had similar trends during specific periods of life. Correlations between toxic, essential, and nonessential elements will be discussed in the following section.

Profiles of Toxic Elements. Lifetime elemental profiles were reconstructed for Cd, Pb, Hg (including THg and MeHg) by plotting the concentration of each element as a function of age or year and can be found in Figure 4.1. Over the entire lifetime of the individual fin whale's life, multiple elements, including those within the same elemental class (e.g., toxic) provided a variety of different trends. Cadmium had a profile consisted of four distinct periods (labeled as period 1, *2, 3, and 4). The age associated with each period can be found in Table 4.2.*

Table 4.2 Temporal periods of elemental profiles.

Period	Age Range (y)	Estimated Year
1	0.5 – 5.0	1913 – 1918
2	5.5 – 10.75	1918 – 1924
3	10.75 – 18.25	1924 – 1931
4	19.25 – 40.25	1932 – 1953

Periods 1 and 4 consisted of concentrations with no visible increase or decreasing trend and are reported as a mean and standard deviation (report as $ng \cdot g^{-1}$), while periods 2 and 3 appeared to have an increasing and proceeding decreasing trend, respectively. Both periods 2 and 3 of concentrations are represented with a corresponding slope ($ng \cdot g^{-1}\ y^{-1}$) and r^2. Cadmium Periods 1 and 4 had means and standard deviations of 162 ± 11.9 and 310 ± 42.5 ng g^{-1}, respectively. Periods 2 and 3 had slopes and r^2 of 48.4 $ng \cdot g^{-1}\ y^{-1}$ and 0.87, and -24.3 $ng \cdot g^{-1}\ y^{-1}$ and 0.53, respectively. During period 2 (5.5 – 10.75 y, ≈ 1918 –

1924) concentrations increased from 157 ng g^{-1} to 460 ng g^{-1}. During period 3 (10.75 – 18.25 y, ≈ 1924 – 1931) the concentration decreased to 240 ng g^{-1}. Interestingly, MeHg has an overall profile similar to Cd with four distinct periods. Periods 1 and 4 had a mean and standard deviation of 7.15 ± 1.20 and 9.70 ± 1.36 ng g^{-1}, respectively. Periods 2 and 3 had a slope and r^2 of 0.725 ng·g^{-1} y^{-1} and 0.63, and -0.114 ng·g^{-1} y^{-1} and 0.14, respectively. During period 2 (5.5 – 10.75 y, ≈ 1918 – ≈ 1924) concentrations increased from 6.98 ng g^{-1} to 9.39 ng g^{-1}. During period 3 (10.75 – 18.25 y, ≈ 1924 – 1931) the concentration decreased to 8.40 ng g^{-1}. The slope of Cd during period 2 was ≈ 67 times greater than the slope of MeHg. For comparison, the concentration of Cd would take ≈ 3 y to double during period 2. For MeHg, it would take ≈ 10 y to double in concentration, suggesting a low rate of exposure. Cd doubled once and was close to doubling a second time at the end of period 2.

The remaining toxic elements did not follow the pattern of four distinct periods and demonstrated a wide range of unique profiles. This may suggest multiple sources of pollution or differences in sequestration. For example, Pb had a mean and standard deviation of 3.06 x 10^4 ± 4,000 ng g^{-1} from birth to approximately 18 years of age. This stable trend was followed by increasing concentrations from ≈ 18.25 to 40.25 y (death) with a slope and r^2 of 4,430 ng·g^{-1} y^{-1} and 0.80, respectively. ^{202}Hg had a decreasing trend from ≈ 0.5 y to 7.75 y with a slope and r^2 of -4.90 ng·g^{-1} y^{-1} and 0.51, respectively. The ^{202}Hg had an increasing trend from ≈ 8.75 y to 40.25 y with a slope and r^2 of 2.82 ng·g^{-1} y^{-1} and 0.85, respectively. THg concentrations showed relatively low variability (i.e. stable) during its first 12 years of life (mean and standard deviation of 66.9 ± 15.1 ng g^{-1}) and was subsequently followed by a period of increasing concentrations until its death at

≈ 40 years of age (slope and r^2 of 3.32 and 0.58, respectively). THg had a concentration of 32.1 ng g^{-1} and doubled approximately every 10 y.

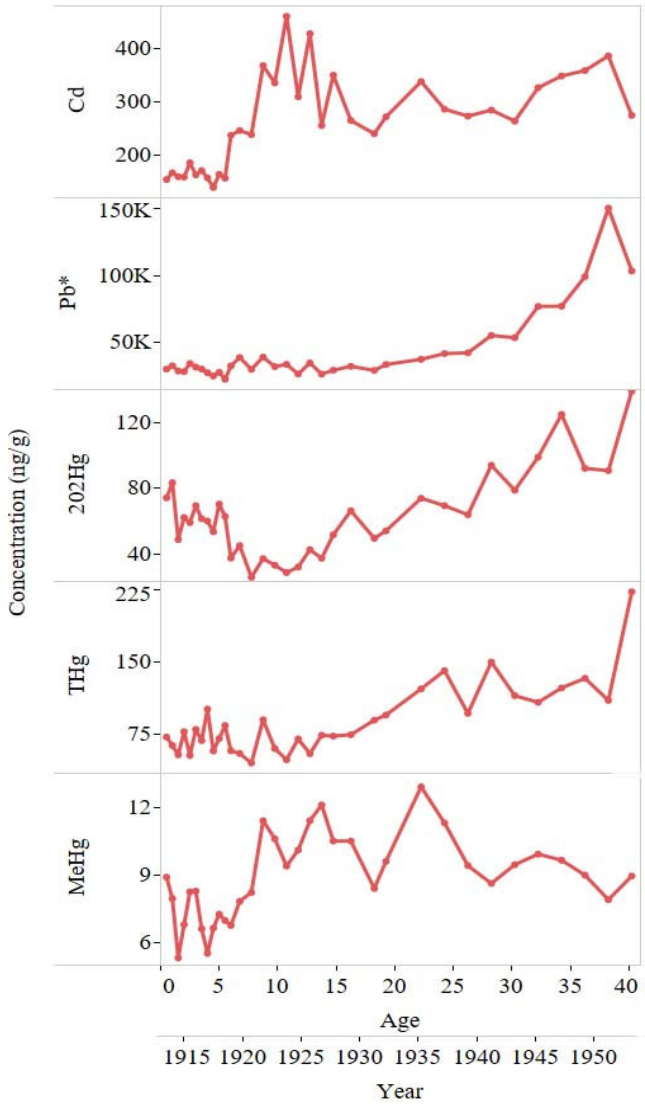

Figure 4.1 Reconstructed lifetime profiles of toxic elements as a function of age and year. Reconstructed profiles (ng/g cerumen) for a 40-year-old fin whale from the Southern Atlantic.

Inorganic and Organic Mercury. Methylmercury and THg concentration comparisons resulted in interesting trends over the entire lifespan of the individual fin whale, Figure 4.2. Inorganic Hg (Hg_i) was detected in a wide range of concentrations

(55.0 ± 25.7 ng g^{-1}) when compared to MeHg (8.90 ± 1.81 ng g^{-1}). concentrations, which remained relatively constant. The Inorganic Hg profile depicts a depression in concentration around 5 y of age which extends to roughly 16 y. This decrease in Hg_i over this 11-y period resulted in an increase in %MeHg from about ≈ 10 to 30%. Concentrations of Hg_i began to increase around the age of 16 y and %MeHg decreased until the death of the individual. This change in Hg_i could be related to variations in concentrations found in the environment or biological processes within the fin whale.

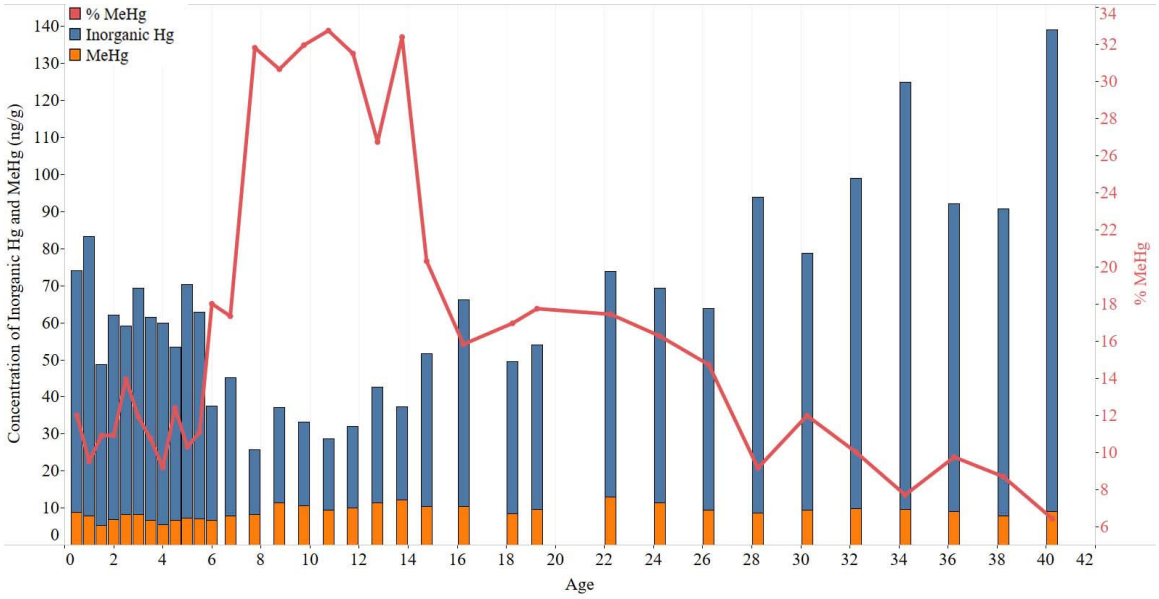

Figure 4.2 Inorganic mercury (Hg_i), methyl mercury (MeHg), and percent MeHg (%MeHg) lifetime profile.

Bioaccumulation Rates of Toxic Elements. Bioaccumulation rates were determined for select toxic elements, including Cd, Pb, and Hg (i.e. ^{202}Hg, THg, and MeHg). Bioaccumulation rates (reported as $n{\cdot}g^{-1}\,y^{-1}$) were produced by summing the concentrations of the current and previous lamina and plotted against the average age (y) were highest for Pb and lowest for MeHg, Figure 4.3. The range in bioaccumulation rates between Pb and MeHg spanned four orders of magnitude. Cd, Pb, ^{202}Hg, THg, and MeHg

had slopes of 222 $ng \cdot g^{-1}\ y^{-1}$, 3.09 x 10^4 $ng \cdot g^{-1}\ y^{-1}$, 45.4 $ng \cdot g^{-1}\ y^{-1}$, 67.1 $ng \cdot g^{-1}\ y^{-1}$, and 7.38 $ng \cdot g^{-1}\ y^{-1}$, respectively, and r^2 ranging from 0.96 to 0.98. For reference, the THg bioaccumulation rate for the previously studied blue whale was 28.2 $ng \cdot g^{-1}\ y^{-1}$, which is ≈ 2.3 times lower than the rate found within this study. This difference in bioaccumulation rate could suggest a decrease in THg within the environment as the blue whale had a later lifespan from 1995 to 2007 or a difference in environmental concentrations between hemispheres.

Profiles of Essential Elements. Similar to toxic elements, essential elements also had a variety of profiles (see Figure 4.4). Interestingly, the profile of calcium was similar to the toxic elements Cd and MeHg. The first period for Ca had a mean and standard deviation of 7.29 x 10^6 ± 2.97 x 10^5 ng g^{-1}. The second period had a slope of 2.43 x 10^6 $ng \cdot g^{-1}\ y^{-1}$ and an r^2 of 0.76. The third period had a slope of -1.16 x 10^6 $ng \cdot g^{-1}\ y^{-1}$ and an r^2 of 0.58. During period 2 (5.5 – 10.75 y, ≈ 1918 – 1924) Ca concentrations increased from 8.29 x 10^6 ng g^{-1} to 2.39 x 10^7 ng g^{-1}. During period 3 (10.75 – 18.25 y, ≈ 1924 – 1931) the concentration decreased to 1.25 x 10^7 ng g^{-1}. Calcium concentrations during period 2 doubled in ≈ 1 y. The fourth period had a mean and standard deviation of 1.34 x 10^7 ± 2.09 x 10^6 ng g^{-1}.

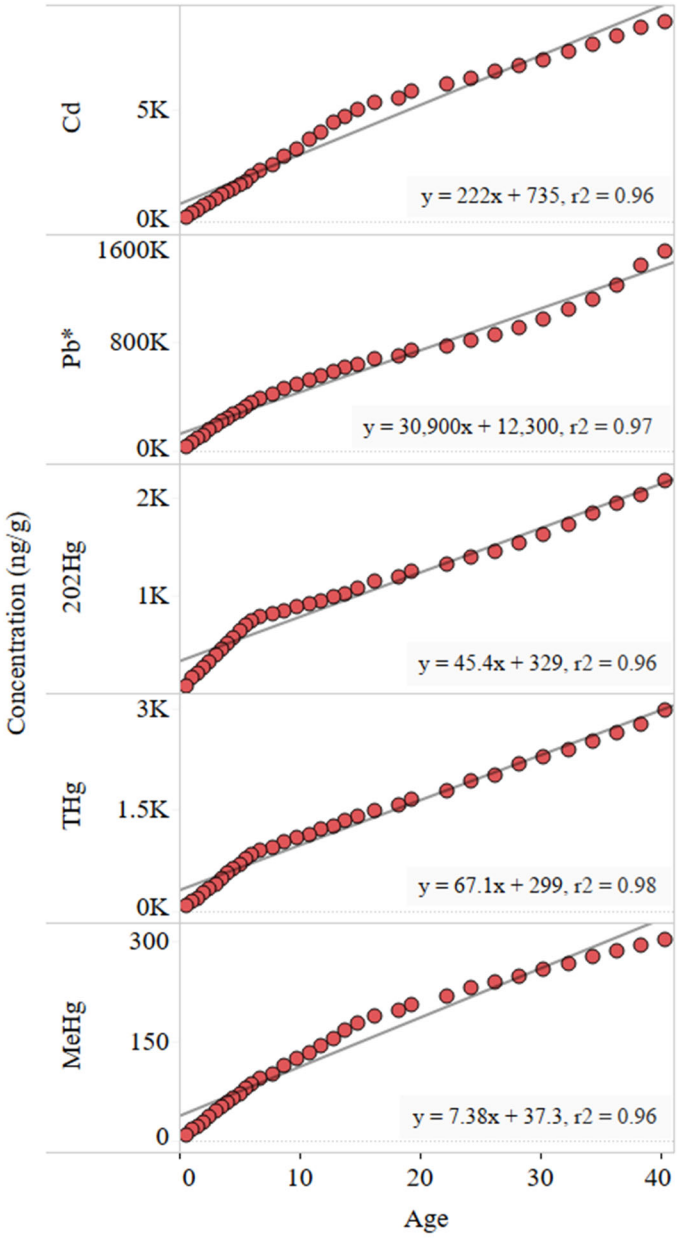

Figure 4.3 Bioaccumulation rates and r^2 of toxic elements including, Cd, Pb, and ^{202}Hg, THg, and MeHg Pb* indicates the sum of lead.

Cobalt, Cu, Mg, Mn, P, Se, Na, and Zn also had similar trends to Ca and their four respective periods are described below. For Co, the first period had a mean and standard deviation of 142 ± 9.73 ng g^{-1}. The second period had a slope of 16.7 ng·g^{-1} y^{-1} and an r^2 of 0.84. The third had a slope of -8.69 ng·g^{-1} y^{-1} and an r^2 of 0.60. The fourth had a mean

and standard deviation of 126 ± 13.4 ng g^{-1}. For Cu, the first period had a mean and standard deviation of 8.47 x10^4 ± 3,520 ng g^{-1}. The second period had a slope of 5,230 ng·g^{-1} y^{-1} and an r^2 of 0.96. The third period had a slope of -2,970 ng·g^{-1} y^{-1} and an r^2 of 0.59. The fourth period had a mean and standard deviation of 7.15 x 10^4 ± 4,230 ng g^{-1}.

For Mg, the first period had a mean and standard deviation of 4.99 x 10^4 ± 4,810 ng g^{-1}. The second period had a slope of 5,180 ng·g^{-1} y^{-1} and an r^2 of 0.88. The third period had a slope of -2,250 ng·g^{-1} y^{-1} and an r^2 of 0.51. The fourth had a mean and standard deviation of 5.28 x 10^4 ± 5,010 ng g^{-1}. For Mn, the first period had a mean and standard deviation of 1,310 ± 77.0 ng g^{-1}. The second period had a slope of 2,950 ng·g^{-1} y^{-1} and an r^2 of 0.85. The third period had a slope of -1,400 ng·g^{-1} y^{-1} and an r^2 of 0.53. The fourth had a mean and standard deviation of 7,100 ± 1,900 ng g^{-1}. For P, the first period had a mean and standard deviation of 5.31 x 10^5 ± 5.13 x 10^4 ng g^{-1}. The second had a slope of 1.23 x 10^6 ng·g^{-1} y^{-1} and an r^2 of 0.79. The third period had a slope of -5.23 x 10^5 ng·g^{-1} y^{-1} and an r^2 of 0.42. The fourth had a mean and standard deviation of 3.71 x 10^6 ± 8.86 x 10^5 ng g^{-1}.

Selenium's first period had a mean and standard deviation of 3.63 x10^4 ± 4.380 ng g^{-1}. Periods 2 and 3 had slopes and r^2 of 5,280 ng·g^{-1} y^{-1} and 0.86 and -1,900 ng·g^{-1} y^{-1} and 0.45, respectively. Period 4 had a mean and standard deviation of 4.09 x 10^4 ± 4,330 ng g^{-1}. For Na, the first period had a mean and standard deviation of 3.36 x 10^4 ± 3,530 ng g^{-1}. The second had a slope of 1.78 x 10^4 ng·g^{-1} y^{-1} and an r^2 of 0.85. The third period had a slope of -9,110 ng·g^{-1} y^{-1} and an r^2 of 0.57. The fourth had a mean and standard deviation of 6.26 x 10^4 ± 8.910 ng g^{-1}. For Zn, the first period had a mean and standard deviation of 2.72 x 10^4 ± 1,500 ng g^{-1}. The second had a slope of 2.22 x 10^4 ng·g^{-1} y^{-1} and an r^2 of

0.83. The third period had a slope of -1.02 x 10^4 ng·g^{-1} y^{-1} and an r^2 of 0.47. The fourth had a mean and standard deviation of 7.32 x 10^4 ± 1.69 x 10^4 ng g^{-1}.

Other essential elemental profiles, including, Cr, I, Fe, Mo, and K, did not appear to have four distinct periods. For example, Cr had a large amount of variation within the first 5.5 y of life represented by a mean and standard deviation of 203 ± 149 ng g^{-1}. In particular, Cr had a large concentration spike of 118 ng g^{-1} at 3 y of age to 593 ng g^{-1} at 3.5 y. Following the first 5.5 y of life (≈ 6 – 40.25 y, death) Cr had a mean and standard deviation of 113 ± 72.6 ng g^{-1}. Similar to Cr, iodine had a large amount of variation within the first 5.5 y of life represented by a mean and standard deviation of 14.0 ± 11.3 ng g^{-1}. Interestingly it had two large spikes in concentration at 1.5 y and 4.5 y. This variability may be due to the ≈ 6 mo. resolution of early lamina. Following the first 5 y of life (≈ 6 – 40.25 y, death), Iodine had a mean and standard deviation of 46.5 ± 18.6 ng g^{-1}. Potassium had a very similar amount of variation within the first ≈ 5.5 y of life represented by a mean and standard deviation of 1.73 x 10^4 ± 5,910 ng g^{-1}. Following the first 5 y of life (≈ 6 – 40.25 y, death), K had a mean and standard deviation of 1.08 x 10^4 ± 2,070 ng g^{-1}. For K, there was also a large spike in concentration around 3 y as concentrations went from 1.57 x 10^4 to 3.03 x 10^4 ng g^{-1}. Iron did not match the above essential elements and had a decreasing trend from an estimated 0.5 to 19.25 y with a slope and r^2 of -752 ng·g^{-1} y^{-1} and 0.52, respectively. Iron concentration then increased from ≈ 19.25 to ≈ 40.25 y (death) with a slope and r^2 of 2,230 ng·g^{-1} y^{-1} and 0.71, respectively. Molybdenum was also alone in its lifetime profile and had a large amount of variability from ≈ 0.5 (birth) to ≈ 19.25 y with a mean and standard deviation of 45.5 ±

10.1 ng g^{-1}. From 19.35 to 40.25 (death) Mo had a slope and r^2 of 0.923 ng·g^{-1} y^{-1} and 0.64, respectively.

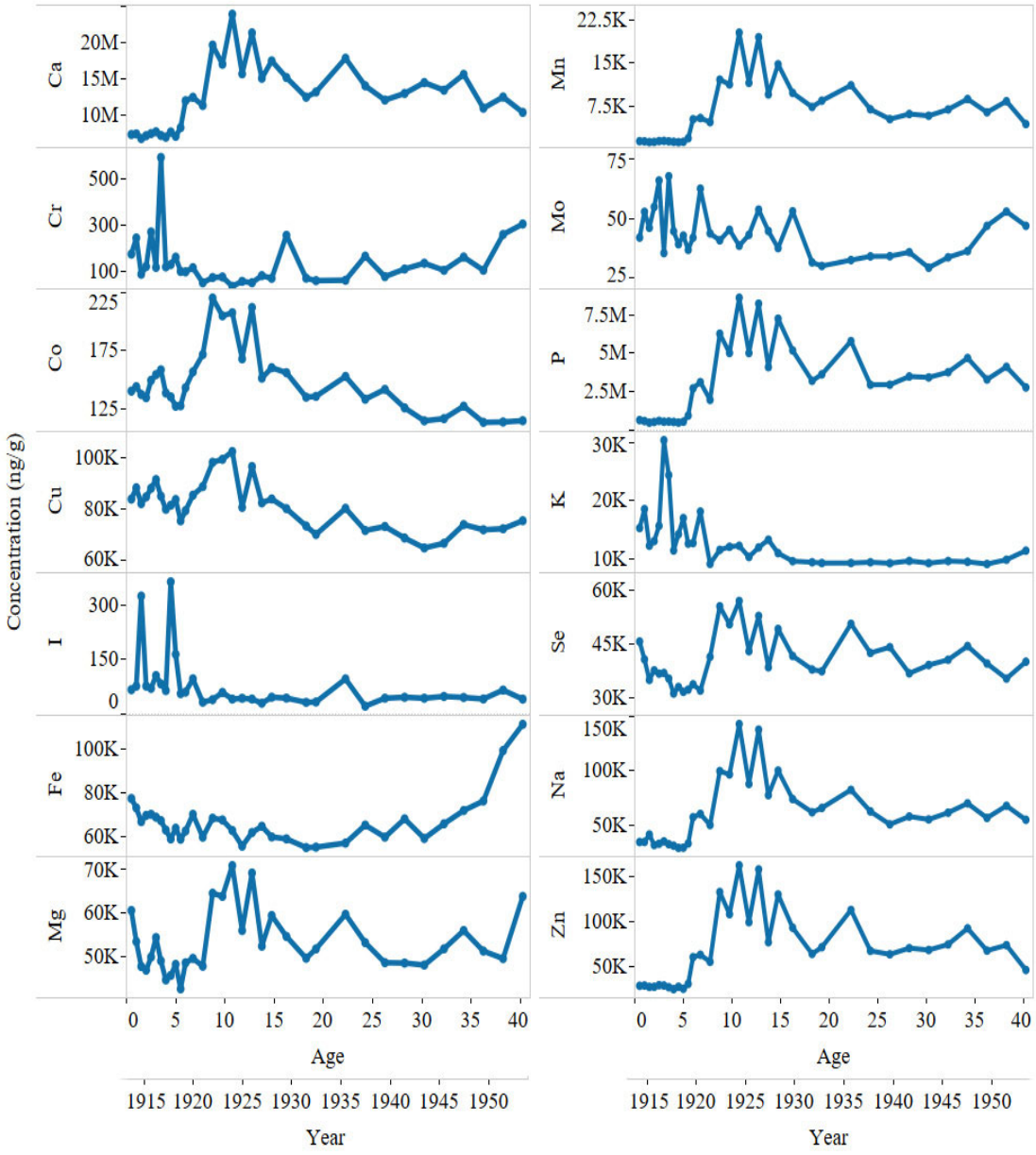

Figure 4.4 Reconstructed lifetime profiles of select essential elements as a function of age and year. Reconstructed profiles (ng g^{-1}cerumen) for a 40-year-old fin whale from the Southern Atlantic.

Profiles of Nonessential Elements. Similar to toxic and essential elemental profiles, nonessential elements also had a variety of trends which can be found in Figure 4.5. Both Li and Ni shared similarities to the four periods found in both toxic and essential elements. Li's first period had a mean and standard deviation of 9.15 ± 4.26 ng g^{-1}. Periods 2 and 3 had slopes and r^2 of 6.16 ng·g^{-1} y^{-1} and 0.86, and -2.83 ng·g^{-1} y^{-1} and 0.59, respectively. During period 2 (5.5 – 10.75 y, ≈ 1918 – 1924) concentrations of Li increased from 5.37 ng g^{-1} to 40.1 ng g^{-1}. During period 3 (10.75 – 18.25 y, ≈ 1924 – 1931) the concentration decreased to 12.2 ng g^{-1}. Ni's first and fourth period had a mean and standard deviation of 1.10 x 10^4 ± 598 and 8,640 ± 753 ng g^{-1}, respectively. Periods 2 and 3 had slopes and r^2 of 874 ng·g^{-1} y^{-1} and 0.96, and -484 ng·g^{-1} y^{-1} and 0.56, respectively. During period 2 (5.5 – 10.75 y, ≈ 1918 – 1924), Ni concentrations increased from 9,933 ng g^{-1} to 1.47 x 10^4 ng g^{-1}. During period 3 (10.75 – 18.25 y, ≈ 1924 – 1931) the concentration decreased to 9,893 ng g^{-1}. The slope of Ni during period 2 was ≈ 142 times greater than the slope of Li.

Interestingly, arsenic's profile had no distinguishing periods or overall trends and had a mean and standard deviation of 135 ± 38.8 ng g^{-1}. Similar to Cr, As is often considered toxic, however, it has also been reported as a necessary element at low concentrations and will be considered nonessential for this study. Unlike other nonessential elements, B's profile had two distinct trends. From birth to ≈ 12.75 y B had a slope and r^2 of -78.0 ng·g^{-1} y^{-1} of 0.55 and was followed by concentrations of limited variability with a mean and standard deviation of 1,800 ± 189 ng g^{-1}. Boron had the highest concentration at 0.5 y (3,257 ng g^{-1}) and never exceeded this concentration over the entire lifespan of the individual fin whale. Lastly, V had a relatively flat profile until

≈ 22.25 y where concentrations had a mean and standard deviation of 200 ± 20.1 ng g^{-1}. This profile was then proceeded by an increasing trend until death with a slope and r^2 of 6.37 ng·g^{-1} y^{-1} and 0.68, respectively. At ≈ 22.25 y the concentration of V was 174 ng g^{-1} and increased to 295 ng g^{-1} at 40.25 y.

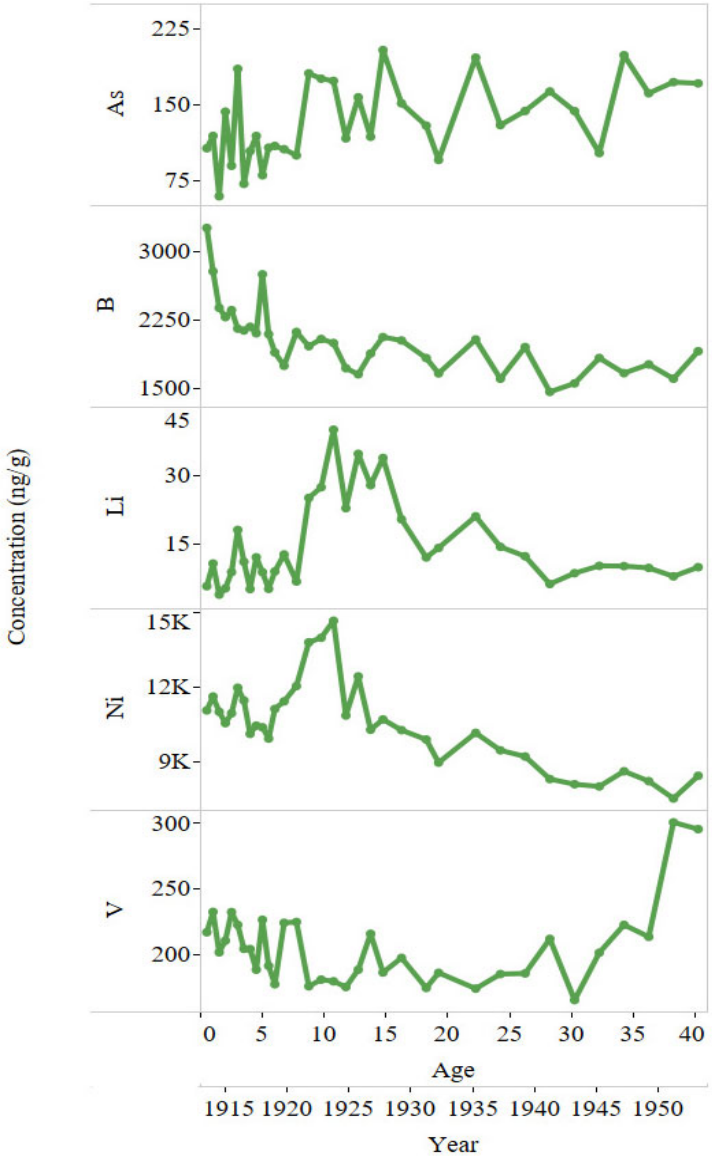

Figure 4.5 Reconstructed lifetime profiles of select non-essential elements as a function of age and year. Reconstructed profiles (ng/g cerumen) for a 40-year-old fin whale from the Southern Atlantic.

Correlations of Toxic, Essential, and Nonessential Elements

Pearson correlations were produced to determine similarity in trends between concentrations over time between all elemental classes. For simplicity, only strong Pearson correlations ($r \geq 0.70$ and $r \leq -0.70$) of toxic, essential, nonessential will be discussed below. In addition to elemental profiles, age, %MeHg, and $Se:Hg_i$ were also included for Pearson correlations. All elemental correlations with age can be found in Figure 4.7. Most elements were positively correlated which could suggest similar accumulation and storage patterns of elements within and outside elemental classes. Only a few cases of negative correlations were identified which could suggest a competition in the accumulation and storage of the element over time.

Typically, elements with their own class showed some strong correlations, though elements with the unique profile of four distinct periods showed evidence of correlations across multiple classes. Toxic elemental profiles were not correlated amongst other toxic elements, except for ^{202}Hg and THg. However, toxic elements were found to be strongly correlated to both essential and nonessential elements. For example, Cd was positively correlated to the following essential elements or age, including, Ca ($r = 0.90$), Mn ($r = 0.90$), P ($r = 0.93$), Se ($r = 0.72$), Na ($r = 0.89$), and Zn ($r = 0.91$). Essential elements were strongly correlated to both essential and nonessential elements. For example, Ca was positively correlated with the following essential and nonessential elements Mg ($r = 0.72$), Mn ($r = 0.97$), P ($r = 0.98$), Se ($r = 0.80$), Na ($r = 0.96$), Zn ($r = 0.98$), and Li ($r = 0.82$).

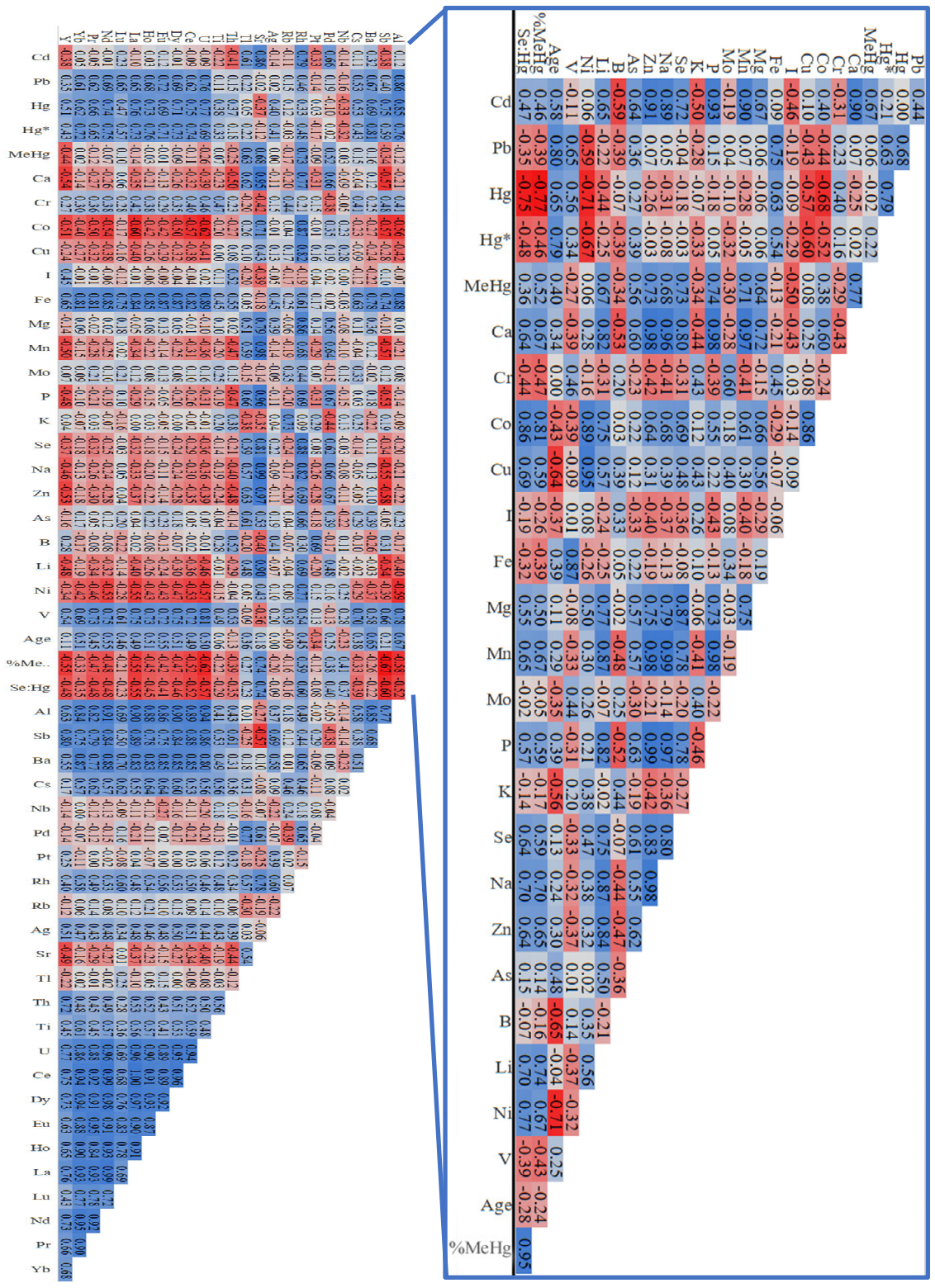

Figure 4.6 Pearson correlations of all elements, age, %MeHg, and Se:Hg. Correlations within the box are discussed within this study. Hg indicates ^{202}Hg and Hg* indicates THg.

Discussion

Through the analysis of a single earplug, elemental profiles were characterized in a single fin whale from the southern hemisphere to reveal relative changes in concentrations as a function of time. Importantly, these changes can occur due to a variety of biological processes, biogeochemical cycles, and anthropogenic activities. Additionally, regional differences and location changes of the whale could also contribute to elemental exposure. In this study, elemental profiles, correlations, bioaccumulation rates of toxic elements, spatiotemporal contributions, age, biological processes (e.g., sexual maturity), biogeochemical cycles (e.g., Fe), and Hg profiles, both inorganic and organic were investigated to address some above the above factors.

Elemental Profiles and Correlations

Elements serve multiple purposes within marine mammals and are subject to change due to their abundance within the environment and an individual's needs. Generally, there is less variability for essential elements as they are often heavily regulated biologically.[165] Additionally, toxic trace elements often accumulate as they have longer biological half-lives compared to other essential elements.[172] Reconstructed elemental profiles often appeared to follow similar trends over the individual's entire lifetime (i.e., those with four distinct periods) and were found to be strongly correlated using Pearson correlations. Correlations were not restricted to the elemental classes assigned in this study, i.e., toxic elements were correlated to both essential and nonessential elements and strong correlations between multiple elemental classes with similar profiles could suggest a similar pathway of sequestration[173], i.e., metallothionein,

or a lack of selectivity.[174] These correlations could also be driven by similar sources of elements within the environment.

Bioaccumulation Rates of Toxic Elements and Spatiotemporal Contributions

Bioaccumulation rates of contaminants are very rare for individuals or populations, particularly for Southern Oceans. Bioaccumulation rates calculated in this study expand on previous rates determined for persistent organic pollutants.[71] This previous study of six whales originating from the northern hemisphere found that bioaccumulation rates of POPs were statistically higher in whales originating from the North Pacific Ocean Basin and that the ocean of origin and when the individual lived were significant factors for the burden of POPs. Using elemental analysis, bioaccumulation rates of three common toxic elements, often considered some of the most important marine pollutants, including, Cd, Pb, and Hg were calculated. For example, alkyl-lead, an anti-knock additive, was initially added to gasoline in the 1920s and was used in most countries until the 1990s.[175] By comparing toxic elemental concentration as a function of time it is possible to determine the rate of exposure and investigate the temporal nature of the contaminant source. Bioaccumulation can also be used to compare individuals, populations, sex, generations through time and space, and differences between species. With limited data, modeling the factors contributing to toxic elemental exposure (e.g., sex, age, diet)[133, 134] is no simple task, however, with additional earplugs from multiple species originating from multiple ocean basins a fixed effect model, previously applied to persistent organic pollutants[71], could provide valuable insight.

Age and Biological Processes

Reconstructed lifetime elemental profiles are a unique opportunity to compare the 48 elements between different life stages (e.g., calf and adult). Differences in concentrations at different age classes were observed in many of the elemental profiles, including toxic, essential, and nonessential elements. Cadmium, MeHg, Ca, Mg, Mn, P, Se, Na, Zn, and Li all had trends where concentrations were lower from birth to ≈ 5 y of age and were followed by an increase until the approximate age of ≈ 10.75 y. This trend was then followed by a decrease in concentration until ≈ 18.25 y and then remained relatively constant throughout the remainder of the individual's life. Due to the similarities in lifetime profiles, the above elements were also found to be strongly positively correlated with r ranging from 0.71 to 0.98. Essential elements are needed in many biological processes and there was no surprise that elements within this class were correlated. This correlation could suggest a common source within the diet, environment, or a lack of selectivity when sequestering elements within the body. Concentrations of some elements at ≈ 5.5 y rose and reached a maximum at ≈ 10.75 y. This rapid increase could be indicative of the individual's accumulation of elements necessary for growth, development, or sexual reproduction.[176, 177] The individual female in this study lived from approximately 1913 to 1954 and likely had an age at sexual maturity closer to ten to eleven years, which matched previous reports from the 1930s.[178] A similar change point was also reported for Zn and Pb in a 2020 study of female walrus teeth, which also grow incrementally over the individual's lifetime.[167] Additional earplugs should be evaluated in both sexes to determine if elemental profiles change due to sexual maturity and other biological processes.

Biogeochemical Cycles

Elements in the marine ecosystem are introduced by a variety of sources[50] and are subject to natural biogeochemical cycles, which could interfere with specific reconstructed elemental profiles found using earplugs. The concentrations of trace elements are limited within the surface water.[42] Baleen whales play an active role in the biogeochemical cycle within the ocean as they have the ability to influence ocean productivity through the direction processing of organic matter.[1, 179, 180] Iron is required for photosynthesis and respiration.[181, 182] Nicol[183] indicated that Southern Ocean krill could contain ≈ 24% of the total Fe from the surface water within its vicinity and that whales' fecal matter originating from the same ocean contained ≈ 1.0×10^7 time the Fe found in surface seawater. Nicol continues to report that whales are concentrating carbon (C) and actively excreting Fe. The accumulation of elements within an individual is dependent on their environment and their own biological needs. The Fe profile, and those similar to it (V, $r = 0.87$), could represent the cycling of materials within the marine ecosystem. The lack of correlation between other biologically and biogeochemically necessary elements is interesting and requires further investigation. The productivity of the ocean is dependent on whales as large predators.[1] Commercial whaling removed a C sink as well as a recycling component of many biogeochemical cycles. By evaluating earplugs, researchers can investigate ocean productivity (i.e., biogeochemical cycles) and anthropogenic impacts as a function of time and space.

Mercury Profiles

The investigation of ^{202}Hg, Hg_i, THg, MeHg, and %MeHg represent a significant characterization of Hg in a baleen whale. This characterization is a unique opportunity to describe Hg as a function of time and to investigate different exposure scenarios or contributions across multiple life stages. ^{202}Hg was positively correlated to THg ($r = 0.79$) and negatively correlated to %MeHg ($r = -0.77$). THg was only positively correlated to age ($r = 0.79$) and could suggest bioaccumulation. MeHg was positively correlated to Ca ($r = 0.77$), Mn ($r = 0.74$), P ($r = 0.74$), Se ($r = 0.73$), and Zn ($r = 0.73$). Cd, Pb, THg, and MeHg were not correlated to any nonessential elements, however, ^{202}Hg was negatively correlated to Ni ($r = -0.71$) and Se:Hg ($r = -0.75$). %MeHg was strongly correlated to $Se:Hg_i$ ($r = 0.95$). MeHg concentrations were detected with little variability over the lifetime of the individual suggesting relatively constant concentrations from 1913 – 1953. Interestingly, the %MeHg profiles increases rapidly at 5 y, driven by the decrease of Hg_i, and returns to the lower contribution around 16 y. The fluctuation of %MeHg suggests that the concentrations of Hg_i were changing throughout the individual's lifespan. Changes in %MeHg could be due to the exposure from the environment, for example, the decrease in Hg_i at approximately 5 y and subsequent increase at approximately 11 y could indicate the individual moved from a more polluted to a less polluted area and then transitioned to a more polluted area, or the area it currently resided in became more polluted. Alternatively, due to the strong positive correlation to essential and nonessential elements, there may be some biological selectivity related to the storage of both inorganic and MeHg. Further studies of individuals living in the same period would be necessary to

determine if the interesting %MeHg profile was due to environmental or biological factors.

Selenium is an essential element for marine mammals as it participates in antioxidant functions and additional processes.[184] Selenium also plays a role in reducing the neurotoxicity of Hg.[185] The neuroprotective mechanism is not fully understood, but many researchers believe that the affinity of Hg to Se is capable of demethylating MeHg.[186, 187] This protective mechanism was first suggested in marine mammals in 1973.[188] Elevated concentrations of Hg and Se have been reported in the livers of marine mammals that showed no overt signs of poisoning.[165] This led to the idea that Se and Hg molar ratios in the liver should be used to assess the health effects of these elements.[44, 189] Animals with $Se:Hg_i$ molar ratios greater than one are considered low risk while ratios less than one are considered to be at high risk of Hg toxicity. In this study, using earwax, this individual fin whale had a mean and standard deviation $Se:Hg_i$ molar ratio of 2,460 ± 1,670. This massive $Se:Hg_i$ molar ratio suggests an excess of Se in comparison to Hg, suggesting that this individual was at little risk of Hg toxicity. In a recent study of elements found in the liver of multiple marine mammal species performed by Hansen et al.[165], found adult bottlenose dolphins (*Tursiops truncatus*) were at higher risk than a humpback whale calf (*Megaptera novaeangliae*). It is important to note that this is a comparison of two different matrices and that further studies are needed to compare liver and earplug elemental concentrations.

Selenium was found to be strongly positively correlated to Cd, MeHg, Ca, Mg, Mn, P, Na, and Zn. The abundance of correlations between elements suggests similar paths of accumulation and storage. Selenium and other elements, post-weaning, are

generally accumulated through food. There is a possibility that the storage of Se is impacted by the presence of other elements. For example, in a study of gray seals (*Halichoerus grypus*) fed low levels of MeHg, they found Hg and Se concentration in the liver increasing in parallel.[190] Se was not provided along with MeHg and was instead taken up from their diet, suggesting an increase in MeHg can cause an increase in the accumulation of Se, however, storage may not be element-specific and likely included the additional correlated elements as well.

Conclusion

The analysis of baleen whale earplugs provides a significant advancement and expansion on the research of elements found within the marine ecosystem as well as the utility of the baleen earplug. The analysis of a single earplug for a fin whale originating from the southern hemisphere, spanning over four decades within the early 20th century, produced more than 40 elemental profiles. These profiles were then used to find correlations between elements, age, %MeHg, and the molar ratio of selenium and mercury. Reconstructed profiles revealed possible changes in elemental profiles due to biological and biogeochemical processes, specifically sexual maturity, and ocean productivity, respectively. In addition to profiles, lifetime bioaccumulation rates of toxic elements were also reconstructed. Toxic elemental profiles also revealed an interesting relationship between inorganic and organic mercury as a function of time. Further analysis of earplugs can provide insight into the productivity and anthropogenic impact within the marine ecosystem.

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Contributions

Zach C Winfield: Statistical analysis, Writing – original draft. Farzaneh Mansouri: Investigation. Danielle D. Crain: Methodology and writing, Martin Shafer: Methodology, formal analysis, Richard Sabin: resources, Stephen J. Trumble: Methodology, Conceptualization, Writing – original draft, Sascha Usenko: Methodology, Conceptualization, Writing – original draft.

Table 4.3 Sum, mean, standard deviation, and range of REE and other elemental concentrations.

Elemental Class	Element	Sum (ng g^{-1})	Mean ± s (ng g^{-1})	Range (ng g^{-1})
REE	^{140}Ce	580	17 ± 12	4.0 - 73
	^{163}Dy	35	1.0 ± 0.91	0.13 - 5.2
	^{151}Eu	16	0.47 ± 0.34	0.057 - 2.0
	^{165}Ho	7.9	0.23 ± 0.18	0.033 - 1.0
	^{139}La	400	12 ± 7.6	2.6 - 46
	^{175}Lu	3.1	0.091 ± 0.071	0.026 - 0.35
	^{146}Nd	320	9.3 ± 7.2	2.5 - 42
	^{141}Pr	84	2.5 ± 1.8	0.47 - 10
	^{173}Yb	17	0.50 ± 0.41	0.12 - 2.4
	^{89}Y	320	9.3 ± 6.5	2.1 - 32
Other	^{27}Al	$3.4x10^6$	$1.0x10^5 \pm 4.4x10^4$	$6.1x10^4$ - $2.7x10^5$
	^{121}Sb	8,700	250 ± 80	140 - 580
	^{138}Ba	2.9×10^4	860 ± 500	450 - 3,300
	^{133}Cs	20	0.60 ± 0.41	0.088 - 1.7
	^{93}Nb	82	2.4 ± 2.1	0.64 - 12
	^{108}Pd	230	7.0 ± 2.0	3.5 - 13
	^{195}Pt	160	4.8 ± 13	0 - 77
	^{103}Rh	140	4.0 ± 0.95	2.9 - 6.5
	^{85}Rb	3,300	97 ± 66	8.7 - 360
	^{109}Ag	310	9.1 ± 3.4	3.7 - 22
	^{88}Sr	4.3×10^5	$1.3x10^4$ ± 8,100	4,400 - 3.6×10^4
	^{205}Tl	31	0.91 ± 0.66	0.20 - 3.8
	^{232}Th	42	1.2 ± 1.1	0.12 - 4.5
	^{49}Ti	1.7×10^4	490 ± 260	170 - 1,200
	^{238}U	520	15 ± 10	3.1 - 58

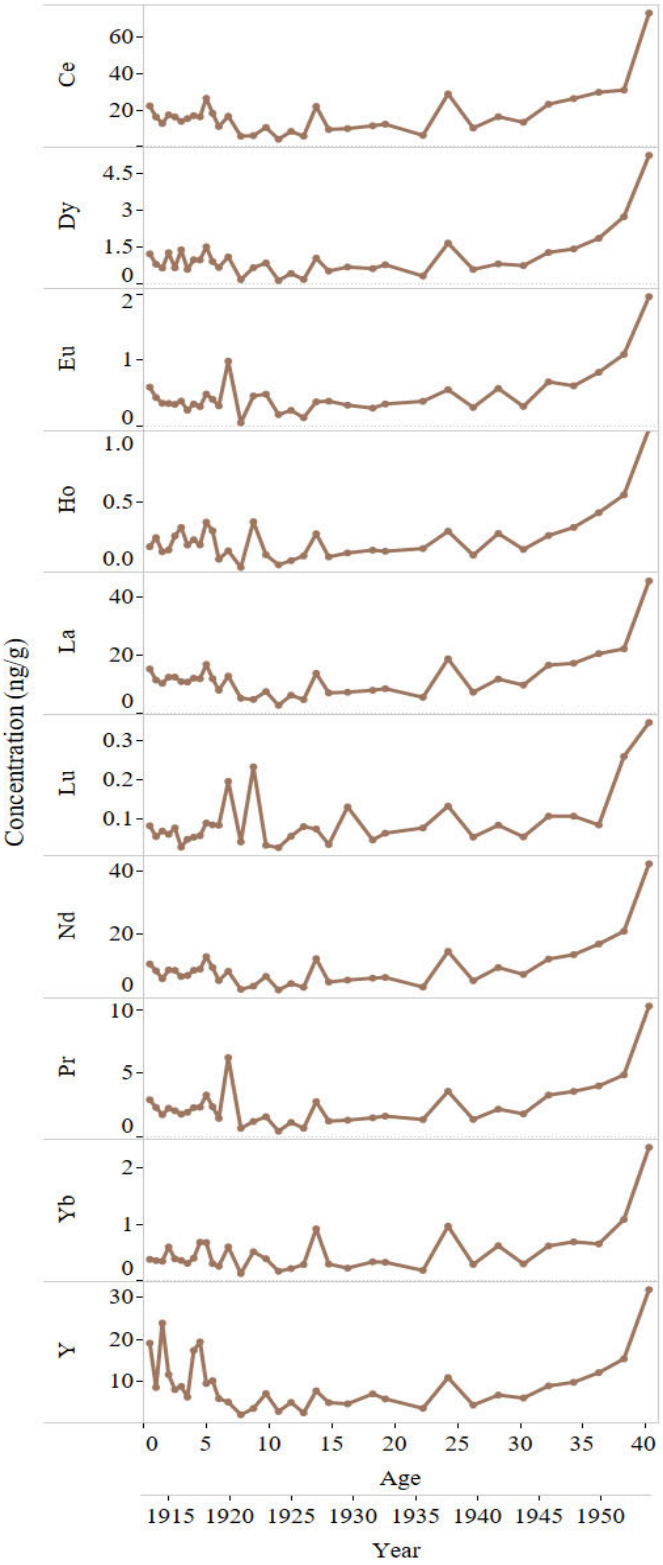

Figure 4.7 Reconstructed REE profiles.

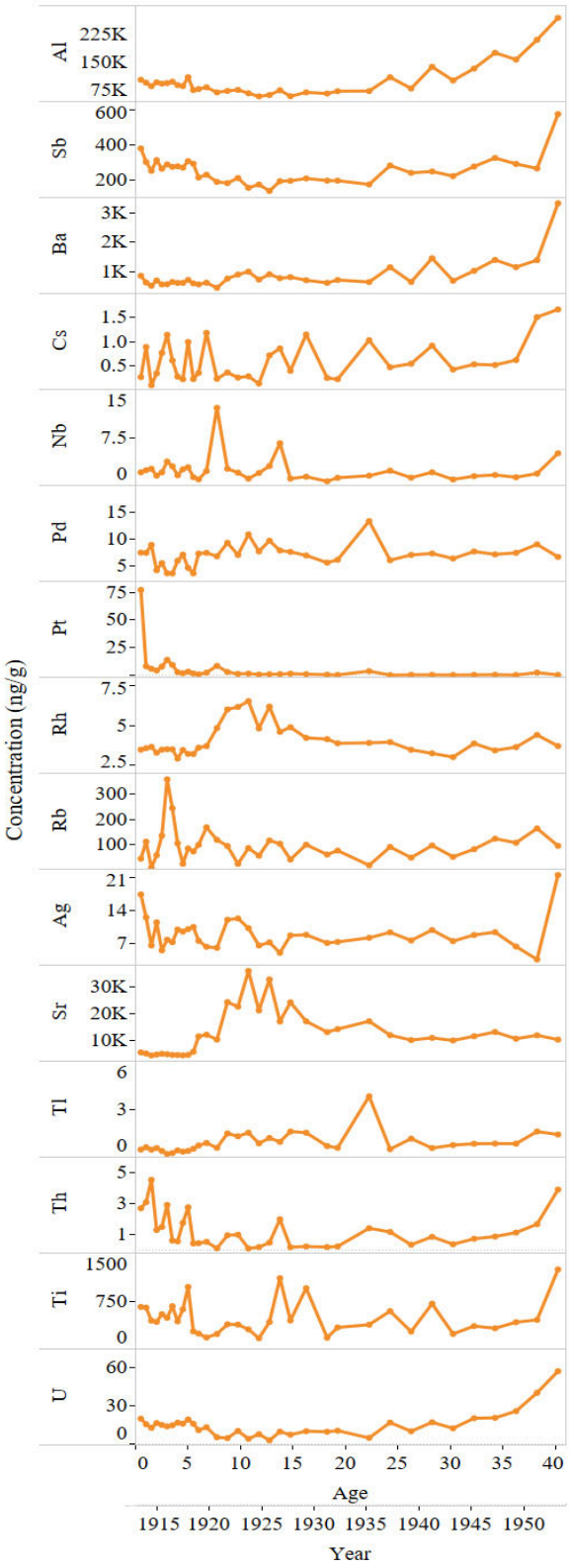

Figure 4.8 Reconstructed elemental profiles of other elements not considered.

CHAPTER FIVE

Conclusion and Future Work

Conclusion and Scientific Significance

In this study, the research sought to investigate multiple stressors and their possible impact on stress in marine mammals by providing a significant contribution to the knowledge gaps within the marine environment. This dissertation sheds light on the complexity of assessing multiple stressors in long-lived free ranging marine mammals and the difficulty associated with linking a health outcome with a known stressor (e.g., contaminants). The dissertation also highlights the utility of bioaccumulation rates and the intricacies of assessing elemental profiles linked to biological and biogeochemical cycles. Though the sample size is limited, this dataset is rare as it provides longitudinal trends of the same individual and allows for the exclusion of confounding factors that cause inter-individual differences. In Chapter Two, the lifetime burdens of POPs and possible health outcomes were investigated using the blubber of harbor porpoises and suggested a possible health outcome due to chronic exposure. In Chapter Three, the techniques used to quantify POPs in blubber (e.g., SPLE and GC-MS) were applied to earplugs. With age estimates determined by counting laminae and POPs analysis, bioaccumulation rates were calculated, and lifetime profiles were reconstructed. This study expanded our understanding of POPs in the northern hemisphere by providing insight into the differences between individuals, species, spatial and temporal trends, and distributions. Lastly, Chapter Four, utilized a single earplug to expand the contaminant

analyte list to include toxic elemental, essential and nonessential elements, as well as profile an individual in the poorly studied southern hemisphere.

Blubber collected from presumably healthy porpoises and an individual with B-cell lymphoma (BCL) was analyzed for contaminants. First, the burden of contaminants was determined, specifically, polychlorinated biphenyls (PCBs), pesticides (such as DDT and its metabolites), and polybrominated diphenyl ethers (PBDEs), found in the blubber of three female harbor porpoises, two pregnant females (one with BCL) and a yearling. This analysis was performed using selective pressurized liquid extraction (SPLE) and gas chromatography-mass spectrometry (GC-MS). Cortisol and progesterone (a sex hormone, related to pregnancy) levels were also measured and compared between the three individuals. This study was the first to report a link between contaminant exposure and BCL in harbor porpoises and encouraged further studies between disease biomarkers and contaminant stressors.

In Chapter Three, the application of SPLE, previously used to both extract and clean up blubber samples in Chapter Two, was then applied to a secretion, earwax. This method, as well as the method used to analyze blubber, was previously developed withing the Usenko Research Group. Contaminant analysis of a previously studied blue whale and five fin whale earplugs from both the North Atlantic and Pacific oceans included PCBs, PBDEs, and chlorinated pesticides (including DDT and its metabolites). Due to a contaminant within the sample extracts, a new method of DDT analysis using gas chromatography-tandem mass spectrometry (GC-MS/MS) was developed. Using SPLE, GC-MS, and GC-MS/MS, contaminant profiles of five fin whales, including profiles 30 years prior to the first reported measurements of contaminants within marine

mammals, were reconstructed. Five bioaccumulation rates were also produced for the fin whales using burden and age. A fixed effect model using age and bioaccumulation suggested that both location (ocean of origin) and time (lifespan) contributed to the overall burden of contaminants.

Expanding on the contaminants found in Chapter Three, the target analyte list for the chemical analysis of baleen whale earplugs was expanded to include toxic metals, i.e., lead (Pb), mercury (Hg), and cadmium (Cd). In addition to toxic metals, essential, nonessential, rare earth elements, and other nonbiologically active elements were also analyzed. Elemental analysis was performed on a single earplug from a female fin whale originating from the Southern Atlantic Ocean. Similar to organic contaminants, inorganic contaminants, and both essential and nonessential elements were plotted as a function of age and year to reconstruct elemental profiles. This was the first-time lifetime profiles were produced of elements. Additionally, bioaccumulation rates of toxic elements were also produced. The study of reconstructed elemental profiles suggests many factors are impacting the accumulation or exposure of elements within a single individual, e.g., biological and biogeochemical processes, spatiotemporal differences, and anthropogenic contributions. Further earplugs would need to be studied to better characterize these factors.

Future Work

The studies within this dissertation serve as a platform for future investigations of stress and stressors within marine mammals. Future work should aim to expand the dataset of contaminants by evaluating multiple plugs and adding additional compounds to the target analyte list (including additional hormones). Additional earplugs would further

improve the model by including data regarding, spatiotemporal trends, species, lifespan, and sex. Datasets should also be used to compare stressors (POPs) to stress response (cortisol). Internal data collected from the earplug should be compared to external datasets, such as pesticide production, noise, and the introduction of new chemical contaminants within the ocean. Lastly, effort should be made to determine health response to chronic stressors.

The first reported bioaccumulation rate and lifetime profile of a blue whale earplug was reported in 2013[142], and the subsequent 2020 publication[71] (i.e., Chapter Three) expanded this original dataset by five additional earplugs and included fin whales of both sexes. A fixed effect model was calculated to determine if lifespan, sex, species, and ocean of origin contributed to the burden. Utilizing just six earplugs originating from the North Atlantic and Pacific Ocean basins, the model suggested that lifespan and ocean of origin were significant. Including additional earplugs with more robust location data may better model contaminant burden within the marine ecosystem. The model could also be improved by including individuals with lifespans beyond those included in the previous study as well as earplugs from different baleen species of varying sex. This overall data could provide insight into stressors within the marine ecosystem.

Chemical analysis of an earplug is frequently limited by the available sample mass of each earplug or lamina. For example, 15 mg and 150 mg are required for hormone and contaminant analysis, respectively. Method development to extract multiple compound classes of interest (i.e., hormones and POPs) should be investigated. Current plans are in place to determine if laminae that underwent hormone extraction could be combined and analyzed for POPs. The number of data points provided by a single

earplug could greatly increase by improving the number of chemical analyses performed on a single sample mass or extract.

The target analyte list should also be expanded to include additional contaminants and biomarkers of stress. The contaminant list could include current-use pesticides and other flame retardants/industrial chemicals (e.g., organophosphate esters, flame retardant, and plasticizer). There are multiple types of stressors and cortisol is only one of many biomarkers for stress (i.e., a glucocorticoid). Many other factors also contribute to the stress of an individual or population and the age estimates provided with earplugs allow for the reconstruction of both stress and stressor profiles. A thorough analysis of the stress and stressors of multiple individuals can provide a wealth of information regarding the marine ecosystem. Hormone analysis using ELISA kits is time-consuming and often restricted to a single analyte per sample plate. ELISA kits are also known to have multiple cross-reactions between other hormones. Chromatographic separation and quantification using mass spectrometry would allow for the simultaneous analysis of multiple hormones as well as the assurance that you are quantifying the compounds of interest with known retention times and fragmentation patterns. The analyte list could be expanded to include additional biomarkers of stress (e.g., corticosterone and aldosterone) and sex hormones (e.g. progesterone and testosterone). By analyzing additional stress hormones, we can investigate specific hormonal responses to stressors with greater accuracy and rapid sample quantification. Sex hormone measurements can also be used to determine an accurate age of sexual maturity of individuals. Progesterone can also be used to both investigate pregnancy rates and model the rates of increase within each

species (i.e., determine the average number of pregnancies an individual has before reaching senescence).

POPs and repeated sub-lethal stressors (e.g., whaling) are two of many possible extrinsic and intrinsic stressors. Additional extrinsic stressors include ship strike, entanglement, noise, psychological factors (e.g., perceived threat). Intrinsic stressors included, pregnancy, lactation, migration, molting, and fasting between breeding and feeding grounds. Lastly, ecological drivers can also contribute to stress and include the loss of keystone species, recurring climate patterns, and climate change.[6] With the age/year estimates available through the analysis of earplugs, it is possible to pair both internal and external datasets of stressors to stress profiles. Following the analysis of both contaminants and cortisol, or other chemical biomarkers of stress, models should be used to determine if contaminant concentrations are contributing to stress.

In a previous study, see Appendix A, Trumble et al.[72] modeled internal baseline-corrected cortisol measurement to external datasets of whaling counts and sea surface temperature (SST) anomalies. The results suggested that stress increased with whaling counts and SST anomalies. Similar to whaling and SST data, it is possible to compare external datasets, such as sightings and acoustic measurements, with reconstructed baseline-corrected stress profiles. Sighting data at common feeding and breeding grounds could be used to determine if stress levels increase following an entanglement. Acoustic sensors embedded in marine mammal tags could provide insight into the exposure to noise over time before the tag is removed. Acoustic data compared to stress profiles could also be used to determine if whales are impacted by ocean noise. Contaminant concentrations should also be compared to the historic use and production of POPs as well as the

introduction of current-use pesticides and other industrial chemicals. If enough data is available via external datasets or internal measurement, models, similar to those reported by Trumble et al.,[72] and Winfield et al.,[71] could be used to determine the significance of the above factors on stress. Significant factors could then be the focus of policymakers and population managers to ensure the survival of baleen species.

Many tissues or excretions are now being studied for biomarkers of disease. For example, human cerumen has recently been used to test for volatile biomarkers of cancer using GC-MS.[191] There is strong evidence that thyroid hormones are suppressed in relation to POP burdens in marine mammals.[192] Further research into the analysis of thyroid hormones, POPs, and the stress response is necessary to determine if the disruption of hormones associated with contaminant exposure leads to additional stress or other adverse health effects. In addition to thyroid health, other diseases could be investigated by screening for other biomarkers. With just a few grams of earwax, researchers can unlock a wealth of current and historic data within the marine ecosystem.

APPENDICES

APPENDIX A

Baleen Whale Cortisol Levels Reveal a Physiological Response to 20th Century Whaling

This article published as Trumble, S. J.; Norman, S. A.; Crain, D. D.; Mansouri, F.; Winfield, Z. C.; Sabin, R.; Potter, C. W.; Gabriele, C. M.; Usenko, S., Baleen whale cortisol levels reveal a physiological response to 20th century whaling. Nature Communications 2018, 9 (1), 4587.[72]

Abstract

One of the most important challenges researchers and managers confront in conservation-based ecology is predicting a population's response to sub-lethal stressors. This is especially true when assessing possible responses of baleen whales to past anthropogenic pressures. Recently developed techniques involving whale earplugs combine age estimates with cortisol measurements to assess spatial and temporal stress/stressor relationships. Here we show a significant positive relationship between baseline-corrected cortisol levels and corresponding whaling counts ($r^2 = 0.78$) ($P = 0.031$) of fin (*Balaenoptera physalus*), humpback (*Megaptera novaeangliae*), and blue (*Balaenoptera musculus*) whales in the Northern Hemisphere spanning the 20th century. We also modeled the impact of alternative demographic and environmental factors and determined that increased anomalies of sea surface temperature over a 46-year mean (1970-2016) were positively associated with cortisol levels ($r^2 = 0.46$). While industrial whaling can deplete populations by direct harvest, our data underscore a widespread stress response in baleen whales that is peripheral to whaling activities.

APPENDIX B

Polychorlinated Biphenyl (PCB) Contaminant in Galveston Bay, Texas: Comparing Concentration and Profiles in Sediment, Passive Samples, and Fish

This article published as Oziolor, E. M.; Apell, J. N.; Winfield, Z. C.; Back, J. A.; Usenko, S.; Matson, C. W., Polychlorinated biphenyl (PCB) contamination in Galveston Bay, Texas: Comparing concentrations and profiles in sediments, passive samplers, and fish. Environmental Pollution 2018, 236, 609-618.[193]

Abstract

The industrialized portion of the Houston Ship Channel (HSC) is heavily contaminated with anthropogenic contaminants, most prominent of which are the polychlorinated biphenyls (PCBs). This contamination has driven adaptive evolution in a keystone species for Galveston Bay, the Gulf killifish (*Fundulus grandis*). We investigated the geographical extent of PCB impacts by sampling 12 sites, ranging from the heavily industrialized upper portion of the HSC to Galveston Island. At each site, PCB concentrations and profiles were determined in three environmental compartments: sediment, water (polyethylene passive samplers), and fish tissue (resident Gulf killifish). We observed a steep gradient of PCB contamination, ranging from 4.00 to 100,000 ng/g organic carbon in sediment, 290–110,000 ng/g lipid in fish, and 4.5–2300 ng/g polyethylene in passive samplers. The PCB congener profiles in Gulf killifish at the most heavily contaminated sites were shifted toward the higher chlorinated PCBs and were highly similar to the sediment contamination profiles. In addition, while magnitude of total PCB concentrations in sediment and total fish contamination levels were highly correlated between sites, the relative PCB congener profiles in fish and

passive samplers were more alike. This strong correlation, along with a lack of dependency of biota-sediment accumulation factors with total contamination rates, confirm the likely non-migratory nature of Gulf killifish and suggest their contamination levels are a good site-specific indicator of contamination in the Galveston Bay area. The spatial gradient of PCB contamination in Galveston Bay was evident in all three matrices studied and was observed effectively using Gulf killifish contamination as an environmentally relevant bioindicator of localized contamination in this environment.

APPENDIX C

A lipid normalization model for the analysis of stable isotopes in baleen whale earplug

This note is accepted and will published as Farzaneh Mansouri, Danielle D. Crain, Zach C. Winfield, Richard Sabin, Charles W. Potter, Ren Zhang, Stephen J. Trumble, Sascha Usenko in the Journal of Marine Mammal Science.

Goals

To address the goal of this study and improve the interpretation of $\delta^{13}C$ values of earwax, lipid correction experiments were conducted on laminae of two fin (*Balaenoptera physalus*, N = 14), two blue (*Balaenoptera musculus,* N = 14), and two humpback (*Megaptera novaeangliae*, N = 14) whale earplugs, originating from animals harvested during the whaling era (< 1972) and from recent stranding whales. Earplugs were delaminated as previously described[72], briefly, each laminae was delaminated and separated from the longitudinal section of earplug using ceramic scalpel under magnification, placed into vials and stored under nitrogen at -80 °C until analysis.

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