Gene expression as a biomonitoring tool? Using a PCR array to determine transcriptomic effects of contaminants in an Arctic seabird species, Black guillemot (*Cepphus grylle)*

By

Yasmeen Zahaby

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science

In

Biology

Carleton University Ottawa, Ontario

Abstract

Increasing economic development in the Arctic leads to higher contaminant burdens in wildlife. Current biomonitoring methods are subpar, due to their inability to quantify complex contaminant mixtures in wild species. This thesis tests the hypothesis that a custom transcriptomic tool can be used to monitor gene expression profiles following exposure from two different contaminant types in "case studies" using Black guillemot livers. Chapter 2 conveyed predictable chemistry profiles with the highest polychlorinated biphenyl (PCB) burdens found at the most contaminated site before remediation. Gene expression results were unreliable due to the age of samples and resulting degraded RNA quality. Chapter 3 showed distinct polycyclic aromatic compound (PAC) and gene expression profiles in birds based on diesel-related contaminants following an oil spill event. Birds collected from the non-spill site showed distinct chemistry and gene expression profiles, indicating exposure to contaminants from unknown sources.

Acknowledgments

First, I would like to thank my incredible advisors Doug Crump, Jason O'Brien, and Jennifer Provencher. Thank you for recruiting me into the lab, despite having no qPCR experience. I ended up writing my thesis on qPCR techniques, so your willingness to teach me was vital. I appreciate your compassion and recommendations to take time away from this project to recharge when needed. I have learned so much during my time in the MolTox lab and have had the opportunity to pass on that knowledge to others.

I would also like to thank Steve Cooke for accepting me into his lab despite my research focus straying from the norm. Steve's lab is managed with an immense amount of compassion and kindness towards his students, which is a huge comfort during the trials and tribulations of graduate school. I appreciate your guidance and thoughtful advice to keep my research in focus.

Thank you to Laurie Chan for the valuable recommendations along the way. I appreciate the attention to detail and suggestions that I would likely have missed.

Thank you to the incredible MolTox team. A special thanks to Suzanne Chiu for troubleshooting and brainstorming with me when my lab work did not go as planned. To Kim Williams, Tasnia Sharin, Tyler Nguyen, Helina Gyasi and Jory Curry; thank you for your support and friendship throughout my graduate school experience and long before that through the development of the ToxChips. I couldn't have asked for a better group to endure long lab sessions with.

Thank you to my parents; Joanne and Mohamed, for believing in me when I had doubts about graduate school. You have been my biggest supporters since day one and I appreciate your interest and enthusiasm for my work. Thank you to my partner Taylor Eggemann and friends for your encouragement and genuine interest in my project. I am so grateful to have had the best support network throughout this degree.

Table of Contents

List of Abbreviations

µg/g	Microgram per gram
ng/g	Nanogram per gram
pg/g	Picogram per gram
ml	milliliter
AMAP	Arctic Monitoring & Assessment Programme
BBDS	Baffin Bay/ Davis Strait
BDL	Below the detection limit
BLGU	Black guillemot
cDNA	Complementary deoxyribonucleic acid
CEACs	Contaminants of emerging Arctic concern
COGRAD	Centre for Oil and Gas Research and Development
Ct	Cycle threshold
DDT	Dichlorodiphenyltrichloroethane
GC/MS	Gas chromatography/mass spectrometry
NL	Newfoundland and Labrador
NWRC	National Wildlife Research Centre
PACs	Polycyclic aromatic compounds
PCA	Principal component analysis
PCBs	Polychlorinated biphenyls
PFOS	Perfluorooctane sulfonate
PLSDA	Partial least squares discriminant analysis
POPs	Persistent organic pollutants
qPCR	quantitative polymerase chain reaction
RIS	RNA Integrity Score
TMM	Trimmed mean of M-values
VIP	Variable Importance in the Projection

List of Figures

Regression coefficients of genes most influential in site predictions. Bars above the horizontal axis relate to Nain predictions, while bars below relate to Postville.

Figure 3.6: PLS regression A) Regression plots were used to show the top 6 performing PACs for which gene expression was used to predict actual PAC concentration in liver samples. These chemicals were successful based on acceptable R2 and slope values. B) Top 6 PAC chemicals with corresponding R2, slope and gene coefficients. Orange cells indicate that the gene is positively correlated (increased exposure leads to increased gene expression) with the chemical and blue cells indicate negative correlation (increased exposure leads to decreased expression) with the chemical.

Statement of Contributions

Chapter 2. Evaluation of a ToxChip PCR array to screen hepatic tissue of Black guillemot (Cepphus grylle) nestlings collected as part of a PCB remediation project at Saglek Bay, NL.

Study design	Yasmeen Zahaby Doug Crump Jason O'Brien Jennifer Provencher
Sample collection	Zou Zou A. Kuzyk Neil Burgess Jason P. Stow Glen A. Fox
Sample collection permits	Nunatsiavut Government
Chemical analysis	Axys Analytical Services Limited
DNA/RNA extraction	Yasmeen Zahaby
ToxChip development	Yasmeen Zahaby Doug Crump
Data analysis	Jason O'Brien Yasmeen Zahaby
Manuscript preparation	Yasmeen Zahaby Doug Crump Jason O'Brien Jennifer Provencher

Chapter 3. Using a ToxChip PCR array to screen hepatic tissue of Black guillemot (Cepphus grylle) collected from a diesel spill site and a non-spill site in the Arctic.

Study design	Yasmeen Zahaby Doug Crump Jennifer Provencher Jason O'Brien
Sample collection	William Ikkusek Edward Sillitt Samantha Pilgrim George Gear
Sample dissection site	Nunatsiavut Research Centre
Sample collection permits	Nunatsiavut Government
Chemical analysis	Centre for Oil and Gas Research and Development (COGRAD)
DNA/RNA extraction	Yasmeen Zahaby
ToxChip development	Yasmeen Zahaby Doug Crump
Data analysis	Jason O'Brien Yasmeen Zahaby
Manuscript preparation	Yasmeen Zahaby Doug Crump Jason O'Brien Jennifer Provencher

Chapter 1: General Introduction

1.1 Contaminants in the Arctic

1.1.1 How do contaminants end up in Arctic wildlife?

For several decades, the Arctic has become a sink for contaminant accumulation in sediment and wildlife tissues, despite its great distance from industrial point sources. Long-range transport through air and ocean currents has resulted in deposition of contaminants in the Arctic at an increasing rate. In addition, point source pollution events have become more frequent as anthropogenic activities expand further north and intensify in some regions. Contaminants are typically deposited into sediment in aquatic and terrestrial environments or remain suspended in the water column. Once in the environment, contaminants can move through the food web as follows: from primary producers (e.g., phytoplankton) to secondary consumers (e.g., aquatic invertebrates) on to species occupying higher trophic levels (e.g., fish, birds). Despite this, the Arctic food chain is relatively short compared to more southern regions, leading to more direct biomagnification (Corsolini & Sarà, 2017).

In seabirds, contaminants are typically integrated into their tissues through ingestion while foraging for prey in sediment or preying on fish and invertebrate species. Many contaminants of concern are highly lipophilic and resist degradation in the environment. Birds utilize oxidative P-450 enzyme activity and can quickly metabolize and easily excrete most PACs (Näf et al., 1992; Waszak et al., 2021). The high metabolic potential of PACs present lower rates of biomagnification compared to other persistent organic pollutants (POPs) (Takeuchi et al., 2009). Importantly, seabirds have greater rates

of bioaccumulation compared to mammals due to their higher energy demands and due to their migration to more polluted areas (Fisk et al., 2001). These factors represent the complexity of contaminant movement, fate, and disposition of chemicals (toxicokinetics) in Arctic wildlife.

1.1.2 The Canadian Arctic and climate change

The Canadian Arctic makes up approximately 40% of the country's landmass (Global Affairs Canada, 2017). The Arctic is known to have extreme environmental conditions that are beyond the range most species can tolerate. The Arctic region is categorized by low productivity and species diversity, as compared to other regions globally. Food web structures in the Canadian Arctic are relatively short, and wildlife communities are dominated by zooplankton, fish, marine mammals, and seabirds. Northern species are highly adapted to surviving long, cold winter months or have migratory strategies where they only reside in the Arctic during the summer months. Within the last few decades, climate change has influenced the survival, movement, and foraging behaviours of Arctic wildlife. The Arctic environment is losing sea-ice at an alarming rate, faster than many models have predicted. Satellite records dating back to 1979 indicate that sea-ice extent reached a record low in 2018 (Kumar et al., 2020). Sea ice thickness has also declined over the past few decades, as old ice melts and new ice replacement slows each year (Liu et al., 2020). Statistical modelling using satellite observational data predicts the first ice-free Arctic summer to occur in 2037, with a margin of error of 7 years (Peng et al., 2020). As a result, Arctic ecosystems are expected to experience changing conditions. For example, rising temperatures within the Arctic are expected to increase the number and spread of infectious diseases to wildlife

and human inhabitants by creating favorable conditions for prokaryotes (Bradley et al., 2005).

Climate change has been shown to disproportionately affect higher latitude regions resulting in faster warming year to year compared to the global average (IPCC, 2022). Additionally, climate change is likely to alter feeding habits, migration, disease resistance and other physiological traits in wildlife. Increasing water and ambient temperature due to climate change in the Arctic can modify the chemistry of a contaminant, often making it more toxic (Schiedek et al., 2007).

1.1.3 Monitoring programs within the Canadian Arctic

Contaminants were detected in Arctic wildlife dating to the 1970s. Holden (1970) reported the presence of 2 organochlorine pesticides (dieldrin and DDT), as well as polychlorinated biphenyls (PCBs) in the blubber of ringed seals from Baffin Island, NU. More detailed evidence of contaminants in Arctic wildlife emerged throughout the 1970s and 1980s. In 1991, the Artic Monitoring & Assessment Programme (AMAP) was established to compile and distill pollution trends within the Arctic. In the same year, the Northern Contaminants Program (NCP) was established by the Canadian Federal Government with the goal of monitoring contaminants with potential impacts on human and wildlife health. In 2001, the Stockholm Convention emerged as a global treaty aimed at reducing the levels of POPs entering the environment, for which both NCP and AMAP were contributors. Canada became one of the first countries to join the Stockholm Convention and remains involved.

Seabirds are good bioindicators of contaminant burdens within the Arctic. Most avian species have migratory routes, which cross international borders (Lescroël et al.,

2016). They also have a wide range of foraging and migration behaviors (Mallory & Braune, 2012; Piatt et al., 2007). Additionally, many seabird species occupy a high trophic level in the Arctic food web (Hobson et al., 2002). These factors contribute to understanding the qualitative health of the bird's environment and enable widespread geographical contaminant monitoring. Avian species are chosen for contaminant analysis based on feeding style, migration patterns, availability for sampling and geographic distribution. Seabird tissue collection for contaminant monitoring began in the mid-1970s in Canada and has continued on a regular basis (Bianchini et al., 2022). Seabird eggs have historically been the most common tissue collected for contaminant analysis. Many seabird species lay more than one egg per clutch, resulting in minimal impacts of species numbers following egg collection. Because seabirds are regularly consumed by local populations, monitoring contaminants in other seabird tissues can help determine potential risks to human health.

1.2 Movement of POPs and concentration trends in seabirds

1.2.1 Persistent organic pollutants (POPs)

Persistent organic pollutants (POPs) are characterized by their resistance to physical, chemical, or biochemical degradation. Thus, this contaminant type has strong potential for global long-range transport and bioaccumulation within wildlife species. Many POPs can remobilize within the environment, particularly from soil or sediment, following changing abiotic factors. Arctic species are particularly vulnerable to these contaminants as most are long-lived, have lipid-rich tissue, and low reproductive output (Barrie et al., 1992). Current contaminant levels remain low in most Arctic wildlife species, but trends indicate some contaminant burdens are increasing through re-

introduction from melting sea ice (Xie et al., 2022). Contaminant burdens are species- and individual-specific, as migration, feeding habits and egg-laying times differ. Many contaminants entering the Arctic environment have endocrine disrupting properties. This is of particular concern for wildlife, as reproduction and environmental adaptation hinge on a well-functioning endocrine system (Letcher et al., 2010). Additionally, each contaminant has different pharmacokinetic parameters, including varying metabolism and uptake rates. This can be further complicated based on age and sex toxicity differences or cumulative effects. To get the full scope of contaminant impacts on the Arctic, both legacy and emerging contaminants need to be routinely monitored within wildlife populations and research needs to be conducted to identify potentially harmful sublethal effects.

1.2.2 Trends of POPs in the Arctic

The concentrations of certain POPs are beginning to decline globally due to a reduction in use and production, while other contaminants are just beginning to emerge as contaminants of concern within the Arctic environment. The Stockholm Convention and its resulting policies have reduced the volume of certain POPs entering the Arctic. This treaty has helped to reduce the number of POPs in many environments, but an assessment by the AMAP found that some POPs are no longer declining at the expected rate in the Arctic (Arctic Monitoring and Assessment Programme, 2010). This is likely due to changing abiotic conditions such as increased chemical production and climate change reducing the extent of summer sea ice. Despite this, recent estimates suggest that temporal trends of some legacy POPs are improving. For example, PCBs show decreasing trends, likely due to national regulations beginning in the 1970s (Vorkamp et

al., 2022). Overall, the AMAP assessment published in 2010 states that legacy POPs are generally decreasing in Canadian Arctic wildlife due to significant restrictions of most global emissions. However, new contaminants of emerging Arctic concern (CEACs) have begun to be detected in wildlife. These contaminants have limited available toxicity data and knowledge gaps relating to multi-stressor interactions and temporal trends are of concern to communities, researchers and risk assessors (Sonne et al., 2021).

1.3 New approach methods for contaminant monitoring

1.3.1 Traditional contaminant testing methods

As the effects of climate change become more evident in the Arctic, there is an increasing need for new methods and tools to monitor and assess sub-lethal contaminant exposure to wildlife. Traditional methods of contaminant screening in animals are costly to perform, focus primarily on laboratory model species and often only reflect exposure to a single chemical. Typically, toxicity testing is conducted on a model species and the resulting data are then used to extrapolate to relevant ecological species (Crump et al., 2021). In 2007, the U.S. Department of Health and Human Services released a report identifying the need for new high-throughput approaches to determine the effects of contaminants (National Research Council et al., 2007). Thus, there is a need for tools, which could provide evidence-based monitoring for ecologically relevant species, and which focus on contaminant mixtures, as these are the most common form of contaminants within the environment.

1.3.2 Toxicogenomics and qPCR as an effective approach to contaminant monitoring

Toxicogenomics is an emerging field within toxicology that deals with the collection, interpretation, and storage of information about gene and protein activity

within cells or tissues of an organism in relation to toxic substances (U.S. Department of Health and Human Services, 2007). Using RNA as a template, we can synthesize corresponding complimentary DNA (cDNA) and determine modulation of gene expression for genes associated with various biological functions. This allows researchers to identify changes related to contaminant exposure in target genes, or the entire transcriptome (Hamadeh et al., 2002). Genes selected for analysis can be modified following exposure to refine estimated effects. These changes to gene expression can be contaminant- or species-specific. Each gene corresponds to a biological process within the tissue that can be altered. Baseline gene expression data can then be compared to tissue collected from an exposed individual to determine which genes are impacted and to what extent. This allows for assessment of effects within species at a mechanistic level.

1.3.3 Transcriptomics as a potential monitoring tool

The advancement of technologies like RNAseq and DNA microarrays allow for large scale analysis of gene expression changes in wildlife. These tools measure variation in gene expression using mRNA, which can then affect translation of proteins and cellular phenotypes that can be altered by ecological processes, contaminant exposure and disease (Alvarez et al., 2015). Recently, transcriptomics has been used to detect and monitor infectious diseases in frogs through whole-transcriptome analysis (RNA Seq) (Campbell et al., 2018), and in birds through whole blood transcriptomics (Jax et al., 2021). Transcriptomics has also been used to determine mechanisms involved in environmental resilience for conservation in fish species (Connon et al., 2018) and for the selection of populations for reintroduction as a conservation tool (He et al., 2012). Toxicogenomic methods were also used to monitor contaminants by determining gene

expression changes in fathead minnows exposed to wastewater effluent (Garcia-Reyero et al., 2011). The refinement and application of transcriptomic and toxicogenomic tools continues to facilitate research into wildlife management and ecotoxicological applications.

1.3.4 The ToxChip transcriptomics tool

The ToxChip tool is a species-specific PCR array developed from a previously annotated gene sequences and corresponding gene-specific primer assays. Genes can be chosen for addition to the ToxChip based on a contaminant of interest and its documented impacts on genes/biological pathways. Using previously annotated coding sequences of target genes similar to the novel target species, this approach is highly adaptable and customizable to species or contaminants of interest. The ToxChip is designed to be used for liver tissue, as this is the main detoxifying organ within all species. The ToxChip enables comparison of gene expression data in exposed and baseline samples to determine potential changes caused by contaminant exposure. Many contaminants impact the same genes or biological pathways; therefore, one ToxChip could be used to determine gene expression in the same species following exposure to a variety of contaminants. The ToxChip approach has been used to screen individual chemicals for risk assessment (Porter et al., 2014) and complex mixtures (Xia et al., 2020; Zahaby et al., 2021). Based on the findings from these papers, the ToxChip approach could aid in long-term contaminant monitoring programs and could be implemented following acute or known exposure to a contaminant to study effects on wildlife.

The ToxChip is a medium-to-high-throughput means to generate evidence-based data on contaminant mixtures using less animals than standard toxicity testing. Given its

customizable potential, the ToxChip could be designed for any species or contaminant of interest. Additionally, the ToxChip is relatively easy to run in most laboratory environments and data can be generated quickly following retrieval of samples. Recently, a ToxChip approach was used to assess gene and pathway effects in response to complex contaminant mixtures in avian species (Xia et al., 2020). The endocrine disrupting nature of many POPs suggests that biological pathways most likely to be impacted by exposure include xenobiotic metabolism, oxidative stress, and hormone pathways.

Another potential use of the ToxChip tool would be to determine contaminant "hotspots" for future monitoring applications. Using machine learning techniques such as partial least squares discriminant analysis (PLSDA) on gene expression and contaminant data, it is possible to predict colony or site of origin for each sample. This method was applied in a recent paper with an 83.3% accuracy at predicting seabird colony of origin based on gene expression (Zahaby et al., 2021).

1.4 Thesis Objectives and Hypotheses

While the concentrations of most legacy pollutants are beginning to decline in the Arctic, new contaminant classes are emerging/increasing because of long-range transport or point source emissions. Human activities are expected to increase as the Arctic becomes more accessible due to climate change and its impacts on the reduction of summer sea ice. A holistic approach to contaminant monitoring in wildlife is needed as many contaminants are found as mixtures, with varying degrees of toxicity to wildlife. Effects of contaminants are driven by many factors including species, chemical concentration, environment, and life history traits. Traditional methods of toxicity testing fail to routinely capture information in ecologically relevant species, and instead

extrapolate using laboratory models, therefore new approaches are needed. Using a transcriptomics approach to determine biological pathways most effected by contaminant exposure allows for more specific endpoint determination and contribution to strategic environmental assessments. Overall, I hypothesize that the chemistry profiles of birds collected from two distinct areas and exposed to two chemical types will influence gene expression profiles and demonstrate contaminant exposure trends. Furthermore, I hypothesize that transcriptomics tools can be used to assist contaminant monitoring in a seabird species.

In Chapter 2, I will use a previously developed Black guillemot (*Cepphus grylle*) ToxChip PCR array on liver samples collected from a Federally Contaminated Site in Saglek Bay, NL. The site was used as a military radar station and underwent remediation for PCB contamination in 2007-2008. Liver samples were collected pre- and post-remediation from nestling Black guillemots at three variably contaminated locations (Beach [most contaminated], Island [moderate], and Reference [least contaminated]). Based on the history of this site, I predict that differences between the sub-sites will be evident based on chemistry and gene expression profiles for both years. Furthermore, I predict that samples collected before the remediation project, in 1999, will have distinct gene expression profiles compared to those collected after the remediation project in 2007, particularly for genes associated with xenobiotic metabolism. The remediation project conducted in 2007 was successful in reducing contaminant burdens in each respective sub-site compared to 1999 (Kuzyk et al., 2003). The ToxChip tool should help determine whether the remediation project in 2007 was successful in terms of gene expression change at the Beach sub-site between both years. To test the thesis hypothesis,

I predict that gene expression profiles can be used to differentiate contaminant burdens between sub-sites and between collection years.

In Chapter 3, I will test the utility of the same Black guillemot ToxChip transcriptomics tool to study how gene expression profiles differ between adult seabirds exposed to oil-related contaminants at a known diesel spill site (Postville, NL) compared to a site with no known acute diesel contamination (Nain, NL). Based on the expected difference in chemistry profiles between the two sites, I predict that liver samples of birds collected from the diesel spill site will have higher burdens of PACs, particularly lower molecular weight PACs known to be associated with diesel fuel (Provencher et al., 2022). This chapter tests the hypothesis that samples collected from the spill site (Postville) will have an increased number of gene alterations compared to samples collected from the non-spill site, particularly for genes associated with xenobiotic metabolism (notably CYP1a5, CYP3a7, UGT1a9 and SULT1e1). I will use liver tissue samples from birds collected at the spill site to understand gene expression profiles following exposure to diesel-related contaminants. To test the general thesis hypothesis, I predict that distinct chemistry profiles at both sites will result in unique gene expression profiles based on PAC source and concentration.

The proposed research will provide insight into the potential use of ‘omics tools to augment contaminant monitoring and detection in an ecologically relevant seabird species (Black guillemot). This project will allow the identification of gene expression effects following contaminant exposure on a temporal and spatial scale in a bioindicator species.

1.5 References

Alvarez, M., Schrey, A. W., & Richards, C. L. (2015). Ten years of transcriptomics in wild populations: What have we learned about their ecology and evolution? *Molecular Ecology*, *24*(4), 710–725. https://doi.org/10.1111/mec.13055

Arctic Monitoring and Assessment Programme. (2010). *AMAP Assessment 2020: POPs and Chemicals of Emerging Arctic Concern: Influence of Climate Change. AMAP*. Retrieved May 26, 2023, from https://www.amap.no/documents/doc/amap-assessment-2020-pops-and-chemicals-of-emerging-arctic-concern-influence-of-climate-change/3580

Barrie, L. A., Gregor, D., Hargrave, B., Lake, R., Muir, D., Shearer, R., Tracey, B., & Bidleman, T. (1992). Arctic contaminants: Sources, occurrence and pathways. *The Science of the Total Environment*, *122*(1–2), 1–74. https://doi.org/10.1016/0048-9697(92)90245-n

Bianchini, K., Mallory, M. L., Braune, B. M., Muir, D. C. G., & Provencher, J. F. (2022). Why do we monitor? Using seabird eggs to track trends in Arctic environmental contamination. *Environmental Reviews*, *30*(2), 245–267. https://doi.org/10.1139/er-2021-0078

Bradley, M., Kutz, S. J., Jenkins, E., & O'Hara, T. M. (2005). The potential impact of climate change on infectious diseases of Arctic fauna. *International Journal of Circumpolar Health*, *64*(5), 468–477. https://doi.org/10.3402/ijch.v64i5.18028

Campbell, L. J., Hammond, S. A., Price, S. J., Sharma, M. D., Garner, T. W. J., Birol, I., Helbing, C. C., Wilfert, L., & Griffiths, A. G. F. (2018). A novel approach to wildlife transcriptomics provides evidence of disease-mediated differential expression and changes to the microbiome of amphibian populations. *Molecular Ecology*, *27*(6), 1413–1427. https://doi.org/10.1111/mec.14528

Connon, R. E., Jeffries, K. M., Komoroske, L. M., Todgham, A. E., & Fangue, N. A. (2018). The utility of transcriptomics in fish conservation. *Journal of Experimental Biology*, *221*(2), jeb148833. https://doi.org/10.1242/jeb.148833

Corsolini, S., & Sarà, G. (2017). The trophic transfer of persistent pollutants (HCB, DDTs, PCBs) within polar marine food webs. *Chemosphere*, *177*, 189–199. https://doi.org/10.1016/j.chemosphere.2017.02.116

Crump, D., Boulanger, E., Farhat, A., Williams, K. L., Basu, N., Hecker, M., & Head, J. A. (2021). Effects on Apical Outcomes of Regulatory Relevance of Early-Life Stage Exposure of Double-Crested Cormorant Embryos to 4 Environmental Chemicals. *Environmental Toxicology and Chemistry*, *40*(2), 390–401. https://doi.org/10.1002/etc.4922

Fisk, A. T., Hobson, K. A., & Norstrom, R. J. (2001). Influence of Chemical and Biological Factors on Trophic Transfer of Persistent Organic Pollutants in the Northwater Polynya Marine Food Web. *Environmental Science & Technology*, *35*(4), 732–738. https://doi.org/10.1021/es001459w

Garcia-Reyero, N., Lavelle, C. M., Escalon, B. L., Martinović, D., Kroll, K. J., Sorensen, P. W., & Denslow, N. D. (2011). Behavioral and genomic impacts of a wastewater effluent on the fathead minnow. *Aquatic Toxicology (Amsterdam, Netherlands)*, *101*(1), 38–48. https://doi.org/10.1016/j.aquatox.2010.08.014

Global Affairs Canada. (2017). *Canada and the Circumpolar Regions*. Government of Canada. https://www.international.gc.ca/world-monde/international_relations-relations_internationales/arctic-arctique/index.aspx?lang=eng

Hamadeh, H. K., Amin, R. P., Paules, R. S., & Afshari, C. A. (2002). An Overview of Toxicogenomics. *Current Issues in Molecular Biology*, *4*(2), Article 2. https://doi.org/10.21775/cimb.004.045

He, X., Johansson, M. L., & Heath, D. D. (2016). Role of genomics and transcriptomics in selection of reintroduction source populations. *Conservation Biology*, *30*(5), 1010–1018.

Hobson, K. A., Fisk, A., Karnovsky, N., Holst, M., Gagnon, J.-M., & Fortier, M. (2002). A stable isotope (δ13C, δ15N) model for the North Water food web: Implications for evaluating trophodynamics and the flow of energy and contaminants. *Deep Sea Research Part II: Topical Studies in Oceanography*, *49*(22), 5131–5150. https://doi.org/10.1016/S0967-0645(02)00182-0

Holden, Av. (1972). *Monitoring organochlorine contamination of the marine environment by the analysis of residues in seals* (HAPAB/73/2382). United States Environmental Protection Agency.

Intergovernmental Panel on Climate Change (IPCC). (2022). *The Ocean and Cryosphere in a Changing Climate: Special Report of the Intergovernmental Panel on Climate Change* (1st ed.). Cambridge University Press. https://doi.org/10.1017/9781009157964

Jax, E., Müller, I., Börno, S., Borlinghaus, H., Eriksson, G., Fricke, E., Timmermann, B., Pendl, H., Fiedler, W., Klein, K., Schreiber, F., Wikelski, M., Magor, K. E., & Kraus, R. H. S. (2021). Health monitoring in birds using bio-loggers and whole blood transcriptomics. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-90212-8

Kumar, A., Yadav, J., & Mohan, R. (2020). Global warming leading to alarming recession of the Arctic sea-ice cover: Insights from remote sensing observations and model reanalysis. *Heliyon*, *6*(7), e04355. https://doi.org/10.1016/j.heliyon.2020.e04355

Lescroël, A., Mathevet, R., Péron, C., Authier, M., Provost, P., Takahashi, A., & Grémillet, D. (2016). Seeing the ocean through the eyes of seabirds: A new path for marine conservation? *Marine Policy*, *68*, 212–220. https://doi.org/10.1016/j.marpol.2016.02.015

Letcher, R. J., Bustnes, J. O., Dietz, R., Jenssen, B. M., Jørgensen, E. H., Sonne, C., Verreault, J., Vijayan, M. M., & Gabrielsen, G. W. (2010). Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish. *The Science of the Total Environment*, *408*(15), 2995–3043. https://doi.org/10.1016/j.scitotenv.2009.10.038

Liu, Y., Key, J. R., Wang, X., & Tschudi, M. (2020). Multidecadal Arctic sea ice thickness and volume derived from ice age. *The Cryosphere*, *14*(4), 1325–1345. https://doi.org/10.5194/tc-14-1325-2020

Mallory, M. L., & Braune, B. M. (2012). Tracking contaminants in seabirds of Arctic Canada: Temporal and spatial insights. *Marine Pollution Bulletin*, *64*(7), 1475–1484. https://doi.org/10.1016/j.marpolbul.2012.05.012

Näf, C., Broman, D., & Brunström, B. (1992). Distribution and metabolism of polycyclic aromatic hydrocarbons (PAHs) injected into eggs of chicken (Gallus domesticus) and common eider duck (Somateria mollissima). *Environmental Toxicology and Chemistry*, *11*(11), 1653–1660. https://doi.org/10.1002/etc.5620111114

National Research Council, Division on Earth and Life Studies, Institute for Laboratory Animal Research, Board on Environmental Studies and Toxicology, & Committee on Toxicity Testing and Assessment of Environmental Agents. (2007). *Toxicity Testing in the 21st Century: A Vision and a Strategy*. National Academies Press.

Peng, G., Matthews, J. L., Wang, M., Vose, R., & Sun, L. (2020). What Do Global Climate Models Tell Us about Future Arctic Sea Ice Coverage Changes? *Climate*, *8*(1), Article 1. https://doi.org/10.3390/cli8010015

Piatt, J. F., Harding, A. M. A., Shultz, M., Speckman, S. G., Van Pelt, T. I., Drew, G. S., & Kettle, A. B. (2007). Seabirds as indicators of marine food supplies: Cairns revisited. *Marine Ecology Progress Series*, *352*, 221–234.

Porter, E., Crump, D., Egloff, C., Chiu, S., & Kennedy, S. W. (2014). Use of an avian hepatocyte assay and the avian Toxchip Polymerse chain reaction array for testing prioritization of 16 organic flame retardants. *Environmental Toxicology and Chemistry*, *33*(3), 573–582. https://doi.org/10.1002/etc.2469

Provencher, J. F., Thomas, P. J., Braune, B. M., Pauli, B., Tomy, G., Idowu, I., O'Hara, P., & Mallory, M. L. (2022). Decadal differences in polycyclic aromatic compound (PAC) concentrations in two seabird species in Arctic Canada. *Science of The Total Environment*, *826*, 154088. https://doi.org/10.1016/j.scitotenv.2022.154088

Schiedek, D., Sundelin, B., Readman, J. W., & Macdonald, R. W. (2007). Interactions between climate change and contaminants. *Marine Pollution Bulletin*, *54*(12), 1845–1856. https://doi.org/10.1016/j.marpolbul.2007.09.020

Sonne, C., Dietz, R., Jenssen, B. M., Lam, S. S., & Letcher, R. J. (2021). Emerging contaminants and biological effects in Arctic wildlife. *Trends in Ecology & Evolution*, *36*(5), 421–429. https://doi.org/10.1016/j.tree.2021.01.007

Takeuchi, I., Miyoshi, N., Mizukawa, K., Takada, H., Ikemoto, T., Omori, K., & Tsuchiya, K. (2009). Biomagnification profiles of polycyclic aromatic hydrocarbons, alkylphenols and polychlorinated biphenyls in Tokyo Bay elucidated by delta13C and delta15N isotope ratios as guides to trophic web structure. *Marine Pollution Bulletin*, *58*(5), 663–671. https://doi.org/10.1016/j.marpolbul.2008.12.022

U.S. Department of Health and Human Services. (n.d.). *Toxicogenomics*. National Toxicology Program. Retrieved May 26, 2023, from https://ntp.niehs.nih.gov/whatwestudy/testpgm/toxicogenomics

Vorkamp, K., Carlsson, P., Corsolini, S., Wit, C. A. de, Dietz, R., Gribble, M. O., Houde, M., Kalia, V., Letcher, R. J., Morris, A., Rigét, F. F., Routti, H., & Muir, D. C. G. (2022). Influences of climate change on long-term time series of persistent organic pollutants (POPs) in Arctic and Antarctic biota. *Environmental Science: Processes & Impacts*, *24*(10), 1643–1660. https://doi.org/10.1039/D2EM00134A

Waszak, I., Jonko-Sobuś, K., Ożarowska, A., & Zaniewicz, G. (2021). Estimation of native and alkylated polycyclic aromatic hydrocarbons (PAHs) in seabirds from the south coast of the Baltic Sea. *Environmental Science and Pollution Research International*, *28*(4), 4366–4376. https://doi.org/10.1007/s11356-020-10653-y

Xia, P., Crump, D., Chiu, S., Chan, H. M., & O'Brien, J. M. (2020). Toxicogenomic Assessment of Complex Chemical Signatures in Double-Crested Cormorant Embryos from Variably Contaminated Great Lakes Sites. *Environmental Science & Technology*, *54*(12), 7504–7512. https://doi.org/10.1021/acs.est.0c02725

Xie, Z., Zhang, P., Wu, Z., Zhang, S., Wei, L., Mi, L., Kuester, A., Gandrass, J., Ebinghaus, R., Yang, R., Wang, Z., & Mi, W. (2022). Legacy and emerging organic contaminants in the polar regions. *Science of The Total Environment*, *835*, 155–376. https://doi.org/10.1016/j.scitotenv.2022.155376

Zahaby, Y., Xia, P., Crump, D., Provencher, J. F., Thomas, P. J., Pauli, B., Braune, B. M., Franckowiak, R. P., Gendron, M., Savard, G., Sarma, S. N., Mallory, M. L., & O'Brien, J. (2021). ToxChip PCR Arrays for Two Arctic-Breeding Seabirds: Applications for Regional Environmental Assessments. *Environmental Science & Technology*, *55*(11), 7521–7530. https://doi.org/10.1021/acs.est.1c00229

2. Chapter 2: Evaluation of a ToxChip PCR array to screen hepatic tissue of Black guillemot (*Cepphus grylle*) nestlings collected as part of a PCB remediation project at Saglek Bay, NL.

2.1 Abstract

Contaminants are continuously produced and transported to remote ecosystems, including the Arctic. It is important to understand the effects complex mixtures pose to local wildlife to ensure effective chemical management strategies and environmental assessments are implemented. Transcriptomics is a potential tool to monitor the effects of contaminants on biological pathways using gene expression profiles. Based on previous work examining polychlorinated biphenyl (PCB) burdens in Black guillemot (*Cepphus grylle*) nestlings collected before and after sediment remediation, we attempt to quantify changes in hepatic gene expression using an existing ToxChip polymerase chain reaction (PCR) array paired with PCB profiles. We found distinct chemistry profiles between the three variably contaminated sub-sites and between collection years (1999 [pre-] and 2007 [post-remediation]). We did not observe any significant trends in gene expression profiles, likely due to the hepatic RNA integrity of the 1999 samples. Future research should explore how archived samples may be used in these types of applications, and contemporary studies should use newly acquired samples to investigate effects of contaminant exposure on gene expression profiles and determine the success of remediation projects in the future.

2.2 Introduction

2.2.1 PCBs and their effects on seabirds

Contaminants are ubiquitous in ecosystems and different groups of contaminants impose variable challenges to wildlife following exposure. Polychlorinated biphenyls (PCBs) were used extensively throughout the 1950s to the 1970s. This class of contaminants, comprising 209 different congeners, were used as coolants, insulating fluids and additives in building materials (Carlsson et al., 2018). Following the gradual restriction of the production and use of PCBs in the 1970s, global emissions began to decline significantly. Despite this reduction in output, PCBs are resistant to degradation and can be remobilized into the environment through changing abiotic conditions (Carlsson et al., 2018). Seabirds are good bioindicators of contamination due to their high trophic position in the Arctic, especially for chemicals such as PCBs given that they bioaccumulate and biomagnify (Antoniadou et al., 2007; Wang et al., 2022). Bird samples (e.g., eggs) are also relatively easy to collect and allow for multi-year monitoring of the same colonies.

Arctic wildlife is especially susceptible to high contaminant burdens due to long lifespans and high body lipid content, which facilitate the bioaccumulation of highly lipophilic contaminants. Cold temperatures in the Arctic favor the persistence of PCBs as they inhibit degradation. Despite remediation efforts at previously contaminated sites in Canada, PCB contamination in seabirds continues to be reported. PCBs were found to be above trace levels (≥0.001 μg/g w.w. (wet weight)) in 98% of bird carcasses collected from various sites in Canada (Braune & Noble, 2009). PCBs can enter an environment

through long range transport and can be remobilized from sediment into the trophic food web years to decades after deposition (Shi et al., 2022).

PCBs have been documented to act as environmental estrogens and can impact populations of breeding shorebirds in areas with high concentrations (Fry, 1995). In juvenile birds, PCBs have been shown to decrease cognitive abilities (Zahara et al., 2015), impair growth and memory (Flahr et al., 2015) and suppress immune function (Grasman et al., 1996). Exposure to a commercial PCB mixture (Aroclor 1254) resulted in an increase in mass and fat and a decrease in plasma thyroid hormones over time in juvenile European starlings exposed at ecologically relevant concentrations (Flahr et al., 2015). In addition, PCBs have been documented to cause immune suppression, reduced hatchability, decreased fertility, embryonic deformities, disruption of growth, and in extreme cases, mortality in birds (Barron et al., 1995).

2.2.2 Transcriptomics as a tool to assist contaminant monitoring

Traditional toxicity testing relies on many animals, is time consuming and expensive, and does not sufficiently address wildlife contaminant sensitivities or contaminants as complex mixtures (Crump et al., 2016). One method to evaluate contaminant burdens in wildlife species is by using transcriptomics. Exposure to contaminants can alter the expression of certain genes. In birds, phase I metabolizing genes, such as CYP1A4/CYP1A5, are involved in xenobiotic metabolism and are typically induced following exposure to contaminants such as PCBs (Head & Kennedy, 2007). Using available literature regarding genes impacted by specific contaminants in both wild and laboratory species, we can determine a subset of genes worth investigating

in response to contaminant exposure that can be evaluated simultaneously using quantitative PCR arrays.

Specific PCR arrays, called ToxChips, were previously developed for two Arctic seabird species (Black guillemot and Thick-billed murre) by our lab at the National Wildlife Research Centre, Ottawa, ON (Zahaby et al., 2021). The goal of that project was to develop an alternative tool to assist with contaminant monitoring in wildlife species that could be employed in real world "case studies". The project outlined the development of the ToxChips and their use on samples collected from an area with a known natural oil seep to determine current baseline gene expression profiles.

2.2.3 Saglek Bay, NL was the site of a former military base

PCB contamination has been reported at several Federally Contaminated Sites in Canada. One such site is Saglek Bay, located in northern Newfoundland and Labrador (NL), Canada. The site has been in use since the 1950s but was modernized to serve as a Long-Range Radar facility. As part of an ecological risk assessment conducted in 1999, sum PCB concentration burdens in Black guillemot nestling livers collected from the area were found to range from 14 to 6200 ng/g (w.w.). PCB burdens decreased in magnitude with distance from the site (Kuzyk et al., 2003). Liver samples were collected at three sub-sites with varying contamination (Beach, Island and Reference) in 1999 and again in 2007. These time points signify sample collection before and after a federally mandated remediation project to remove all contaminated soil from the Beach site exceeding 50 µg/g (Ficko et al., 2013). Livers collected from females at the Beach site were enlarged 36% relative to females collected from the Reference site in 1999. Furthermore, Beach and Island nestlings showed elevated EROD activities and reduced retinol concentrations.

Birds collected from the Beach site in 2007 showed a 6-fold decrease in total PCB concentrations compared to 1999 samples from the same sub-site (Brown et al., 2009). There are currently 440 federally contaminated sites with known or suspected PCB contamination in Canada (Treasury Board of Canada Secretariat, 1994). Contaminant monitoring in wildlife species following a remediation effort is important to determine its success.

One potential way to evaluate the success of remediation efforts is to utilize molecular biology tools, such as ToxChip PCR arrays. One important consideration, however, is that while there is transcriptome annotation information available for laboratory model species, such as the chicken or Japanese quail, there is a knowledge gap regarding transcriptome annotation for many wildlife species. With evidence that PCBs impacted nestlings collected in 1999 at Saglek Bay in a dose-dependent manner, we investigated whether samples could be differentiated between sub-sites and years based on gene expression and/or contaminant data (Brown et al., 2009). This information will allow more in-depth knowledge regarding the potential use of transcriptomic tools in monitoring or remediation initiatives.

I hypothesize that the chemistry profiles of birds collected from three variably contaminated sites at two time points will affect ToxChip gene expression profiles in these birds and demonstrate contaminant exposure trends. Furthermore, I hypothesize that transcriptomic tools can be used for contaminant monitoring in wildlife species. Based on existing empirical data from Saglek Bay, chemistry profiles were shown to differ between the sub-sites, as well as between collection years. I predict that samples collected before the remediation project in 1999 will have distinct gene expression

profiles compared to those collected after the remediation project in 2007, particularly for genes associated with the xenobiotic metabolism pathway. Gene expression data derived from the Black guillemot ToxChip tool should help determine if the remediation project in 2007 was successful in removing most of the PCB contamination at Saglek Bay beach. To test the thesis hypothesis, I predict that gene expression profiles can be used to differentiate contaminant burdens between sub-sites and between collection years.

2.3 Materials and Methods

2.3.1 Study site and sample preparation

Saglek Bay (58°29'N, 62°40'W) is an inlet located in northeast Newfoundland and Labrador. This bay is approximately 67 kilometres in length and is connected to the Labrador Sea. This site was home to a U.S. Air Force communication and radar site between the 1950s and 1970s. In 1971, the U.S. Air Force transferred control of the site to the Canadian Forces, who subsequently closed the site. This former military site remained closed until the late 1990s, when it was commissioned for clean-up under the Federal Contaminated Sites Action Plan. The site was deemed to have been contaminated by PCBs, which are harmful contaminants found in the electrical equipment used at the time.

Black guillemot (*Cepphus grylle*) nestlings were collected from three sub-sites within Saglek Bay with varying contamination levels. The Beach group, located no more than 4 km away from the contaminated site, contains 2 sampling sites (East and West); the Island group, located approximately 6 km from the Beach site, contains 2 sampling sites (Rocky and Little Bluebell); the Reference group, located approximately 18 km from the beach site, contains 3 sampling sites (Glitsch, Black & Gull) (Figure 2.1). A

total of 6 juvenile guillemots were collected from each sub-site in 1999 (n=18) and again in 2007 (n=18) to understand the contaminant burden and potential effects in birds before and after the remediation project.

Figure 2.1: Map of Saglek Bay, NL with 3 sub-sites: Beach (site of PCB contamination), Islands & Reference.

One chick was taken per sampled nest at ages between 21 and 36 days old. Body size measurements, mass and an immune response test were taken for each chick, as well as a blood sample. Chicks were then decapitated and necropsied, and gender was determined. The left liver lobe was placed in a cryovial for later contaminant analysis and preserved in liquid nitrogen within 10 minutes of the chick's death. The right lobe was placed in an acetone/hexane rinsed tube and immediately frozen at -20°C for biochemical analysis (Kuzyk et al., 2003). A portion of the left lobe was homogenized and kept for biochemical analysis at the National Wildlife Research Centre (NWRC) in Ottawa, ON. For the current project, archived left liver lobes were retrieved from –80°C freezers at the NWRC in 2021. Total RNA was extracted following the manufacturer's protocols for the

RNeasy Mini extraction kit (Qiagen) with DNase on-column digest. The resulting RNA concentration was determined using a QIAxpert (Qiagen). Several liver samples collected in 1999 were only available as homogenates (n=5). A retroactive study permit was obtained from the Nunatsiavut Government prior to sample manipulation (NGRAC-15594228).

2.3.2 Chemical analysis of livers collected in 1999 and 2007

Contaminant analyses were conducted by Axys Analytical Services Limited, Sydney, BC for each right liver lobe collected in 1999. Each sample was assayed for 84 PCB congeners and metabolites by capillary gas chromatography/mass spectrometry (GC/MS). A detailed description of contaminant analysis for these samples was described by (Kuzyk et al., 2003). Detection limits for PCB congeners and pesticides were sample specific and are described below. Each right liver lobe from 2007 was assayed for 91 PCB congeners by Axys Analytical Services Limited, Sydney, BC. A detailed methodology for the contaminant analysis of these samples is provided by (Brown et al., 2009).

Coplanar PCBs were extracted from homogenized tissue, ground, and dried. The powder was then placed in a glass chromatographic column and eluted with 1:1 dichloromethane/hexane. The eluate was subsampled for lipid determination and the remaining extract was concentrated, loaded onto a gel permeation column, and eluted with 1:1 dichloromethane/hexane. The final 1:1 dichloromethane/hexane extract was analyzed by high resolution GC/MS.

In 1999, a total of 45 PCB congeners were detected in one or more samples. Recoveries of eight PCB congeners from spiked reference materials averaged 101±12%

and results from the certified reference material (cod liver oil) (N.I.S.T Certified Reference Material 1588) were within margins of error. Detection limits ranged from 1.3 to 6.7 pg/g. Spike recoveries for coplanar PCBs ranged from 91-105%. Only one blank contained a trace amount (0.17 pg/g), and analytical duplicates were within 9.1%.

In 2007, the analytical method used C-13 labeled PCB standards added at the commencement of analysis. Any congeners less than the detection limit were replaced with a random number between the detection limit and zero (Brown et al., 2009). Analytes quantified against the C-13 labeled standard are recovery corrected in the samples. Recoveries of 53 and 25 PCB congeners from 4 and 3 samples of spiked reference materials averaged 104±1.6% and 103±1.5%, respectively. Blanks were below detection for all congeners. For livers, PCB concentrations were expressed as wet weight (w.w.) for both collection years.

2.3.3 Application of ToxChip PCR array

A previously developed ToxChip array for the Black guillemot was used for all samples in this project. The steps for development have been outlined previously (Crump et al., 2016; Zahaby et al., 2021). The ToxChip array contains 32 target genes, and 3 samples are run on each 96 well array. Approximately 30 mg of liver tissue was excised from the left lobe of each bird, and total RNA was extracted following the manufacturer's protocols for the RNeasy extraction kit with a QIAcube (Qiagen) and with a DNAse I on-column treatment to remove genomic DNA contamination. The resulting concentration and purity of RNA was determined using a QIAxpert (Qiagen). All samples had purity values A260/A280 ≥ 1.8. Half the volume of each RNA sample was aliquoted, and the remaining volume was stored at −80 °C. Approximately 1000 ng of total RNA from each

sample was reverse transcribed to cDNA using a QuantiTect kit (Qiagen) with modifications described by Porter et al., 2014. The resulting cDNA was diluted 1:10 in Diethyl pyrocarbonate (DEPC) treated water. Array wells were spotted with 5 µl of optimally concentrated primers using a QIAgility machine (Qiagen) prior to the addition of SYBR Green with ROX Mastermix (Qiagen) and 5 µl diluted cDNA. Total reaction volume in each well was 25 µl. The arrays were run on a Mx3005P qPCR machine (Agilent). The thermocycle program included an activation step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and ending with a dissociation curve segment of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s.

2.3.4 Data analysis for PCB chemicals and gene expression

Samples collected in 1999 were analyzed at a lower resolution compared to 2007 samples. Due to a lack of detection limits for each PCB congener in 1999, detection limits were derived based on available data. The PCB value with the highest detection limit in all samples for each PCB congener was determined, and any value under the highest was set to 10% of its value (the new detection limit). Next, we removed any PCB congener where all samples contained values below the detection limit (BDL). All remaining PCBs for which 50% of the values were BDL were removed. For the remaining PCBs, any value BDL was replaced with 10% of that value. This method was used for samples from 1999 only because samples from 2007 had detection limits available. After filtering, 9 PCBs remained in 5 co-elution groups (PCB 153, 138/163/164, 180, 182/187, 170/190). All data filtering and manipulation was completed in R Studio prior to analysis.

PCR cycle threshold (Ct) data were normalized using the two housekeeping genes included on the ToxChip (RPL4 & EEF1a1). The fold change of target gene abundance was calculated using the $2^{-\Delta Ct}$ method. This method assumes the housekeeping genes to have constant expression levels between samples and experimental conditions (Hasanpur et al., 2022)

Genes with high Ct values (> 35) or with no detectable Ct (No Ct) in ≥ 50% of samples were removed from analysis (9 genes were removed during this step). Remaining Ct values > 35 were ignored from filtering as there is confidence in gene expression based on previous work (Zahaby et al., 2021), and higher Ct values were therefore deemed valuable for analysis in this case. Any remaining No Ct was replaced with the experimental average. One sample was not included in the analysis as it produced an outlier in the gene expression data (SGL_99_29).

All statistics were conducted in R Studio or R Commander. Principal component analysis (PCA) is a multivariate analysis technique used to reduce the dimensionality of a dataset and emphasize variation. Data are visualized on a two-dimensional graph with the first principal component accounting for the most variation. Samples, or loadings, tend to cluster based on projected similarity. Linear Discriminant Analysis (LDA) was used to show separability among sub-sites and among collection years based on chemistry profiles.

Partial least squares discriminant analysis (PLSDA) was used as a predictive modelling tool. In this chapter, PLSDA was used with a Leave One Out principal which trained the algorithm to predict sub-site or collection year of a sample based on its gene expression profile. A PCA was also used to visualize gene expression data.

2.4 Results

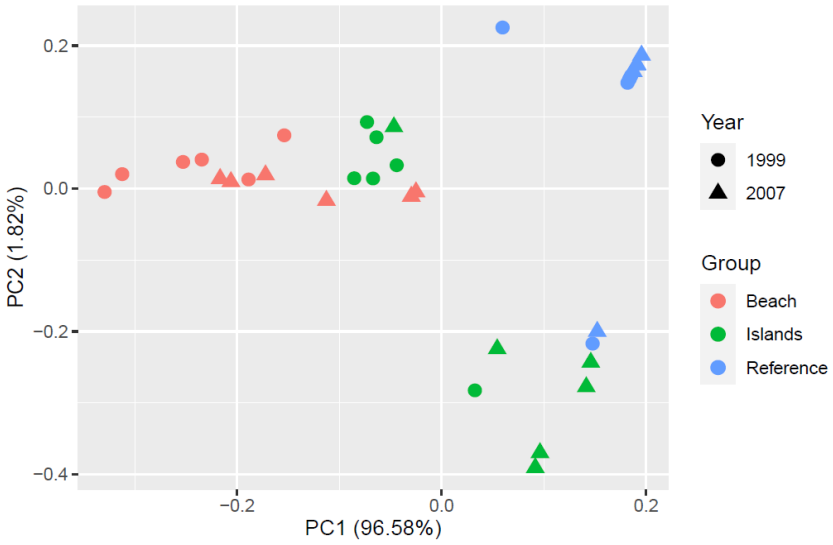
2.4.1 Black guillemots and PCB burdens

Black guillemot nestlings (n=36) were collected from three sub-sites at Saglek Bay, NL in 1999 and again in 2007. The chicks collected in 1999 and 2007 were approximately 21 & 28 days old, respectively, at the time of collection. The sex ratio of birds collected in 1999 was 50% female (9/18) and 50% male (9/18). In 2007, the birds collected were 44% female (8/18) and 56% male (10/18). Saglek Bay, NL is a Federally Contaminated Site with elevated levels of PCB soil contamination. The sub-sites included in the collection were variably contaminated. The Beach site represents the source of contamination and is thus highly contaminated, the Island sites are moderately contaminated and are off shore from the Beach, and the Reference sites are the furthest from the source and the least contaminated (Figure 2.1). A total of 84 PCB congeners were measured in each liver, but only 9 congeners were included in this analysis due to an abundance of non-detects in many of the samples. All PCB congeners included are non-dioxin like (U.S. Environmental Protection Agency, 2015). The nestling with the highest liver sum PCB burden from 1999 (SGL_99_17) had 6477 ng/g (w.w.). In 2007, the nestling with the highest sum PCB burden (SGL_07_22) had 1143 ng/g (w.w.) in its liver. Both birds with the highest sum PCB burdens from each sampling year were collected from the Beach site. The nestling with the lowest sum PCB burden (SGL_99_28) had 24.58 ng/g (w.w.) in 1999 and in 2007 the nestling with the lowest sum PCB (SGL_07_5) had 5 ng/g (w.w.). Both chicks with the lowest sum PCB burden were from the Reference site.

2.4.2 PCB chemistry data profiles

The PCA for chemistry data (both years + all sites) shows clear clustering of Beach samples, regardless of year. Samples from the Island sub-site tend to cluster together based on collection year. Most Reference samples cluster together, with a few outlier samples from both 1999 and 2007 (Figure 2.2a). PC1 accounts for most of the variation among samples (96.6%). Most samples collected in 1999 clustered within the higher concentrated branches of the heatmap (61%) (Figure 2.2b). Alternatively, 61% of samples collected in 2007 clustered with the lower concentrated branches.

A)



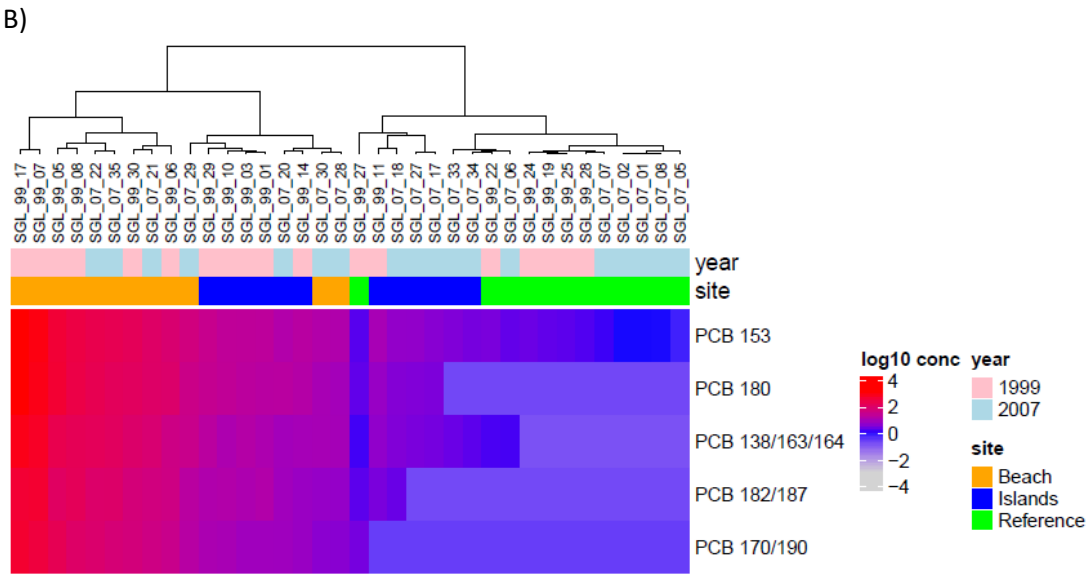


Figure 2.2: Chemistry Profile. A) Principal component analysis (PCA) was used to show chemistry data for both experimental years (1999 & 2007) and each site (Beach, Island, Reference). PC1 accounts for most variation among the data. B) Heatmap of log10 transformed chemistry data (ng/g w.w.) data for Saglek Bay, NL samples from both sample years and all sub-sites.

2.4.3 Gene expression profiles

The PCA for gene expression data (both years & all sub-sites) shows clear clustering based on collection year (Figure 2.3). The PCA shown used housekeeping gene normalized gene expression data using RPL4 & EEF1a1. Unfortunately, the distinction based on collection year is likely due to the poor quality RNA used during qPCR analysis. For samples collected in 1999, 67% had RNA Integrity Scores (RIS) below 5. This indicates degraded RNA quality and therefore reduced gene expression amplification during quantitative PCR (Padhi et al., 2018).

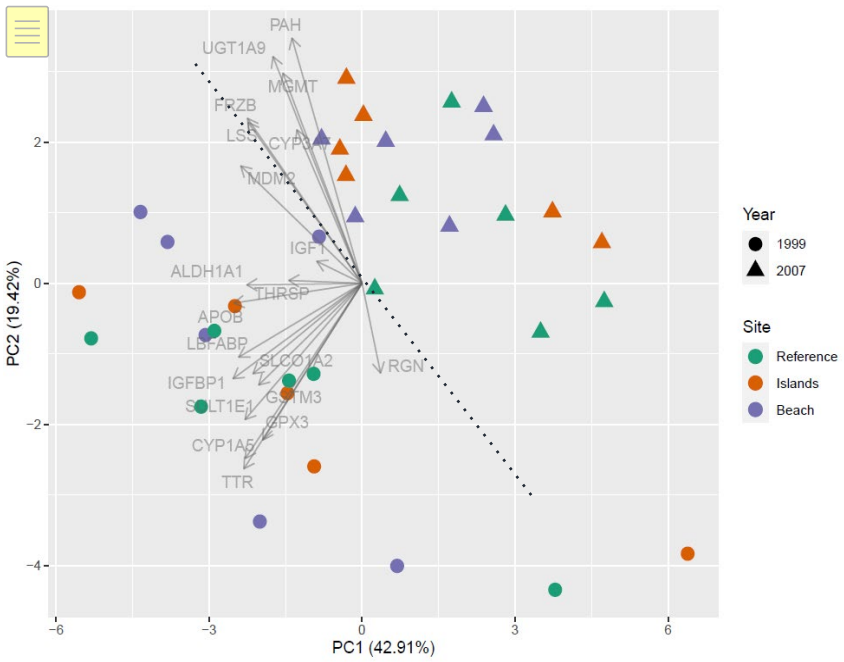


Figure 2.3: PCA shows gene expression data for both collection years (1999 & 2007) and all three collection sites (Beach, Island & Reference). Years separate clearly on the plot, but sites do not. Principal component 1 accounts for 42.91% of the variation in gene expression. Dotted line was added after analysis for visual year differentiation.

2.4.4 Predictive power for sub-site and collection year using linear discriminant analysis

Linear discriminant analysis (LDA) was used to predict sub-site and collection year of a sample based on chemistry data. Using samples from all sub-sites and collection years, the model predicted a sample with 69.4% accuracy for year and predicted sub-site with 77.7% accuracy. For samples collected in 1999 and 2007, the algorithm had an accuracy of 72.2% and 66.6%, respectively. Beach samples were predicted correctly 83.3% of the time, while Island and Reference samples were predicted correctly 75% of the time (Figure 2.4b). LDA was used instead of PLSDA for these data due to the low number of quantifiable PCB congeners.

A)

Observed	Predicted		Confusion Matrix and Statistics
	1999	2007	Accuracy: 0.6944
1999	13	6	95% Confidence Interval: (0.5189, 0.8365)
2007	5	12	p-value (Acc > NIR): 0.01441

B)

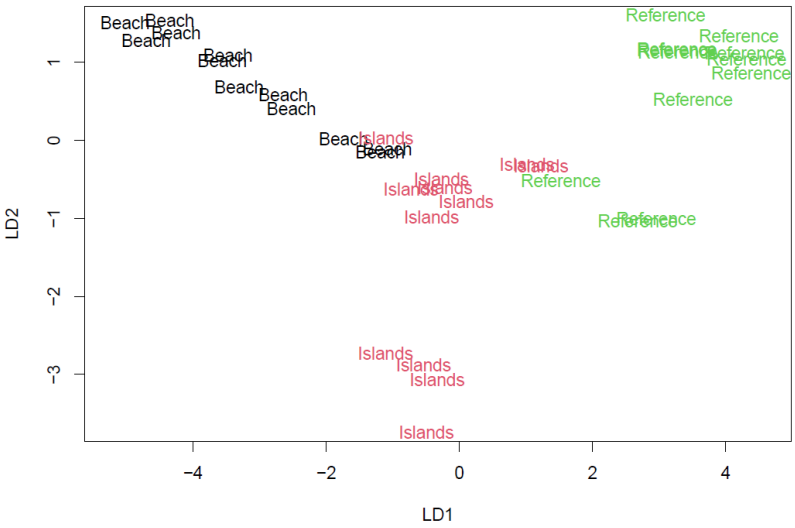


Figure 2.4: Chemistry grouping. A) Results from a linear discriminant analysis (LDA) used to predict collection year based on chemistry data. Acc > NIR means that the accuracy is greater than the "no information rate". B) LDA biplot was used to show good separation between sub-sites (Beach: black, Islands: red, Reference: green) based on chemistry data.

PLSDA was completely accurate at predicting collection year based on gene expression data. The model was correct every time (100% accuracy) (Figure 2.5).

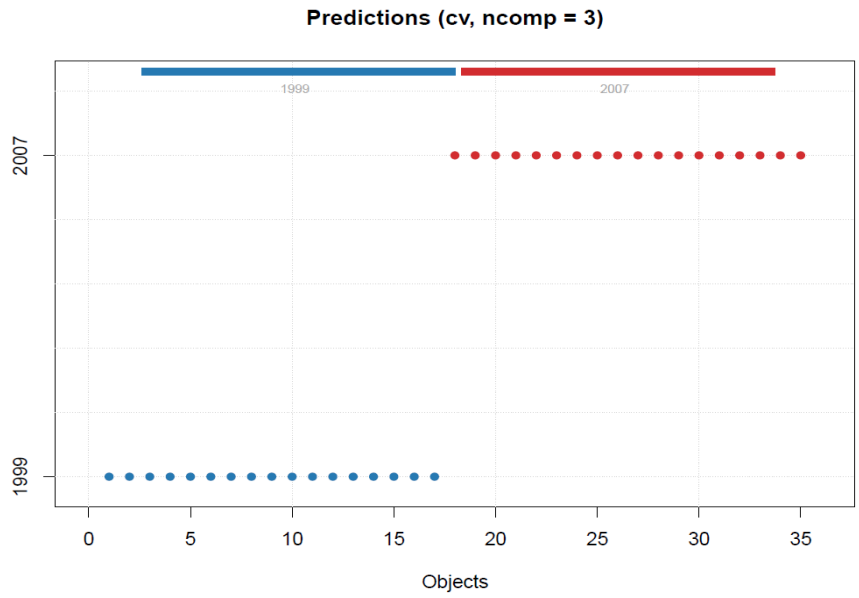


Figure 2.5: Partial least squares discriminant analysis (PLSDA) was used to show predictions of sample year based on gene expression data. The algorithm predicted the correct year 100% of the time, for each year.

The PLSDA prediction model of sample sub-site based on gene expression failed to predict any sites correctly (Figure S2.2). The model predicted that a Reference site sample was a Beach site sample 66.6% of the time. The PLSDA prediction model for 2007 sample sub-site based on gene expression data generated similar results (Figure S2.3). The model failed to predict any Beach or Island sites correctly. The model predicted a Reference site sample correctly 33.3% of the time. The model predicted an Island site was a Beach site 66.6% of the time.

Overall, the PLSDA model was 100% accurate at predicting the collection year of samples based on gene expression data. Alternatively, the model was not successful at predicting collection sub-site based on gene expression. The model was not able to predict collection site for either year with accuracy.

2.5 Discussion

To determine if differential gene expression could be used as a tool for effects-based monitoring, Black guillemot nestling livers with known PCB concentrations were retrieved from a national tissue bank and run on a custom ToxChip PCR array. Gene expression profiles were used to determine the effects of chemistry on biological pathways and trends relating to the degree of contaminant exposure. Samples were collected in 1999 and again in 2007 (before and after sediment remediation at the source). Three sub-sites (Beach, Island and Reference) represented spatial variation in PCB contamination and followed a predictable concentration gradient. The Beach site had the highest PCB burdens, regardless of collection year. Several PCB congeners included in the analysis, namely 138, 153 and 180, are known to be dominant in bird tissues worldwide. This is likely due to their high bioaccumulation potential and low metabolic degradation rate (Quinn et al., 2013). All PCBs which made it through the filtering process are considered non-dioxin like or ortho substituted. This class of PCBs are typically less toxic but more abundant than dioxin-like, and have lower degradation potential (Henry & DeVito, 2003).

2.5.1 Chemistry data shows trends in site and remediation across years

Using the chemistry data for samples from all three sub-sites and from both collection years, we were able to determine distinct PCB concentration profiles in nestling livers. Samples from 1999 follow a predictable gradient along PC1, which accounts for 96.58 % of the variability (Figure 2.2a). Beach samples cluster together, as do Reference samples, indicating that there are similar chemistry profiles based on sub-site. Beach and Island samples cluster closer together on the plot and Reference samples

cluster independently. This means that Beach and Island samples from 1999 tend to have distinct chemistry profiles from Reference samples.

Hierarchical cluster analysis attempts to identify relatively homogeneous groups of variables based on selected characteristics. The heatmap (Figure 2.2b) shows a predictable gradient of concentrations based on sub-site, with Beach samples having the highest PCB concentrations and Reference samples having the lowest, overall. Most of the samples collected in 1999 (11/18 or 61%) were present in the upper half of PCB concentrations on the heatmap. Previously, sub-site contaminant burden differences were reported and an exponential decrease in PCB concentrations from the Beach sites to the Reference sites was observed. The average Beach and Island group sum PCB concentrations were elevated 33- and 3-fold compared to the Reference sample average, respectively (Kuzyk et al., 2003).

Samples from 2007 show a similar sub-site gradient with Beach samples clustering to the left and Reference samples to the right of the PCA plot. Hierarchical cluster analysis shows there are still high concentrations of PCBs in birds from the Beach site collected in 2007 (Figure 2.2b). Most samples collected in 2007 (11/18 or 61%) were present in the lower half of PCB concentrations on the heatmap. This means that the difference in PCB concentrations was more prominent between sub-sites than it was between collection years. A notable reduction (6-fold) in the average PCB burden of birds from the Beach sites was found between 1999 and 2007 (Brown et al., 2009).

Linear discriminant analysis (LDA) was used to predict collection year and sub-sites based on chemistry data. The LDA predicted a sample with 69.4% accuracy for year

and predicted sub-site with 77.7% accuracy. The biplot for LDA shows good separability for sub-sites based on chemistry data (Figure 2.4a).

Based on the heatmap of chemical contaminants for both years and all three sites, there is still significant contamination in at least 4 individuals (samples 21, 22, 29 & 35) from the Beach site in 2007 (Figure 2.2b). High concentrations of the 9 PCB congeners were also reported in 5 individuals from the Reference site (samples 1, 2, 5, 7, 8) in 2007. This indicates that despite the sediment remediation project at the Beach site, there is still PCB contamination in wildlife at the site furthest from the source, following the remediation project's completion. PCBs are incredibly resistant to degradation and can travel long distances through air and ocean currents; therefore, it is unsurprising that PCB contamination remains measurable in wildlife at the Reference sites post-remediation (Tomza-Marciniak et al., 2019).

In terms of chemistry profiles, individuals from the Beach sub-site clustered together, as did individuals from the Reference sub-site on the PCA, regardless of year (Figure 2.2a). If the remediation project was a complete success, we would expect to see distinct clustering based on sub-sites for both years. Because differences in chemistry profiles were found on a temporal and spatial scale, the next step in this research was to use PCR array analysis to determine if chemistry had a direct effect on gene expression trends.

2.5.2 Gene expression profiles were distinct based on collection year

Samples collected in 1999 show no clear sub-site clustering trends based on gene expression. Differences between sub-sites were not evident on the PCA plot based on gene expression but samples separate clearly along PC1 based on year (Figure 2.4).

While there is a difference in gene expression profiles between the two years, samples collected from all three sub-sites had similar profiles in 1999 and 2007. This makes sense, as the most contaminated sub-site before remediation would likely still be the most contaminated sub-site after remediation, even though concentrations were reduced. Gene expression profiles can still be useful indicators of exposure despite the continued presence of contamination.

While contaminant analysis was performed within months of sample retrieval, gene expression analysis on frozen livers was conducted 22 and 14 years after collection. Although the samples remained frozen during this time, RNA is extremely sensitive to degradation through improper storage or freeze-thaw cycles. RNA quality can be quantified using an RNA Integrity Score (RIS) value. Generally, RIS values ≥ 5 are considered acceptable for qPCR amplification (Fleige & Pfaffl, 2006). The average RIS value for samples collected in 1999 and 2007 was 4.4 and 7.1, respectively (Table S2.1). For this reason, we are unable to determine with certainty if the gene expression profiles are accurate. It is likely that if RNA is degraded, qPCR amplification efficiency would be negatively affected resulting in reduced or no amplification of target genes. While the RIS values for 2007 samples were generally high enough to be considered intact, a PCA analysis of samples from this collection year alone showed no clustering of samples by sub-site based on gene expression (Figure S2.3).

Sample year was predicted with 100% accuracy using gene expression alone (Figure 2.5a). This further confirms that while it may appear that there is distinction between gene expression profiles of both collection years, it is more likely that the RNA integrity of samples resulted in this separation.

2.5.3 Gene expression profiles were not indicative of contaminant levels

These results show clear separation of collection year based on gene expression. The gene profiles of birds from both years are distinct from each other, yet sub-sites are not. There is no clear pattern in the heavily contaminated samples from 1999, as Beach and Reference samples appear non-distinct (Figure 2.3).

In this instance, differential gene expression cannot be used to identify remediated sites compared to non-remediated sites. The distinct separation of collection year gene expression profiles is likely due to poor RNA quality. Overall, RNA quality for 1999 was significantly degraded compared to samples from 2007 and would therefore have reduced amplification in quantitative PCR. The ToxChip tool cannot be used to identify spatial contaminant gradients of PCBs successfully in this context. This analysis would likely be more successful using newer and less degraded tissue.

While the remediation project may have been successful in terms of removing PCB contamination based on previous reporting, there is no strong evidence of gene expression or contaminant difference between the three variably contaminated sub-sites in the collection year following remediation. Overall, chemistry data proved to be useful for sub-site and collection year trend identification. Chemistry profiles showed a predictable gradient with Beach sites containing the highest concentrated samples and Reference the lowest, for both years. PCB contamination was reduced in samples from all three sub-sites in 2007 based on the heatmap, although PCB concentration trends based on sub-sites were more noticeable than based on year (Figure 2.2b).

2.6 Limitations and Future Considerations

Overall, the findings of this study do not support the hypothesis that transcriptomic approaches can be used as a tool for PCB effects monitoring in archived samples. The RNA integrity was not ideal, particularly for samples collected in 1999. RIS value determination revealed that most samples from this year scored below the acceptable level for accurate qPCR transcript amplification and therefore, results from the ToxChip PCR array are not reliable for differential gene expression assessment. Samples collected in 2007 proved to be less degraded and overall had RIS values that were acceptable for qPCR application. Nevertheless, results from 2007 samples alone had no distinct sub-site clustering of gene expression profiles (Figure S2.3). Overall, we were unable to determine whether chemistry data influenced gene expression profiles in this chapter. PCB bioaccumulation depends on many factors including sex, age, diet and time of residence in PCB contaminated areas (Tomza-Marciniak et al., 2019). Therefore, it is difficult to compare various metrics in an uncontrolled experiment (wild bird populations). Most PCBs measured were not included in this analysis due to a low number of detections across samples. Additionally, analytical methods have come a long way since these samples were initially processed, particularly those collected in 1999. This means we are omitting a great deal of potentially important data, as some PCBs are more potent than others, even at low concentrations (Ishikawa et al., 2007; Simon et al., 2007). Furthermore, individual PCB congeners have varying impacts on organisms. At high concentrations, PCB 153 and PCB 180 exposure resulted in hypoactivity in rats (Johansen et al., 2011) and higher accumulation rates compared to other congeners in American kestrel (Elliott et al., 1991; Grimm et al., 2015). PCB 180 was found to be

obesogenic in humans (Yu et al., 2021). PCB 138 and PCB 180 impair cognitive function in perinatal humans (Llansola et al., 2010). The PCR array was designed using adult liver tissue samples to identify target genes, while the birds used in this project were juveniles. There may be certain pathways/genes that are not expressed in nestlings, which would lead to their ineffectiveness as biomarker genes. Finally, there could be natural biological variation between birds from variably contaminated sites from both years.

The array used comprises only 29 target genes, which does not account for the full scope of biological pathways in this seabird species. In the future, it would be worthwhile to investigate a larger gene panel or the entire transcriptome to increase the diversity of biological pathways measured and ensure that critical gene-contaminant interactions are not overlooked. Overall, this "case study" was opportunistic in nature. Samples were collected as part of a previous study, and analytical chemistry techniques have advanced significantly since these samples were examined. Sample handling and storage, as well as analytical chemistry methodology, would have been conducted differently had we anticipated this project earlier.

2.7 References

Antoniadou, V., Konstantinou, I. K., Goutner, V., Sakellarides, T. M., Albanis, T. A., & Bintoudi, E. (2007). PCB levels and accumulation patterns in waterbird eggs and in their prey at Lake Kerkini, a north-eastern Mediterranean wetland of international importance. *Archives of Environmental Contamination and Toxicology*, *53*(2), 249–260. https://doi.org/10.1007/s00244-006-0176-2

Barron, M. G., Galbraith, H., & Beltman, D. (1995). Comparative reproductive and developmental toxicology of PCBs in birds. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *112*(1), 1–14. https://doi.org/10.1016/0742-8413(95)00074-7

Braune, B. M., & Noble, D. G. (2009). Environmental contaminants in Canadian shorebirds. *Environmental Monitoring and Assessment*, *148*(1), 185–204. https://doi.org/10.1007/s10661-007-0150-0

Brown, T. M., Sheldon, T. A., Burgess, N. M., & Reimer, K. J. (2009). Reduction of PCB Contamination in an Arctic Coastal Environment: A First Step in Assessing Ecosystem Recovery after the Removal of a Point Source. *Environmental Science & Technology*, *43*(20), 7635–7642. https://doi.org/10.1021/es900941w

Carlsson, P., Breivik, K., Brorström-Lundén, E., Cousins, I., Christensen, J., Grimalt, J. O., Halsall, C., Kallenborn, R., Abass, K., Lammel, G., Munthe, J., MacLeod, M., Odland, J. Ø., Pawlak, J., Rautio, A., Reiersen, L.-O., Schlabach, M., Stemmler, I., Wilson, S., & Wöhrnschimmel, H. (2018). Polychlorinated biphenyls (PCBs) as sentinels for the elucidation of Arctic environmental change processes: A comprehensive review combined with ArcRisk project results. *Environmental Science and Pollution Research International*, *25*(23), 22499–22528. https://doi.org/10.1007/s11356-018-2625-7

Elliott, J. E., Kennedy, S. W., Jeffrey, D., & Shutt, L. (1991). Polychlorinated biphenyl (PCB) effects on hepatic mixed function oxidases and porphyria in birds—II. American kestrel. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 99(1–2), 141–145. https://doi.org/10.1016/0742-8413(91)90090-G

Ficko, S. A., Luttmer, C., Zeeb, B. A., & Reimer, K. (2013). Terrestrial ecosystem recovery following removal of a PCB point source at a former pole vault line radar station in Northern Labrador. *Science of The Total Environment*, *461–462*, 81–87. https://doi.org/10.1016/j.scitotenv.2013.04.075

Flahr, L. M., Michel, N. L., Zahara, A. R. D., Jones, P. D., & Morrissey, C. A. (2015). Developmental Exposure to Aroclor 1254 Alters Migratory Behavior in Juvenile European Starlings (*Sturnus vulgaris*). *Environmental Science & Technology*, *49*(10), 6274–6283. https://doi.org/10.1021/acs.est.5b01185

Fleige, S., & Pfaffl, M. W. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine*, *27*(2), 126–139. https://doi.org/10.1016/j.mam.2005.12.003

Fry, D. M. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environmental Health Perspectives*, *103*(7), 165–171.

Grasman, K. A., Fox, G. A., Scanlon, P. F., & Ludwig, J. P. (1996). Organochlorine-associated immunosuppression in prefledgling Caspian terns and herring gulls from the Great Lakes: An ecoepidemiological study. *Environmental Health Perspectives*, *104*(suppl 4), 829–842. https://doi.org/10.1289/ehp.96104s4829

Grimm, F. A., Hu, D., Kania-Korwel, I., Lehmler, H.-J., Ludewig, G., Hornbuckle, K. C., Duffel, M. W., Bergman, Å., & Robertson, L. W. (2015). Metabolism and metabolites of polychlorinated biphenyls. Critical Reviews in Toxicology, 45(3), 245–272. https://doi.org/10.3109/10408444.2014.999365

Hasanpur, K., Hosseinzadeh, S., Mirzaaghayi, A., & Alijani, S. (2022). Investigation of chicken housekeeping genes using next-generation sequencing data. *Frontiers in Genetics*, *13*, 827538. https://doi.org/10.3389/fgene.2022.827538

Head, J. A., & Kennedy, S. W. (2007). Same-sample analysis of ethoxyresorufin-O-deethylase activity and cytochrome P4501A mRNA abundance in chicken embryo hepatocytes. *Analytical Biochemistry*, *360*(2), 294–302. https://doi.org/10.1016/j.ab.2006.10.033

Henry, T. R., & DeVito, M. J. (2003). Non-dioxin-like PCBs: effects and consideration in ecological risk assessment. Washington, DC: Ecological Risk Assessment Support Center, Office of Research and Development, *US Environmental Protection Agency*.

Ishikawa, Y., Noma, Y., Mori, Y., & Sakai, S. (2007). Congener profiles of PCB and a proposed new set of indicator congeners. *Chemosphere*, *67*(9), 1838–1851. https://doi.org/10.1016/j.chemosphere.2006.05.075

Johansen, E. B., Knoff, M., Fonnum, F., Lausund, P. L., Walaas, S. I., Wøien, G., & Sagvolden, T. (2011). Postnatal exposure to PCB 153 and PCB 180, but not to PCB 52, produces changes in activity level and stimulus control in outbred male Wistar Kyoto rats. *Behavioral and Brain Functions*, *7*(1), 18. https://doi.org/10.1186/1744-9081-7-18

Kuzyk, Z. Z. A., Burgess, N. M., Stow, J. P., & Fox, G. A. (2003). Biological Effects of Marine PCB Contamination on Black Guillemot Nestlings at Saglek, Labrador: Liver Biomarkers. *Ecotoxicology*, *12*(1), 183–197. https://doi.org/10.1023/A:1022550709962

Llansola, M., Montoliu, C., Boix, J., & Felipo, V. (2010). Polychlorinated Biphenyls PCB 52, PCB 180, and PCB 138 Impair the Glutamate−Nitric Oxide−cGMP Pathway in Cerebellar Neurons in Culture by Different Mechanisms. *Chemical Research in Toxicology*, *23*(4), 813–820. https://doi.org/10.1021/tx900440q

Padhi, B. K., Singh, M., Rosales, M., Pelletier, G., & Cakmak, S. (2018). A PCR-based quantitative assay for the evaluation of mRNA integrity in rat samples. *Biomolecular Detection and Quantification*, *15*, 18–23. https://doi.org/10.1016/j.bdq.2018.02.001

Porter, E., Crump, D., Egloff, C., Chiu, S., & Kennedy, S. W. (2014). Use of an avian hepatocyte assay and the avian Toxchip Polymerse chain reaction array for testing prioritization of 16 organic flame retardants. *Environmental Toxicology and Chemistry*, *33*(3), 573–582. https://doi.org/10.1002/etc.2469

Quinn, L. P., Roos, C., Pieters, R., Løken, K., Polder, A., Skaare, J. U., & Bouwman, H. (2013). Levels of PCBs in wild bird eggs: Considering toxicity through enzyme induction potential and molecular structure. *Chemosphere*, *90*(3), 1109–1116. https://doi.org/10.1016/j.chemosphere.2012.09.016

Shi, J., Huang, L., Sanganyado, E., Mo, J., Zhao, H., Xiang, L., Wong, M. H., & Liu, W. (2022). Spatial distribution and ecological risks of polychlorinated biphenyls in a river basin affected by traditional and emerging electronic waste recycling in South China. *Ecotoxicology and Environmental Safety*, *243*, 114010. https://doi.org/10.1016/j.ecoenv.2022.114010

Simon, T., Britt, J. K., & James, R. C. (2007). Development of a neurotoxic equivalence scheme of relative potency for assessing the risk of PCB mixtures. *Regulatory Toxicology and Pharmacology*, *48*(2), 148–170. https://doi.org/10.1016/j.yrtph.2007.03.005

Tomza-Marciniak, A., Pilarczyk, B., Witczak, A., Rząd, I., & Pilarczyk, R. (2019). PCB residues in the tissues of sea ducks wintering on the south coast of the Baltic Sea, Poland. *Environmental Science and Pollution Research*, *26*(11), 11300–11313. https://doi.org/10.1007/s11356-019-04586-4

Treasury Board of Canada Secretariat. (1994, January 1). *Federally Contaminates Sites Inventory*. Government of Canada. https://www.tbs-sct.gc.ca/fcsi-rscf/home-accueil-eng.aspx

U.S. Environmental Protection Agency. (2015, November 2). *Non-Dioxin-Like PCBs: Effects and Consideration In Ecological Risk Assessment* [Reports and Assessments]. https://www.epa.gov/risk/non-dioxin-pcbs-effects-and-consideration-ecological-risk-assessment

Wang, Q., Xie, C., Long, C., Yang, W., Wang, Y., Xu, W., Zhang, L., & Sun, Y. (2022). Bioaccumulation and Biomagnification of Polychlorinated Biphenyls and Dichlorodiphenyltrichloroethane in Biota from Qilianyu Island, South China Sea. *Toxics*, *10*(6), 324. https://doi.org/10.3390/toxics10060324

Xie, Z., Zhang, P., Wu, Z., Zhang, S., Wei, L., Mi, L., Kuester, A., Gandrass, J., Ebinghaus, R., Yang, R., Wang, Z., & Mi, W. (2022). Legacy and emerging organic contaminants in the polar regions. *Science of The Total Environment*, *835*, 155–376. https://doi.org/10.1016/j.scitotenv.2022.155376

Yu, C., Wen, Q., Ren, Q., Du, Y., & Xie, X. (2021). Polychlorinated biphenyl congener 180 (PCB 180) regulates mitotic clonal expansion and enhances adipogenesis through modulation of C/EBPβ SUMOylation in preadipocytes. *Food and Chemical Toxicology*, *152*, 112–205. https://doi.org/10.1016/j.fct.2021.112205

Zahaby, Y., Xia, P., Crump, D., Provencher, J. F., Thomas, P. J., Pauli, B., Braune, B. M., Franckowiak, R. P., Gendron, M., Savard, G., Sarma, S. N., Mallory, M. L., & O'Brien, J. (2021). ToxChip PCR Arrays for Two Arctic-Breeding Seabirds: Applications for Regional Environmental Assessments. *Environmental Science & Technology*, *55*(11), 7521–7530. https://doi.org/10.1021/acs.est.1c00229

Zahara, A. R. D., Michel, N. L., Flahr, L. M., Ejack, L. E., & Morrissey, C. A. (2015). Latent cognitive effects from low-level polychlorinated biphenyl exposure in juvenile European starlings (Sturnus vulgaris). *Environmental Toxicology and Chemistry*, *34*(11), 2513–2522. https://doi.org/10.1002/etc.3084

3. Chapter 3: Using a ToxChip PCR array to screen hepatic tissue of Black guillemot (*Cepphus grylle*) collected from a diesel spill site and a non-spill site in the Arctic

3.1 Abstract

Economic development, transportation and oil exploration are increasing in the Arctic, and there is concern regarding increased oil-related contaminants entering into this sensitive environment. Polycyclic aromatic compounds (PACs) are the main component in oil-related contaminants and can be monitored in wildlife species following exposure. In 2020, a diesel spill occurred off the coast of Postville, NL. Using a ToxChip PCR array tool, we evaluate hepatic polycyclic aromatic compound (PAC) burdens and gene expression profiles to help determine biological pathways effected by exposure to diesel in a seabird species, Black guillemot. Distinct diesel-related contaminant profiles in birds collected from the spill site were determined, characterized by the presence of naphthalene, pyrene and dibenzothiophene. Despite the difference in chemistry profiles between sites, the gene expression profiles were not as distinct from the non-spill site as expected. However, using PLS regression modeling revealed that expression of certain target genes on the ToxChip were good predictors of observed chemical concentrations. These results indicate that birds from the non-spill site were also exposed to PACs. Future research could use samples from a known low contamination site to compare results; however, finding acceptable reference/baseline sites with little to no contamination is becoming increasingly difficult globally.

3.2 Introduction

3.2.1 Climate change and its effects on shipping in the Arctic

While climate change is increasingly impacting every part of the world, the Arctic is experiencing the effects faster and at a greater magnitude than anywhere else (Rantanen et al., 2022). Consequences of climate change in the Arctic include increased surface air and ocean temperatures, decreased sea ice extent and duration throughout the year and increased shipping activity (IPCC, 2022). Additionally, average sea ice extent in the Arctic was the lowest recorded in history during the summer of 2019 (Yadav et al., 2020). Reduced sea ice has led to increased shipping traffic in the Arctic. Overall distance travelled by ships in the Canadian Arctic nearly tripled between 1990 and 2015 (Dawson et al., 2018). In the last decade, shipping traffic has increased by more than 75% in the Arctic. Currently, the largest portion of ship traffic is made up of cargo and research vessels, but private vessels are the fastest growing group of vessels in the region.

3.2.2 Sources and exposure patterns of PACs

As shipping and oil exploration/extraction increases, there is concern regarding accidental spills of oil offshore. Events such as the Deepwater Horizon or Exxon Valdez spills can cause direct and indirect harm to wildlife for years after the event (Esler et al., 2002; G. Troisi et al., 2016; Zuberogoitia et al., 2006). PACs are the main component in oil-related compounds and tend to be present in the environment as complex mixtures. There are an estimated 100,000 PAC congeners in global circulation (Hobson et al., 2002). PACs and other degradation-resistant contaminants settle in sediment and can be released back into the water column (Chen et al., 2018). Among the persistent organic pollutants (POPs) of concern in the Arctic, PACs continue to infiltrate through natural

and anthropogenic sources. PACs enter the environment through air and ocean currents and can be introduced from point sources through accidental release of oil into the water and shorelines. PACs can be categorized into two sub-groups based on molecular weight and source: petrogenic or pyrogenic. Petrogenic PACs tend to be formed over geologic timescales and are typically alkylated. Pyrogenic PACs are released through incomplete combustion activities and are typically found in the parent form. Currently, the main source of atmospheric PACs entering the global environment are from biomass burning (60.5%), followed by open field biomass burning (13.6%) and petroleum consumption by motor vehicles (12.8%) (Shen et al., 2013). Within the Arctic, natural seeps accounted for 95% of hydrocarbons entering the environment in 2007 (Arctic Monitoring and Assessment Programme, 2010). The Arctic is also host to large quantities of untapped oil and natural gas, which will become more accessible. An estimated 90 billion barrels of oil remain to be extracted within the Arctic region (Bird et al., 2008). A more regionally specific estimate states there are 10 billion barrels of oil within the Baffin Bay-Davis Strait (BBDS) region (Gautier et al., 2009).

3.2.3 Seabirds as bioindicators of PACs in the environment

Birds are particularly good sentinels for contaminant exposure within the Arctic. Seabirds occupy a relatively high trophic position and impacts of contaminants are well documented in both laboratory and wild avian species. Direct oiling of seabirds has been shown to result in hypothermia, emaciation, inhibited weight gain, dehydration, and death (Bianchini et al., 2021). In addition, in vitro and early-life stage studies using PACs have shown reduced hatching success, increased developmental abnormalities, altered biochemical responses, and embryotoxicity (Albers, 2006; Brunström et al., 1991).

Toxicity of oil on birds depends on myriad variables including congener, feeding and migration, species, life stage, dose, and duration of exposure. Evidence points to several highly impacted biological pathways including glucose, protein, and fatty acid metabolism (King et al., 2021). Seabirds in the Arctic are likely to have low exposure to PAC contamination through ingestion or inhalation of PAC molecules. During an acute exposure event such as an oil spill, however, seabirds will be exposed dermally and through ingestion as they preen. PACs are present in the environment as complex mixtures comprising of many congeners. Congeners have varying toxicity and it is therefore difficult to measure their effects individually. There is a need for innovative tools to assist with contaminant monitoring and economic planning in the Arctic.

The overall goal of this research is to determine what biological pathways are most impacted in seabirds exposed to PACs following a diesel spill event. To do so, we focused on the site of a diesel oil spill (Postville, NL) and a non-spill site (Nain, NL) in 2020. Gene expression profiles of birds from the diesel spill site will be compared to birds collected from a site with no known contamination. We will also test the applicability of a new transcriptomic tool to identify collection site based on chemistry and gene expression data for each bird. This information will help infer sites of interest for future contaminant monitoring projects. This chapter includes two collection sites; Postville, NL is the site of an accidental 3000 L diesel spill and Nain, NL is a non-spill site 200 km northeast of Postville.

Based on the expected difference in chemistry profiles between the two sites, I predict that birds collected from the diesel spill site will have higher PAC burdens, particularly lower molecular weight PACs associated with diesel fuel (Provencher et al.,

2022). This chapter follows the prediction that samples collected from the spill site (Postville) will have a higher number of altered genes compared to samples collected from the non-spill site, particularly for genes involved in xenobiotic metabolism and hormone pathways. We will be able to use birds collected from the spill site to understand gene expression profiles following exposure to diesel-related contaminants. To test the thesis hypothesis, I predict that distinct chemistry profiles at both sites will result in distinct gene expression profiles based on PAC source.

3.3 Materials and Methods

3.3.1 Study sites and sample preparation

In June of 2020, a diesel spill of approximately 3,000 liters was detected off the coast of Postville, NL (54°54'N, 59°48'W). Adult Black guillemot individuals (n=16) were shot and collected in October 2020 by local hunters and staff members from Environment and Climate Change Canada. Livers from each bird were removed and flash frozen, and the remaining bird carcass was subsequently frozen. The livers from each bird were sent to the National Wildlife Research Centre (NWRC), Ottawa, ON for storage and bird carcasses were later dissected during a workshop at the Nunatsiavut Research Centre. The left liver lobe was used for gene expression analysis using a previously developed ToxChip PCR array and the right liver lobe was used for PAC contaminant analysis at the Centre for Oil and Gas Research and Development (COGRAD), Winnipeg, MB. For each sample, 52 PACs were measured. PAC concentrations were expressed in ng/g lipid weight (l.w.).

Nain, NL (56°32'N, 61°43'W) is located approximately 200 km NE of Postville (Figure 3.1). Nain was chosen as a suitable benchmark site due to its distance from

Postville, relative lack of contaminants in the surrounding environment and presence of a local research station. In October 2020, adult Black guillemots (n=15) were collected from Nain in the same manner as Postville. Adult Black guillemots were collected from both sites again in 2021 (n=10).

Figure 3.1: Map of Nain, NL and Postville, NL. Postville was the site of a diesel spill in June 2020.

3.3.2 Chemical analysis of livers collected from Nain and Postville in 2020

The analysis of PACs and their alkylated congeners was completed at COGRAD, a CALA-accredited and ISO 17025 certified facility following published methods in Idowu et al. (2018). Briefly, approximately 5 grams of liver homogenate was weighed on a Sartorius Cubis MSE225P100-DI (Fisher Scientific) and mixed with diatomaceous earth. The mixture was loaded in an accelerated solvent extraction (ASE) cell, spiked with C-13 labelled internal standards and the dead volume of the cell was filled with Ottawa Sand. Procedural blanks, standard reference material (NIST SRM 2974a) and duplicates were included for each sample run. Samples were extracted under high pressure conditions and dichloromethane (DCM) was used as the extraction solvent. The

extract volume was evaporated down to 2.6 mL under ultra-high-purity nitrogen, and mixed with hexane (1:1 v/v) to a final volume of 5.2 mL. We used 200 µL of this to gravimetrically determine percent lipid. Removal of lipids from the extracts was achieved using automated gel permeation chromatography (GPC; J2-scientific AccuPrepMPS™, Columbia, Missouri, USA). The final extract was again reduced down to 1 mL under nitrogen and loaded on a silica/alumina column for final clean-up by adsorption chromatography. The PAC containing fraction was eluted with 25 mL DCM/Hexane (1:1, v/v), and reduced in volume by rotary evaporation and UHP nitrogen. The final extracts were stored under refrigerated conditions at 4 °C in amber glass vials prior to instrumental analysis. An Agilent 7890 gas chromatograph coupled with a triple quadrupole mass spectrometer fitted with an electron ionization (EI) source was used for the acquisition of MS/MS spectra with helium as the carrier gas at a constant flow rate of 1.2 mL/min. Sample (1 µL) was injected with a PAL RSI 85 autosampler at 250 °C in splitless mode. The mean, standard deviation and ranges of all PACs tested for (SOM) are reported in ng/g lipid weight (l.w.). QA/QC included the use of duplicates (10% of seabird liver samples were run in duplicate), blanks at the start and end of each GC–MSMS run (approx. 2 blanks for each run of ~30 samples) and the use of certified reference material (NIST CRM 2974a).

3.3.3 Application of ToxChip PCR array to Postville and Nain, NL samples

A previously developed ToxChip array for the Black guillemot was used for all collected liver samples in this project. The steps for ToxChip development have been outlined previously (Crump et al., 2016; Zahaby et al., 2021). The ToxChip array contains 32 target genes, and 3 samples are run on each 96 well array. The arrays were

run on a Mx3005P Stratagene (Richmond Scientific) qPCR machine. Approximately 30 mg of liver tissue was excised from the left lobe of each bird, and total RNA was extracted following the manufacturer's protocols for the RNeasy extraction kit with the QIAcube (Qiagen) with a genomic DNA contamination on column treatment. The resulting concentration and purity of RNA was determined using the QIAxpert (Qiagen). All samples had purity values A260/A280 ≥ 1.8. Half the volume of each RNA sample was aliquoted, while the remaining volume was stored at −80 °C. Approximately 1000 ng of total RNA from each sample was reverse transcribed to cDNA using the QuantiTect kit (Qiagen) with modifications described by Porter et al., 2014. The resulting cDNA was diluted 1:10 in DEPC water. Array wells were spotted with 5 µl of optimally concentrated primers using a QIAgility machine (QIAGEN) prior to the addition of SYBR Green with ROX Mastermix (QIAGEN) and 5 µl diluted cDNA. The total reaction volume in each well was 25 µl. The arrays were run on a Mx3005P qPCR machine (Agilent). The thermocycle program included an activation step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and ending with a dissociation curve segment of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s.

3.3.4 Data analysis for PACs and gene expression

The limit of detection for each PAC congener was determined during analysis by COGRAD. Two PAC congeners did not have detection limits (Retene and C4 Fluorene). To account for this, we determined the average detection limits for all other compounds which did have detection limits to be 3% of the lowest reported value and set this value as the "new" detection limit for Retene and C4 Fluorene. PACs with >50% of samples below zero were removed from analysis. For remaining PACs, any value below the

detection limit (BDL) was replaced with a value of 10% of the detection limit. This left 23 individual PACs for use in the analysis after filtering.

Array data for each individual bird was retrieved from a Mx3005P Stratagene qPCR machine. PCR cycle threshold (Ct) data were transformed into $2^{-\Delta Ct}$ followed by normalization using the trimmed mean of M-values (TMM) method via the calcNormFactors function from the edgeR library in R Studio. This normalization method has been used in several recent studies evaluating hepatic transcriptomic signatures in seabirds (Xia et al., 2020; Zahaby et al., 2021). The parameters of logratioTrim and sumTrim for TMM normalization were assigned with 0.5 and 0.1, respectively. Relative gene expression of each gene was calculated by dividing the normalized gene expression by the mean normalized expression of that gene across all individuals.

Genes with no detectable Ct (No Ct) in ≥ 50% of samples were removed from analysis (7 genes were removed during this step). Ct values > 35 were ignored from filtering as there is confidence in gene expression based on previous work (Zahaby et al., 2021), and higher Ct values were therefore deemed valuable for analysis in this case. Any remaining No Ct values present after the initial filtering were replaced with the experimental average. One sample was not included in the data analysis for gene expression because it contained too many non-detect Ct values (PG_20_15).

All statistics were conducted in R Studio or R Commander. Principal component analysis (PCA) is a multivariate analysis technique used to reduce the dimensionality of a dataset and emphasize variation. Data is visualized on a two-dimensional graph with the first principal component accounting for the most variation. Samples, or loadings, tend to cluster based on projected similarity. Partial least squares discriminant analysis (PLSDA)

is used as a predictive modelling tool. In this chapter, PLSDA was used with a Leave One Out cross validation, which trained an algorithm to predict collection site of a sample based on its chemistry and gene expression profile. Variable Importance in the Projection (VIP) gene scores evaluate the importance of each variable (gene) in the projection (prediction).

Partial least squares (PLS) regression was used to determine if gene expression levels were predictive of PAC concentrations, using Leave One Out cross validation. The results are evaluated by comparing the "actual observed concentrations" to the "predicted concentrations" of the models. To build the model, first all genes on the array were used, then just genes with a VIP score > 1. VIP scores determine the importance of each variable in the projection used in a PLS model and can be used for predictor variable (gene) selection (Xia et al., 2020). Based on an acceptable slope (> 0.5) and R^2 value (> 0.4), 6 individual PACs met these criteria. Gene coefficients indicate the contribution that each gene makes to the chemical concentration predictions. A positive value indicates positive correlation while a negative value indicates negative correlation.

3.4 Results

3.4.1 Black guillemots and PAC chemistry data

In June 2020, a diesel spill of approximately 3000 litres was reported off the coast of Postville, NL. Nain, NL is approximately 220 km NW of Postville and is considered a non-spill site for contaminant burden comparison. In October 2020, a team of experienced hunters and staff was tasked with collecting adult Black guillemot individuals for liver contaminant analysis from Nain and Postville. A total of 30

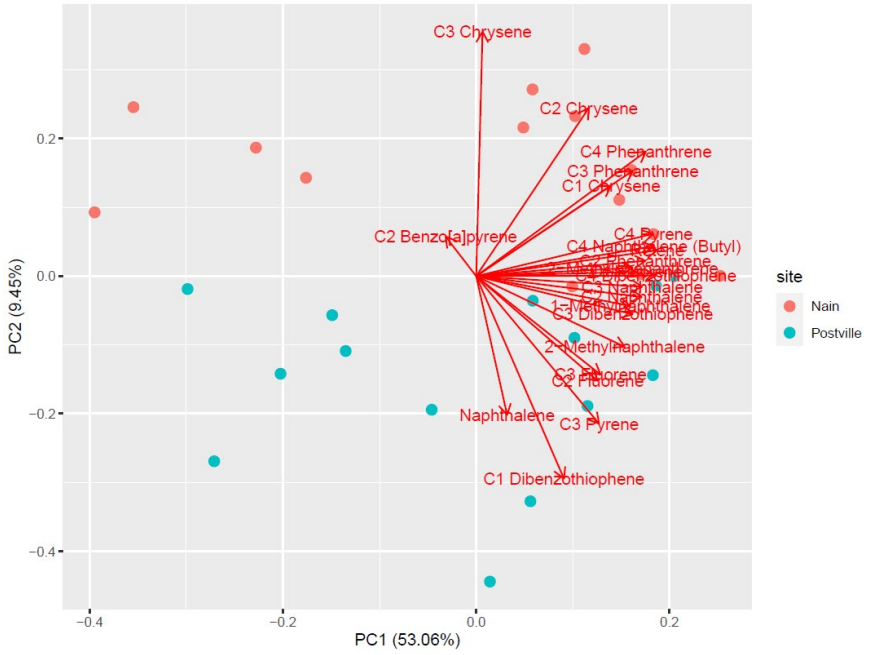
individuals were shot and collected offshore at the spill and non-spill sites, 15 individuals from each site.

A total of 52 PACs were measured in individual bird liver tissue. Based on the filtering parameters discussed above in the methods section, 23 PACS were included in the analysis for this project. The bird with the highest sum PAC burden (NG_20_06) had 374.23 ng/g l.w. The bird with the lowest sum PAC burden (PG_20_09) had 4.44 ng/g l.w.

3.4.2 PAC chemistry data profiles

The PCA for chemistry data shows a clear separation between Nain and Postville samples, indicating that the chemistry profiles are distinct between the sites (Figure 3.2a). PACs related to diesel (naphthalene, C1 dibenzothiophene, C3 pyrene) (Jia & Batterman, 2010; Marr et al., 1999; Rhee et al., 1998) influence the site difference for Postville samples the most. For Nain, C3 Chrysene is the most influential PAC for site differentiation. The hierarchical cluster analysis within the heatmap shows Nain samples have relatively high concentrations of certain PACs (C2 Benzo(a)pyrene (BaP), Naphthalene, C3 naphthalene) (Figure 3.2b). Many of the Nain samples are the most highly contaminated (i.e., contain the highest concentrations of the most PAC congeners). PLSDA predicted a sample correctly with 85.7% accuracy using chemistry data for both Nain and Postville (Figure 3.3). Using only VIP contaminants did not improve the predictive power of the algorithm.

A)



B)

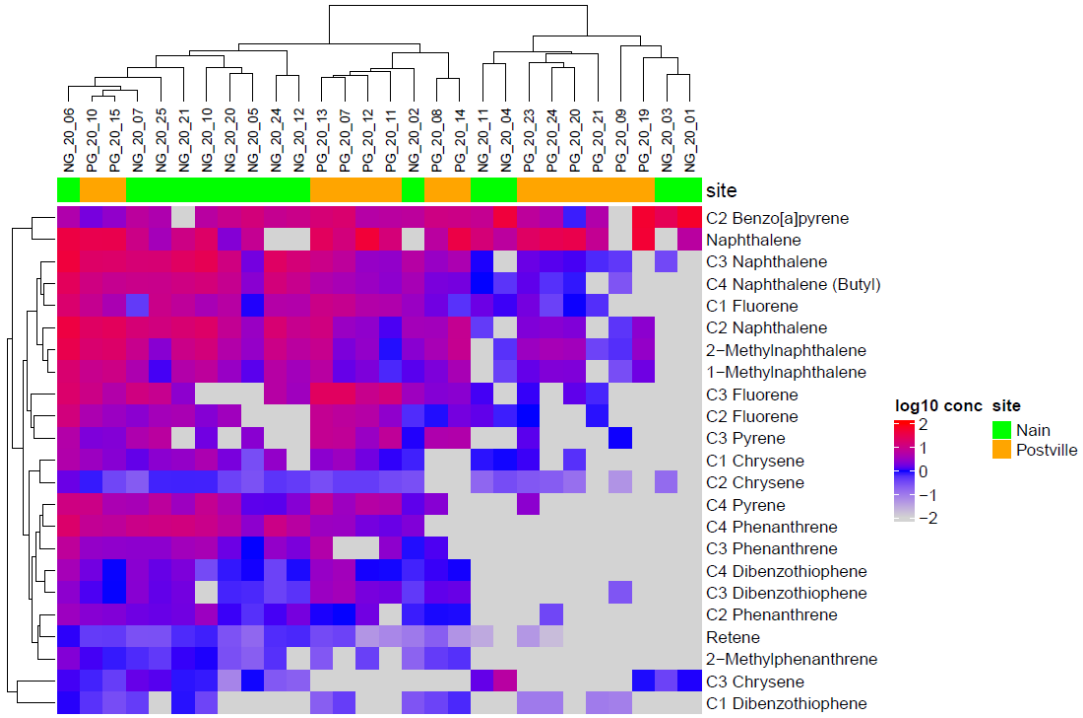


Figure 3.2: Chemistry Profile. A) Principal component analysis (PCA) shows chemistry data for both sites (Nain & Postville) collected in 2020. PC1 accounts for approximately half (53.06%) of the variation among the data. The data presented are centered and scaled. B) Heatmap of log10 transformed chemistry data (ng/g l.w.) for samples from Nain, NL and Postville, NL collected in 2020.

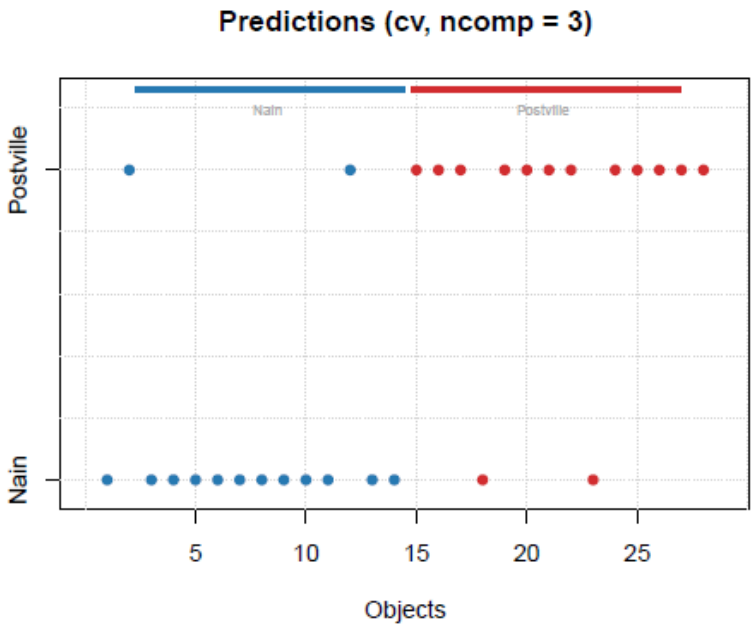


Figure 3.3: PLSDA shows predictions of sample site based on chemistry data. The algorithm predicted the correct site 85.7% of the time for Nain & Postville samples.

3.4.3 Gene expression profiles

The PCA for gene expression data shows a clear separation between Nain and Postville samples along PC1. Genes related to thyroid hormone response (TTR) and oxidative stress (GSTM3) influence the site difference for the samples from Postville. LBFABP influenced the Nain gene expression profile the most. Overall, the majority of ToxChip genes were not found to be heavily influenced by site, based on the loading direction being parallel to the dotted line (Figure 3.4).

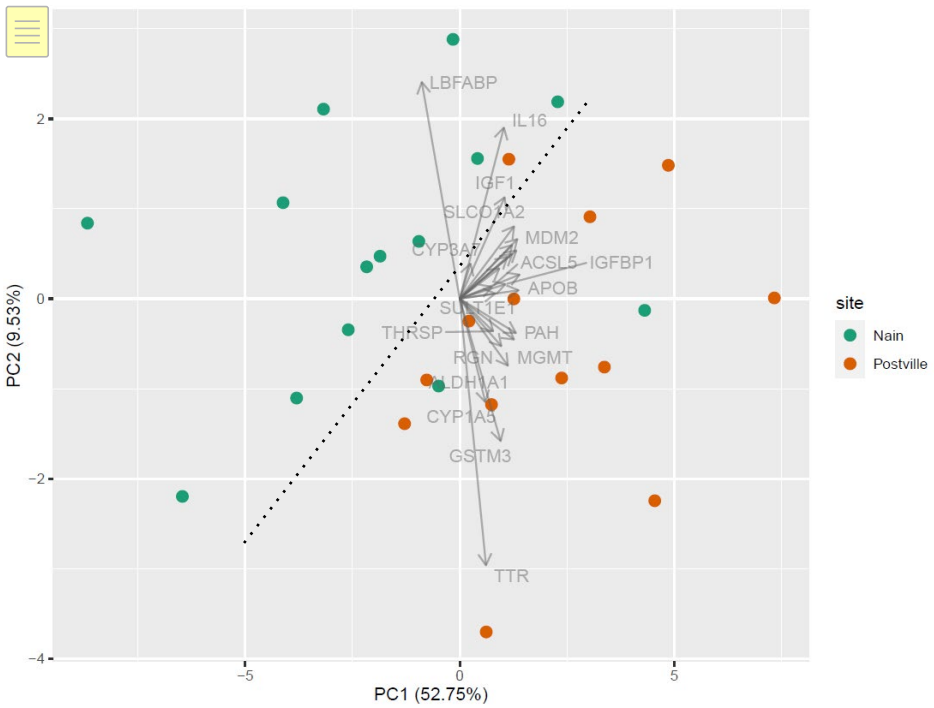
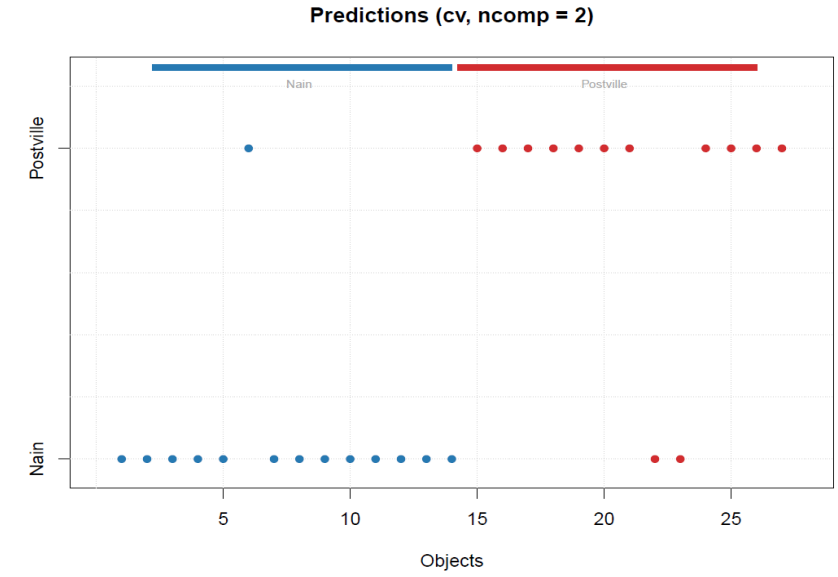


Figure 3.4: Principal component analysis (PCA) shows gene expression data for both sites (Nain & Postville) collected in 2020. PC1 accounts for approximately half (52.75%) of the variation among the data. The data are normalized using TMM. Dotted line was added after analysis for visual year differentiation.

The PLSDA model was able to predict the origin site of a sample based on gene expression data (Figure 3.5a). The model predicted a sample correctly with 92.8% accuracy for Nain and 84.6% accuracy for Postville using gene expression data. Using only VIP genes did not improve the predictive power of the algorithm. Regression coefficients allow for the identification of genes most important for the model's predictability (Figure 3.5b). For Nain samples, LBFABP was most influential for determining samples originating from that site. Another important gene for Nain sample determination was SULT1e1, SLCO1a2 and IL16 (Figure 3.5b). The gene most influential for determining Postville samples was TTR. RGN and MGMT were also important genes for Postville site determination.

A)



B)

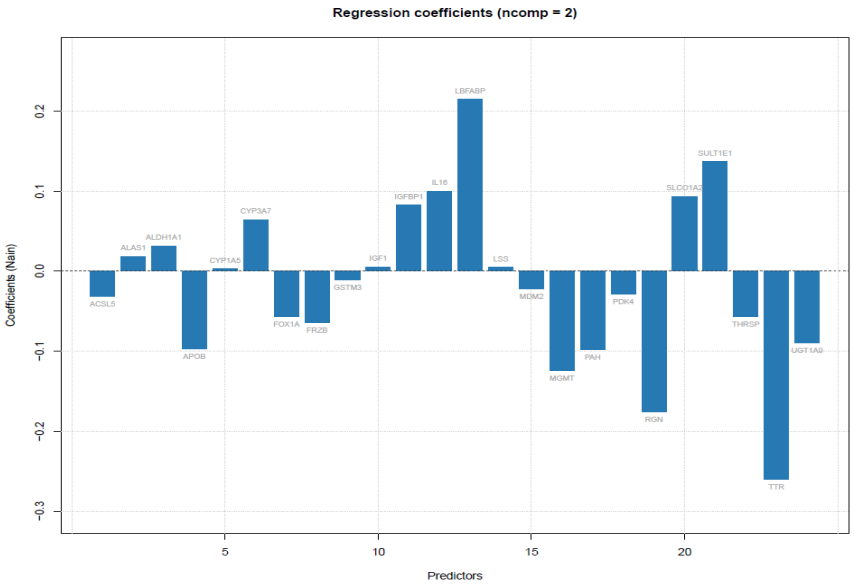


Figure 3.5: Predictions based on Gene Expression. A) PLSDA shows predictions of sample site based on TMM normalized gene expression data. The algorithm predicted the correct site 92.8% of the time for Nain and 84.6% of the time for Postville samples. B) Regression coefficients of genes most influential in site predictions. Bars above the horizontal axis relate to Nain predictions, while bars below relate to Postville.

3.4.4 Predictive power using PLS regression

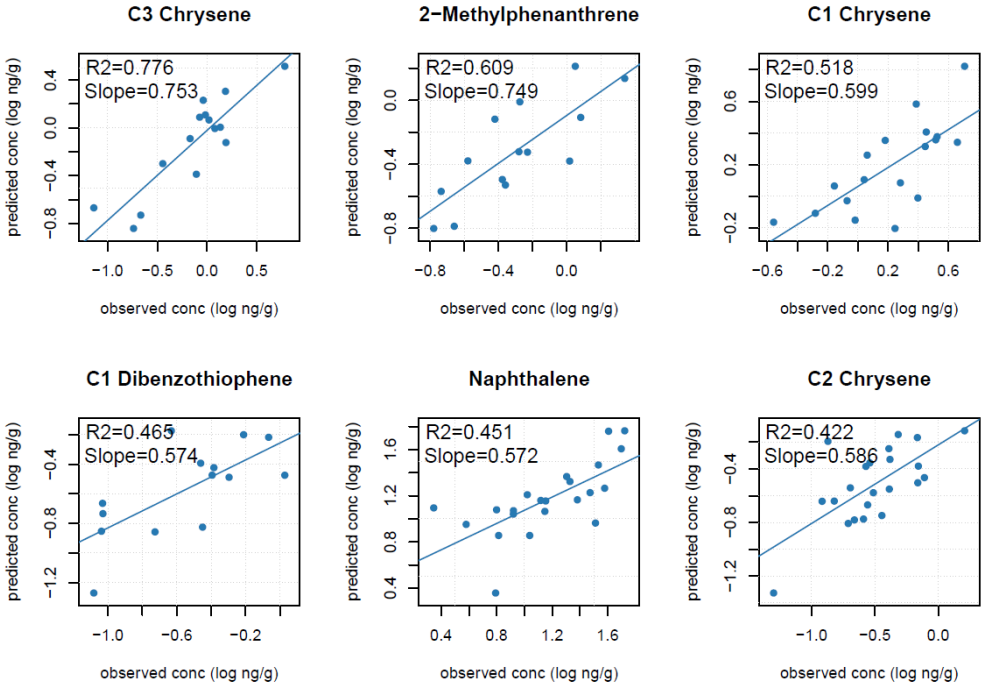
The PLSDA predictive model was effective at using chemical and gene expression data to predict the site of origin of a sample. Based on this success, we applied a PLS regression model to test if we could determine individual chemical concentrations in a sample using VIP genes. Only genes with VIP scores > 1 were used for this model. Out of 23 individual PACs, 6 passed the PLS criteria threshold, and their concentrations were predicted with relatively good accuracy based on gene expression. C3 chrysene had the best predictive value with an R^2 of 0.776 and a slope of 0.753 (Figure 3.6a). A higher slope may indicate better regression fitting between predicted and observed concentrations of that chemical. Because there is so much variability in wild samples and many confounding factors, an R^2 value of 0.5 or greater is considered satisfactory (Díez et al., 2013; Ho et al., 2021).

The phase I metabolizing gene, CYP1a5, was positively correlated with 5 out of 6 top performing PACs. CYP1a5 made the largest contribution to 2-Methylphenanthrene concentration prediction with a gene coefficient of 0.795 (Figure 3.6b). Other genes important for chemical concentration prediction are involved in xenobiotic metabolism (CYP3a7, SULT1e1), thyroid hormone response (THRSP) and cellular processes/calcium homeostasis (RGN). For many of the genes related to concentration prediction, there was overlap of predicted chemicals. Some genes were important for a single chemical prediction (FOX1a, IGF1, PAH, LSS) with gene coefficient values above 0.4.

Overall, CYP1a5 was the best at predicting chemical concentration with gene coefficients ranging from 0.291 to 0.795. Other genes including RGN, CYP3a7, THRSP and SULT1e1 demonstrated strong correlation trends with at least 3 chemicals. The

results of the PLS regression show that 5 of the 6 best performing chemicals were also among the lower half in terms of chemical concentration in birds on the chemical heatmap (Figure 3.2b), namely C1, C2 & C3 chrysene, 2-methylphenanthrene and C1 dibenzothiophene.

A)



B)

			Gene Coefficients																
Chemical	R2	Slope	CYP1A5	THRSP	LBFABP	PDK4	FOX1A	IGF1	PAH	LSS	IGFBP1	CYP3A7	RGN	SULT1E1	TTR	GSTM3	ALAS1	ACSL5	MGMT	SLCO1A2
C3 Chrysene	0.776	0.753	-	-0.101	-	-	0.634	-	0.540	-	0.043	0.500	-	-0.314	-	-	-0.256	-0.408	-0.363	-
2-Methylphenanthrene	0.609	0.749	0.795	0.621	-	-	-	-	-	-	-	-0.012	-0.375	-0.445	-	-0.246	-0.082	-	-	-
C1 Chrysene	0.518	0.599	0.432	0.302	-	-	-	0.595	-	-	-	-0.295	-0.627	-0.194	-	-0.391	-	-	-	-
C1 Dibenzothiophene	0.465	0.574	0.291	-	0.235	0.122	-	-	-	-	-	-0.172	-0.312	-	-0.255	-	-	-	-	-
Naphthalene	0.451	0.572	0.644	-0.210	-0.162	-	-	-	-	-	-	-	-	-	0.126	-	-	-	-	-0.118
C2 Chrysene	0.422	0.586	0.432	-	0.208	0.154	-	-	-	0.426	-	-0.164	-0.638	-	-0.180	-	-	-	-	-

Figure 3.6: PLS regression A) Regression plots were used to show the top 6 performing PACs for which gene expression was used to predict actual PAC concentration in liver samples. These chemicals were successful based on acceptable R2 and slope values. B) Top 6 PAC chemicals with corresponding R2, slope and gene coefficients. Orange cells indicate that the gene is positively correlated (increased exposure leads to increased gene expression) with the chemical and blue cells indicate negative correlation (increased exposure leads to decreased expression) with the chemical.

3.5 Discussion

To determine if gene expression profiles could be used to infer contaminant exposure following a diesel spill, black guillemot adult liver samples from two distinct sites were collected in 2020. Postville, NL was the site of a diesel spill, which resulted in approximately 3000 L being released offshore in the summer of 2020. Adult black guillemots were collected from the area surrounding the spill to understand the role of diesel-related PACs on gene expression patterns following acute exposure. Samples from a relatively nearby non-spill site (Nain) were collected at the same time to serve as a comparison dataset. For this project, it was hypothesized that livers collected from Postville would demonstrate a clear diesel-related PAC chemistry profile compared to those collected from the non-spill site. Genes related to xenobiotic metabolism and thyroid hormone response were expected to be most impacted in the spill site livers due to their known alteration in response to PAC contaminants (Fernie et al., 2019; Shimada & Fujii-Kuriyama, 2004).

3.5.1 Postville and Nain chemistry profiles were mostly dominated by petrogenic PACs

Lower molecular weight PACs (composed of four aromatic rings) are typically associated with a petrogenic source. As such, PAC profiles in Postville were expected to be dominated by naphthalene, fluorene, acenaphthylene and phenanthrene. A study looking at 5 different diesel oil compositions found that these PACs, along with pyrene, were the highest concentrated in all diesel samples tested (Marr et al., 1999). Naphthalene and C3 pyrene were important in the distinction of Postville samples in the present study (Figure 3.2a). As these PACs are typically found in diesel, their detection in Postville black guillemot liver samples confirms that the diesel spill had an impact on sampled

birds in the area. C3 dibenzothiophene also strongly influences the Postville sample chemistry profile and is typically found in coal and fossil fuels (Clark & Pazdernik, 2016). Overall, while diesel-related PACs did dominate the Postville chemistry profile, PACs related to diesel were also found in Nain and therefore were less predictive of Postville samples than expected. These results suggest that birds from Nain are exposed to a variety of PACs, including those related to diesel oil.

Despite the lack of an acute PAC contamination in Nain, NL, the results of this project show that there is some contamination present in many of the birds collected from this site in 2020. Many samples collected from Nain have the highest PAC burdens (Figure 3.2). This is surprising given Nain is the reference non-spill site. PAC contamination can originate from a variety of sources, and the most concentrated PACs in birds from Nain are ubiquitous in the environment (e.g., alkylated BaP, naphthalene) (McCormick et al., 2022; Metre et al., 2009). C3 chrysene strongly influenced the Nain chemistry profile and was only detected in three Postville samples, suggesting that birds from Nain are being exposed to a petrogenic PAC source (Figure 3.2b). Despite this strong influence, concentrations of C3 chrysene were low in birds from Nain. This pattern was also observed in 4 bird species sampled from the BBDS region (Provencher et al., 2020). Petrogenic (C3) chrysene is a constituent of coal tar, and such lower molecular weight PACs are more volatile and can be reconstituted into the environment long after deposition (Wong et al., 2004). Contaminants with the highest concentrations in the most Nain samples included C2 BaP and naphthalene, both known carcinogens (Moradi et al., 2022). C2 BaP is a petrogenic PAC, possibly from underground bitumen. PAC composition in the Arctic has shown to be mostly petrogenic in ocean and surface

sediments (Harvey et al., 2014; Ma et al., 2017). Parent compounds tend to be pyrogenic, therefore the influence of naphthalene on Nain sample identification indicates likely exposure to combustion byproducts, typically gasoline (Jia & Batterman, 2010). Shipping traffic is prevalent in Nain, which could explain the exposure to gasoline fumes in these birds. Additionally, parent naphthalene has many sources within the environment, so exact origin of exposure can only be hypothesized. Notably, BaP was not found in significant concentrations within any of the bird livers measured. This is likely due to its high capacity for CYP1a1 mediated metabolism (Troisi et al., 2006; Näf et al., 1992). Of the 16 priority PAHs typically studied, only naphthalene was found in high concentrations in these bird livers (Figure 3.2b). These 16 priority PAHs, set by the U.S. EPA in 1983, have been the focus of many ecotoxicological studies in recent years (Pereira et al., 2009). All are parent or pyrogenic in nature.

3.5.2 Based on gene profiles, Nain and Postville samples show distinct separation

Similar to the chemistry profiles, the PCA for gene expression data shows a clear separation between Nain and Postville samples. The dotted line on the gene expression PCA is used to help visualize the separation of sites. Genes related to thyroid hormone response (TTR) and oxidative stress (GSTM3) have a strong influence on Postville samples (Figure 3.4). This is likely due to the presence of diesel-related PACs, which have been shown to impact hormone homeostasis (Lee et al., 2017) and can contribute to oxidative stress through P-450 enzyme activity (Näf et al., 1992). Transthyretin (TTR) is a transport protein involved in thyroid hormone movement to target organs within a bird (Duan et al., 1991; Egloff et al., 2011). This gene has been shown to down-regulate in chicken hepatocytes following exposure to flame retardants (Su et al., 2014) and in

zebrafish larvae following exposure to perfluorooctane sulfonate (PFOS) (Shi et al., 2009). Glutathione S-transferases mu3 (GSTM3) is involved in detoxification and its expression has been found to increase following exposure to mercury in female birds (Gibson et al., 2014) and following exposure to BaP in mice (Li et al., 2020). The aryl hydrocarbon receptor (AhR) is activated by PACs and other contaminants. Activation of the AhR leads to its translocation to the nucleus and binding of the AhR complex to xenobiotic response elements within the DNA. These are commonly found in the promoters of AhR target genes, such as CYP1A (Guyot et al., 2013). A xenobiotic metabolism related gene, which is typically upregulated following AhR activation (CYP1a5) (Crump et al., 2017; Zhang et al., 2016) was important for the Postville gene expression profile. Interestingly, fewer genes involved in xenobiotic metabolism influenced the Postville profiles than expected.

In Nain samples, liver basic fatty acid binding protein (LBFABP) influenced the gene profile of these birds the most (Figure 3.4). This gene is mainly involved in lipid homeostasis and has been shown to be down-regulated in birds following PAC exposure (Bianchini et al., 2021; Mundy et al., 2019). A study found that an orally dosed PAC mixture containing chrysene reduced LBFABP gene expression in Sanderling, resulting in pre-migratory fueling impairment (Bianchini et al., 2021). Interleukin 16 (IL16), which is involved in immune response, shows some importance in the differentiation of Nain samples. Following exposure to BaP, IL16 expression increased in mice (Kerley-Hamilton et al., 2012). Notably, genes involved in xenobiotic metabolism did not differentiate the spill site from the non-spill site as expected. This likely means that birds from Nain are being exposed to contaminants that would alter xenobiotic metabolism in

the liver or that inter-individual variation in gene expression profiles plays a larger role than expected. This provides further evidence that Nain birds have distinct gene expression profiles and have exposure to PACs, despite being a non-spill site.

Most genes (parallel with the dotted line) are not strongly influenced by site, but rather show natural variability among samples (Figure 3.4). This sample variability is unsurprising in wildlife samples, as each bird has distinct feeding and contaminant exposure tendencies.

3.5.3 Predictive power using PLS regression

Overall, the performance of the PLS regression was better than anticipated. The chemical plots show good linear relationships between the predicted and observed chemical concentrations (Figure 3.6a). While only 6 out of 23 chemicals were top performing in this model, these results are in line with previously reported data using wild bird samples (Xia et al., 2020).

CYP1a5 is a well-known biomarker for PAC exposure in birds (Perez-Umphrey et al., 2018; Wiens, 2013). The results of the PLS regression support this as it was a major positive predictor for 5 out of the 6 PACs included in the analysis. This means that as PAC concentrations increase, so does the expression of the xenobiotic metabolism-related gene, CYP1a5. Interestingly, CYP3a7 and RGN show negative correlations with most PAC chemicals. This result is unexpected as in most studies to date, these genes show positive correlations or are upregulated following PAC exposure (Crump et al., 2017; Kreitsberg et al., 2023). C3 chrysene was the top performing chemical and demonstrates an interesting trend compared to the others. For THRSP, C3 chrysene is negatively correlated while it is positively correlated with CYP3a7. These results are an

anomaly compared to the other chemicals, which show the opposite trend. This chemical also strongly influenced the difference among sites on the chemistry PCA (Figure 3.2a). Chrysene is typically found in petroleum products, specifically coal tar (Biswas & Ghosh, 2014).

Compared to the log transformed chemical concentrations, most of the chemicals with the best predictive performance had the lowest concentration burdens in these birds (Figure 3.2b). C1 dibenzothiophene and C3 chrysene had the lowest overall abundance in birds from both sites, yet both chemicals were among the top 6 performers for concentration prediction using gene expression. Some genes were relatively good at predicting concentrations for several chemicals, while other genes were good at predicting concentrations for single chemicals. When determining if we can use gene expression alone to predict the concentration of a chemical in a bird, CYP1a5 stands out as a top performer. Depending on the chemical of interest, other genes could be used with accuracy. Biomarker genes have been used in several environmental monitoring applications (Bonisoli-Alquati, 2014; Piña et al., 2007) and to identify chemical biodegradation in wildlife (Gedalanga et al., 2014). Notably, the chemicals which were the most predictive of sample site origin also had the most predictable concentrations based on gene expression.

3.6 Limitations and Future Considerations

The findings of this study support the hypothesis that chemistry profiles will impact gene expression in a predictable manner following exposure to PACs. Diesel-related PACs influenced gene expression profiles, particularly for thyroid hormone responsive and oxidative stress genes, in birds collected from Postville. In this scenario,

transcriptomics can be a useful approach to monitor contaminant mixtures in a seabird species. Contaminants most influential in terms of distinguishing Postville samples were diesel related. Nevertheless, several Nain samples had high PAC concentrations from a variety of sources (parent and alkylated). One potential limitation to this analysis is the high metabolic transformation potential of many PACs (Troisi et al., 2006). PACs are expected to be metabolized and excreted quickly in seabirds, which could greatly affect their detection and quantification. Thus, a more accurate PAC burden, following exposure to the spill, might be attainable if samples were collected as soon as possible following a spill event in the future. Using a different tissue type, such as eggs, would allow for accurate PAC burden identification because PACs are transferred maternally, and metabolic capacity is low in eggs.

Genes involved in thyroid hormone response, oxidative stress and xenobiotic metabolism had the most influence on Postville gene expression profiles. However, the comparative influence on xenobiotic metabolism genes was less pronounced than expected in Postville samples compared to Nain. This is possibly the result of Nain birds being exposed to contaminants not measured in this project, which could alter genes related to xenobiotic metabolism. The scope of this project does not consider other complex chemical mixtures present in the birds. For future work, it would be beneficial to broaden the scope of chemicals included for analysis to include those of emerging concern in the Arctic, such as methylmercury (Chastel et al., 2022; Albert et al., 2021). One potential limitation to the use of gene array data for this chapter was the high frequency of Ct values for the housekeeping genes that were above the ideal range. The housekeeping genes on the ToxChip (RPL4 and EEF1a1) typically show consistent

expression in bird liver tissue (Hasanpur et al., 2022). This was not the case, and housekeeping gene Cts were regularly ≥ 30. Due to the housekeeping gene instability, it was decided to employ trimmed mean of M values (TMM) normalization. This is a different normalization method than the one used in Chapter 2 and needs to be considered as a variable given TMM is most often used with larger gene sets. Another limitation was that samples were not randomized during qPCR, and site-specific samples were run on the same PCR array. This could have resulted in batch-effects related to gene expression profiles. Future studies should endeavour to include samples from different sites/treatments on the same ToxChip given that 3 samples can be screened per array. Additionally, other assays such as ethoxyresorufin O-deethylase (EROD) could be conducted using tissue from the same livers added to the ToxChip to provide further insight into the effects of environmental contaminants.

Evaluating the impact of chemical mixtures on individual gene expression is difficult because many different chemicals impact the same genes. This means that it is difficult to tease out individual effects of chemicals on specific genes within the mixture. With a larger sample size, the PLS regression model would likely result in better predictions of chemical concentration based on gene expression. Additionally, the model does not account for the common co-occurrence of chemicals, which throws off predictions. Overall, diesel exposure was identifiable via chemical signature biomarkers as well as through gene biomarker induction. Chemical concentration predictions were more successful than anticipated, specifically using genes such as CYP1a5, RGN, THRSP, SULT1e1 and CYP3a7.

3.7 References

Albers, P. H. (2006). Birds and polycyclic aromatic hydrocarbons. *Avian and Poultry Biology Reviews*, *17*(4), 125–140. USGS Publications Warehouse.

Albert, C., Helgason, H. H., Brault-Favrou, M., Robertson, G. J., Descamps, S., Amélineau, F., Danielsen, J., Dietz, R., Elliott, K., Erikstad, K. E., Eulaers, I., Ezhov, A., Fitzsimmons, M. G., Gavrilo, M., Golubova, E., Grémillet, D., Hatch, S., Huffeldt, N. P., Jakubas, D., … Fort, J. (2021). Seasonal variation of mercury contamination in Arctic seabirds: A pan-Arctic assessment. *Science of The Total Environment*, *750*, 142201. https://doi.org/10.1016/j.scitotenv.2020.142201

Balmer, J.E., Hung, H., Yu, Y., Letcher, R.J., & Muir, D.C.G. (2019). Sources and environmental fate of pyrogenic polycyclic aromatic hydrocarbons (PAHs) in the Arctic. *Emerging Contaminants*, *5*, 128–142.

Bianchini, K., Crump, D., Farhat, A., & Morrissey, C. A. (2021). Polycyclic Aromatic Hydrocarbons Alter the Hepatic Expression of Genes Involved in Sanderling (Calidris alba) Pre-migratory Fueling. *Environmental Toxicology and Chemistry*, *40*(7), 1981–1989. https://doi.org/10.1002/etc.5056

Bird, K. J., Charpentier, R. R., Gautier, D. L., Houseknecht, D. W., Klett, T. R., Pitman, J. K., Moore, T. E., Schenk, C. J., Tennyson, M. E., & Wandrey, C. J. (2008). *Circum-Arctic Resource Appraisal: Estimates of Undiscovered Oil and Gas North of the Arctic Circle*. U.S. Geological Survey. https://pubs.usgs.gov/fs/2008/3049/

Biswas, S., & Ghosh, B. (2014). Chrysene. In *Encyclopedia of Toxicology* (pp. 959–962). Elsevier. https://doi.org/10.1016/B978-0-12-386454-3.00286-4

Bonisoli-Alquati, A. (2014). Avian genetic ecotoxicology: DNA of the canary in a coalmine. *Current Zoology*, *60*(2), 285–298. https://doi.org/10.1093/czoolo/60.2.285

Brunström, B., Broman, D., & Näf, C. (1991). Toxicity and EROD-inducing potency of 24 polycyclic aromatic hydrocarbons (PAHs) in chick embryos. *Archives of Toxicology*, *65*(6), 485–489. https://doi.org/10.1007/BF01977361

Chastel, O., Fort, J., Ackerman, J. T., Albert, C., Angelier, F., Basu, N., Blévin, P., Brault-Favrou, M., Bustnes, J. O., Bustamante, P., Danielsen, J., Descamps, S., Dietz, R., Erikstad, K. E., Eulaers, I., Ezhov, A., Fleishman, A. B., Gabrielsen, G. W., Gavrilo, M., … Yannic, G. (2022). Mercury contamination and potential health risks to Arctic seabirds and shorebirds. *Science of The Total Environment*, *844*, 156944. https://doi.org/10.1016/j.scitotenv.2022.156944

Chen, Q., Reisser, J., Cunsolo, S., Kwadijk, C., Kotterman, M., Proietti, M., Slat, B., Ferrari, F. F., Schwarz, A., Levivier, A., Yin, D., Hollert, H., & Koelmans, A. A. (2018). Pollutants in Plastics within the North Pacific Subtropical Gyre. *Environmental Science & Technology*, *52*(2), 446–456. https://doi.org/10.1021/acs.est.7b04682

Clark, D. P., & Pazdernik, N. J. (2016). Synthetic Biology. In *Biotechnology* (pp. 419–445). Elsevier. https://doi.org/10.1016/B978-0-12-385015-7.00013-2

Crump, D., Farhat, A., Chiu, S., Williams, K. L., Jones, S. P., & Langlois, V. S. (2016). Use of a Novel Double-Crested Cormorant ToxChip PCR Array and the EROD Assay to Determine Effects of Environmental Contaminants in Primary Hepatocytes. *Environmental Science & Technology*, *50*(6), 3265–3274. https://doi.org/10.1021/acs.est.5b06181

Crump, D., Williams, K. L., Chiu, S., Zhang, Y., & Martin, J. W. (2017). Athabasca Oil Sands Petcoke Extract Elicits Biochemical and Transcriptomic Effects in Avian Hepatocytes. *Environmental Science & Technology*, *51*(10), 5783–5792. https://doi.org/10.1021/acs.est.7b00767

Dawson, J., Pizzolato, L., Howell, S. E. L., Copland, L., & Johnston, M. E. (2018). Temporal and Spatial Patterns of Ship Traffic in the Canadian Arctic from 1990 to 2015. *ARCTIC*, *71*(1), Article 1. https://doi.org/10.14430/arctic4698

De Souza, C. V., & Corrêa, S. M. (2016). Polycyclic aromatic hydrocarbons in diesel emission, diesel fuel and lubricant oil. *Fuel*, *185*, 925–931. https://doi.org/10.1016/j.fuel.2016.08.054

Díez, C. M., Gaut, B. S., Meca, E., Scheinvar, E., Montes-Hernandez, S., Eguiarte, L. E., & Tenaillon, M. I. (2013). Genome size variation in wild and cultivated maize along altitudinal gradients. *New Phytologist*, *199*(1), 264–276. https://doi.org/10.1111/nph.12247

Duan, W., Achen, M. G., Richardson, S. J., Lawrence, M. C., Wettenhall, R. E. H., Jaworowski, A., & Schreiber, G. (1991). Isolation, characterization, cDNA cloning and gene expression of an avian transthyretin. *European Journal of Biochemistry*, *200*(3), 679–687. https://doi.org/10.1111/j.1432-1033.1991.tb16232.x

Egloff, C., Crump, D., Chiu, S., Manning, G., McLaren, K. K., Cassone, C. G., Letcher, R. J., Gauthier, L. T., & Kennedy, S. W. (2011). In vitro and in ovo effects of four brominated flame retardants on toxicity and hepatic mRNA expression in chicken embryos. *Toxicology Letters*, *207*(1), 25–33. https://doi.org/10.1016/j.toxlet.2011.08.015

Esler, D., Bowman, T., Trust, K., Ballachey, B., Dean, T., Jewett, S., & O'Clair, C. (2002). Harlequin duck population recovery following the "Exxon Valdez" oil spill: Progress, process and constraints. *Marine Ecology-Progress Series - MAR ECOL-PROGR SER*, *241*, 271–286. https://doi.org/10.3354/meps241271

Fernie, K. J., Marteinson, S. C., Chen, D., Palace, V., Peters, L., Soos, C., & Smits, J. E. G. (2019). Changes in thyroid function of nestling tree swallows (Tachycineta bicolor) in relation to polycyclic aromatic compounds and other environmental stressors in the Athabasca Oil Sands Region. *Environmental Research*, *169*, 464–475. https://doi.org/10.1016/j.envres.2018.11.031

Friocourt, M. P., Berthou, F., & Picart, D. (2008). Dibenzothiophene derivatives as organic markers of oil pollution†. *Toxicological & Environmental Chemistry*. https://doi.org/10.1080/02772248209356979

Gannon, M., Gilday, D., & Rifkind, A. B. (2000). TCDD induces CYP1A4 and CYP1A5 in chick liver and kidney and only CYP1A4, an enzyme lacking arachidonic acid epoxygenase activity, in myocardium and vascular endothelium. *Toxicology and Applied Pharmacology*, *164*(1), 24–37. https://doi.org/10.1006/taap.1999.8864

Gautier, D. L., Bird, K. J., Charpentier, R. R., Grantz, A., Houseknecht, D. W., Klett, T. R., Moore, T. E., Pitman, J. K., Schenk, C. J., Schuenemeyer, J. H., Sørensen, K., Tennyson, M. E., Valin, Z. C., & Wandrey, C. J. (2009). Assessment of Undiscovered Oil and Gas in the Arctic. *Science*, *324*(5931), 1175–1179. https://doi.org/10.1126/science.1169467

Gedalanga, P. B., Pornwongthong, P., Mora, R., Chiang, S.-Y. D., Baldwin, B., Ogles, D., & Mahendra, S. (2014). Identification of biomarker genes to predict biodegradation of 1,4-dioxane. *Applied and Environmental Microbiology*, *80*(10), 3209–3218. https://doi.org/10.1128/AEM.04162-13

Gibson, L. A., Lavoie, R. A., Bissegger, S., Campbell, L. M., & Langlois, V. S. (2014). A positive correlation between mercury and oxidative stress-related gene expression (GPX3 and GSTM3) is measured in female Double-crested Cormorant blood. *Ecotoxicology*, *23*(6), 1004–1014. https://doi.org/10.1007/s10646-014-1243-5

Glattke, T. J., Chacón-Patiño, M. L., Hoque, S. S., Ennis, T. E., Greason, S., Marshall, A. G., & Rodgers, R. P. (2022). Complex Mixture Analysis of Emerging Contaminants Generated from Coal Tar- and Petroleum-Derived Pavement Sealants: Molecular Compositions and Correlations with Toxicity Revealed by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *Environmental Science & Technology*, *56*(18), 12988–12998. https://doi.org/10.1021/acs.est.2c00582

Guyot, E., Chevallier, A., Barouki, R., & Coumoul, X. (2013). The AhR twist: Ligand-dependent AhR signaling and pharmaco-toxicological implications. *Drug Discovery Today*, *18*(9–10), 479486.https://doi.org/10.1016/j.drudis.2012.11.014

Harvey, H. R., Taylor, K. A., Pie, H. V., & Mitchelmore, C. L. (2014). Polycyclic aromatic and aliphatic hydrocarbons in Chukchi Sea biota and sediments and their toxicological response in the Arctic cod, Boreogadus saida. *Deep Sea Research Part II: Topical Studies in Oceanography*, *102*, 32–55. https://doi.org/10.1016/j.dsr2.2013.07.013

Hasanpur, K., Hosseinzadeh, S., Mirzaaghayi, A., & Alijani, S. (2022). Investigation of chicken housekeeping genes using next-generation sequencing data. *Frontiers in Genetics*, *13*, 827538. https://doi.org/10.3389/fgene.2022.827538

He, C., Zuo, Z., Shi, X., Sun, L., & Wang, C. (2012). Pyrene exposure influences the thyroid development of Sebastiscus marmoratus embryos. *Aquatic Toxicology (Amsterdam, Netherlands)*, *124–125*, 28–33. https://doi.org/10.1016/j.aquatox.2012.07.007

Hodson, P. V., Wallace, S. J., de Solla, S. R., Head, S. J., Hepditch, S. L. J., Parrott, J. L., Thomas, P. J., Berthiaume, A., & Langlois, V. S. (2020). Polycyclic aromatic compounds (PACs) in the Canadian environment: The challenges of ecological risk assessments. *Environmental Pollution*, *266*, 115–165. https://doi.org/10.1016/j.envpol.2020.115165

Honda, M., & Suzuki, N. (2020). Toxicities of Polycyclic Aromatic Hydrocarbons for Aquatic Animals. *International Journal of Environmental Research and Public Health*, *17*(4), 1363. https://doi.org/10.3390/ijerph17041363

Hylland, K. (2006). Polycyclic Aromatic Hydrocarbon (PAH) Ecotoxicology in Marine Ecosystems. *Journal of Toxicology and Environmental Health, Part A*, *69*(1–2), 109–123. https://doi.org/10.1080/15287390500259327

Idowu, I., Francisco, O., Thomas, P. J., Johnson, W., Marvin, C., Stetefeld, J., & Tomy, G. T. (2018). Validation of a simultaneous method for determining polycyclic Aromatic compounds and alkylated isomers in biota. *Rapid Communications in Mass Spectrometry: RCM*, *32*(3), 277–287. https://doi.org/10.1002/rcm.8035

Intergovernmental Panel on Climate Change (IPCC). (2022). The Ocean and Cryosphere in a Changing Climate: Special Report of the Intergovernmental Panel on Climate Change (1st ed.). Cambridge University Press. https://doi.org/10.1017/9781009157964

Jia, C., & Batterman, S. (2010). A Critical Review of Naphthalene Sources and Exposures Relevant to Indoor and Outdoor Air. *International Journal of Environmental Research and Public Health*, *7*(7), Article 7. https://doi.org/10.3390/ijerph7072903

Kerley-Hamilton, J. S., Trask, H. W., Ridley, C. J. A., DuFour, E., Lesseur, C., Ringelberg, C. S., Moodie, K. L., Shipman, S. L., Korc, M., Gui, J., Shworak, N. W., & Tomlinson, C. R. (2012). Inherent and Benzo[a]pyrene-Induced Differential Aryl Hydrocarbon Receptor Signaling Greatly Affects Life Span, Atherosclerosis, Cardiac Gene Expression, and Body and Heart Growth in Mice. *Toxicological Sciences*, *126*(2), 391–404. https://doi.org/10.1093/toxsci/kfs002

King, M. D., Elliott, J. E., & Williams, T. D. (2021). Effects of petroleum exposure on birds: A review. *Science of The Total Environment*, *755*, 142834. https://doi.org/10.1016/j.scitotenv.2020.142834

Kreitsberg, R., Nääb, L., Meitern, R., Carbillet, J., Fort, J., Giraudeau, M., & Sepp, T. (2023). The effect of environmental pollution on gene expression of seabirds: A review. *Marine Environmental Research*, *189*, 106067. https://doi.org/10.1016/j.marenvres.2023.106067

Lee, J.-S., Kim, E.-Y., & Iwata, H. (2009). Dioxin activation of CYP1A5 promoter/enhancer regions from two avian species, common cormorant (Phalacrocorax carbo) and chicken (Gallus gallus): Association with aryl hydrocarbon receptor 1 and 2 isoforms. *Toxicology and Applied Pharmacology*, *234*(1), 1–13. https://doi.org/10.1016/j.taap.2008.09.007

Lee, S., Hong, S., Liu, X., Kim, C., Jung, D., Yim, U. H., Shim, W. J., Khim, J. S., Giesy, J. P., & Choi, K. (2017). Endocrine disrupting potential of PAHs and their alkylated analogues associated with oil spills. *Environmental Science: Processes & Impacts*, *19*(9), 1117–1125. https://doi.org/10.1039/C7EM00125H

Li, G., Xiong, H., Saeed, K., Ma, R., Xing, Y., Bi, Y., Li, C., Huang, J., & Zhang, Y. (2020). Comparative toxicity analysis of corannulene and benzo[a]pyrene in mice. *Toxicology Letters*, *331*, 130–142. https://doi.org/10.1016/j.toxlet.2020.05.002

Ma, Y., Halsall, C. J., Xie, Z., Koetke, D., Mi, W., Ebinghaus, R., & Gao, G. (2017). Polycyclic aromatic hydrocarbons in ocean sediments from the North Pacific to the Arctic Ocean. *Environmental Pollution*, *227*, 498–504. https://doi.org/10.1016/j.envpol.2017.04.087

Marr, L. C., Kirchstetter, T. W., Harley, R. A., Miguel, A. H., Hering, S. V., & Hammond, S. K. (1999). Characterization of Polycyclic Aromatic Hydrocarbons in Motor Vehicle Fuels and Exhaust Emissions. *Environmental Science & Technology*, *33*(18), 3091–3099. https://doi.org/10.1021/es9812271

McCormick, S., Snawder, J. E., Chen, I.-C., Slone, J., Calafat, A. M., Wang, Y., Meng, L., Alexander-Scott, M., Breitenstein, M., Johnson, B., Meadows, J., & Fairfield Estill, C. (2022). Exposure assessment of polycyclic aromatic hydrocarbons in refined coal tar sealant applications. *International Journal of Hygiene and Environmental Health*, *242*, 113971. https://doi.org/10.1016/j.ijheh.2022.113971

Metre, P. C. V., Mahler, B. J., & Wilson, J. T. (2009). PAHs Underfoot: Contaminated Dust from Coal-Tar Sealcoated Pavement is Widespread in the United States. *Environmental Science & Technology*, *43*(1), 20–25. https://doi.org/10.1021/es802119h

Moradi, M., Hung, H., Li, J., Park, R., Shin, C., Alexandrou, N., Iqbal, M. A., Takhar, M., Chan, A., & Brook, J. R. (2022). Assessment of Alkylated and Unsubstituted Polycyclic Aromatic Hydrocarbons in Air in Urban and Semi-Urban Areas in Toronto, Canada. *Environmental Science & Technology*, *56*(5), 2959–2967. https://doi.org/10.1021/acs.est.1c04299

Muir, D. C. G., & de Wit, C. A. (2010). Trends of legacy and new persistent organic pollutants in the circumpolar arctic: Overview, conclusions, and recommendations. *Science of The Total Environment*, *408*(15), 3044–3051. https://doi.org/10.1016/j.scitotenv.2009.11.032

Mundy, L. J., Williams, K. L., Chiu, S., Pauli, B. D., & Crump, D. (2019). Extracts of Passive Samplers Deployed in Variably Contaminated Wetlands in the Athabasca Oil Sands Region Elicit Biochemical and Transcriptomic Effects in Avian Hepatocytes. *Environmental Science & Technology*, *53*(15), 9192–9202. https://doi.org/10.1021/acs.est.9b02066

Näf, C., Broman, D., & Brunström, B. (1992). Distribution and metabolism of polycyclic aromatic hydrocarbons (PAHs) injected into eggs of chicken (Gallus domesticus) and common eider duck (Somateria mollissima). Environmental Toxicology and Chemistry, 11(11), 1653–1660. https://doi.org/10.1002/etc.5620111114

Pereira, M. G., Walker, L. A., Wright, J., Best, J., & Shore, R. F. (2009). Concentrations of polycyclic aromatic hydrocarbons (PAHs) in the eggs of predatory birds in Britain. *Environmental science & technology*, *43*(23), 9010-9015.

Perez-Umphrey, A. A., Bergeon Burns, C. M., Stouffer, P. C., Woltmann, S., & Taylor, S. S. (2018). Polycyclic aromatic hydrocarbon exposure in seaside sparrows (Ammodramus maritimus) following the 2010 Deepwater Horizon oil spill. *Science of The Total Environment*, *630*, 1086–1094. https://doi.org/10.1016/j.scitotenv.2018.02.281

Piña, B., Casado, M., & Quirós, L. (2007). Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. *TrAC Trends in Analytical Chemistry*, *26*(11), 1145–1154. https://doi.org/10.1016/j.trac.2007.09.009

Porter, E., Crump, D., Egloff, C., Chiu, S., & Kennedy, S. W. (2014). Use of an avian hepatocyte assay and the avian Toxchip Polymerse chain reaction array for testing prioritization of 16 organic flame retardants. *Environmental Toxicology and Chemistry*, *33*(3), 573–582. https://doi.org/10.1002/etc.2469

Provencher, J. F., Thomas, P. J., Pauli, B., Braune, B. M., Franckowiak, R. P., Gendron, M., Savard, G., Sarma, S. N., Crump, D., Zahaby, Y., O'Brien, J., & Mallory, M. L. (2020). Polycyclic aromatic compounds (PACs) and trace elements in four marine bird species from northern Canada in a region of natural marine oil and gas seeps. *Science of The Total Environment*, *744*, 140959. https://doi.org/10.1016/j.scitotenv.2020.140959

Rantanen, M., Karpechko, A. Y., Lipponen, A., Nordling, K., Hyvärinen, O., Ruosteenoja, K., Vihma, T., & Laaksonen, A. (2022). The Arctic has warmed nearly four times faster than the globe since 1979. *Communications Earth & Environment*, *3*(1), Article 1. https://doi.org/10.1038/s43247-022-00498-3

Rhee, S.-K., Chang, J. H., Chang, Y. K., & Chang, H. N. (1998). Desulfurization of Dibenzothiophene and Diesel Oils by a Newly Isolated Gordona Strain, CYKS1. *Applied and Environmental Microbiology*, *64*(6), 2327–2331.

Shen, H., Huang, Y., Wang, R., Zhu, D., Li, W., Shen, G., Wang, B., Zhang, Y., Chen, Y., Lu, Y., Chen, H., Li, T., Sun, K., Li, B., Liu, W., Liu, J., & Tao, S. (2013). Global Atmospheric Emissions of Polycyclic Aromatic Hydrocarbons from 1960 to 2008 and Future Predictions. *Environmental Science & Technology*, *47*(12), 6415–6424. https://doi.org/10.1021/es400857z

Shi, X., Liu, C., Wu, G., & Zhou, B. (2009). Waterborne exposure to PFOS causes disruption of the hypothalamus–pituitary–thyroid axis in zebrafish larvae. *Chemosphere*, *77*(7), 1010–1018. https://doi.org/10.1016/j.chemosphere.2009.07.074

Shimada, T., & Fujii-Kuriyama, Y. (2004). Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and1B1. *Cancer Science*, *95*(1), 1–6. https://doi.org/10.1111/j.1349-7006.2004.tb03162.x

Su, G., Letcher, R. J., Crump, D., Farmahin, R., Giesy, J. P., & Kennedy, S. W. (2014). Photolytic Degradation Products of Two Highly Brominated Flame Retardants Cause Cytotoxicity and mRNA Expression Alterations in Chicken Embryonic Hepatocytes. *Environmental Science & Technology*, *48*(20), 12039–12046. https://doi.org/10.1021/es503399r

Troisi, G., Barton, S., & Bexton, S. (2016). Impacts of oil spills on seabirds: Unsustainable impacts of non-renewable energy. *International Journal of Hydrogen Energy*, *41*(37), 16549–16555. https://doi.org/10.1016/j.ijhydene.2016.04.011

Troisi, G. M., Bexton, S., & Robinson, I. (2006). Polyaromatic Hydrocarbon and PAH Metabolite Burdens in Oiled Common Guillemots (Uria aalge) Stranded on the East Coast of England (2001−2002). *Environmental Science & Technology*, *40*(24), 7938–7943. https://doi.org/10.1021/es0601787

Wiens, J. A. (2013). *Oil in the Environment: Legacies and Lessons of the Exxon Valdez Oil Spill*. Cambridge University Press.

Wnorowski, A., Harnish, D., Jiang, Y., Celo, V., Dabek-Zlotorzynska, E., & Charland, J.-P. (2022). Assessment and Characterization of Alkylated PAHs in Selected Sites across Canada. *Atmosphere*, *13*(8), Article 8. https://doi.org/10.3390/atmos13081320

Wong, F., Harner, T., Liu, Q.-T., & Diamond, M. L. (2004). Using experimental and forest soils to investigate the uptake of polycyclic aromatic hydrocarbons (PAHs) along an urban-rural gradient. *Environmental Pollution*, *129*(3), 387–398. https://doi.org/10.1016/j.envpol.2003.12.006

Xia, P., Crump, D., Chiu, S., Chan, H. M., & O'Brien, J. M. (2020). Toxicogenomic Assessment of Complex Chemical Signatures in Double-Crested Cormorant Embryos from Variably Contaminated Great Lakes Sites. *Environmental Science & Technology*, *54*(12), 7504–7512. https://doi.org/10.1021/acs.est.0c02725

Yadav, J., Kumar, A., & Mohan, R. (2020). Dramatic decline of Arctic sea ice linked to global warming. *Natural Hazards*, *103*(2), 2617–2621. https://doi.org/10.1007/s11069-020-04064-y

Zahaby, Y., Xia, P., Crump, D., Provencher, J. F., Thomas, P. J., Pauli, B., Braune, B. M., Franckowiak, R. P., Gendron, M., Savard, G., Sarma, S. N., Mallory, M. L., & O'Brien, J. (2021). ToxChip PCR Arrays for Two Arctic-Breeding Seabirds: Applications for Regional Environmental Assessments. *Environmental Science & Technology*, *55*(11), 7521–7530. https://doi.org/10.1021/acs.est.1c00229

Zhang, Y., Dong, S., Wang, H., Tao, S., & Kiyama, R. (2016). Biological impact of environmental polycyclic aromatic hydrocarbons (ePAHs) as endocrine disruptors. *Environmental Pollution*, *213*, 809–824. https://doi.org/10.1016/j.envpol.2016.03.050

Zuberogoitia, I., Martínez, J., Iraeta, A., Azkona, A., Zabala-Albizua, J., Jiménez, B., Merino, R., & Gómez, G. (2006). Short-term effects of the prestige oil spill on the Peregrine Falcon (Falco peregrinus). *Marine Pollution Bulletin*, *52*, 1176–1181. https://doi.org/10.1016/j.marpolbul.2006.02.016

4. Chapter 4: General Conclusions

4.1 Thesis Summary

New and legacy contaminants are continually entering the into Arctic environment. Persistent organic pollutants (POPs) tend to bioaccumulate and resist degradation. Exposure to POPs can result in a wide range of adverse effects in wildlife species including reduced breeding success and behavioural abnormalities (Letcher et al., 2010). Wildlife species in the Arctic are particularly vulnerable to POP exposure due to their long life spans and slow reproduction time, allowing for a longer bioaccumulation time range. The full effects of complex mixtures on wildlife are poorly understood and current methods of toxicity testing fail to account for species- and mixture-specific effects. Monitoring contaminants in wildlife is further complicated by the existence of multiple stressors, which can act in synergy (Folt et al., 1999). Using toxicogenomics to identify biological pathways altered following exposure to complex mixtures can increase our understanding of concentration- and species-specific endpoints (Xia et al., 2020; Zahaby et al., 2021). Seabirds can act as good bioindicator species for contaminant burdens due to their high trophic position and known migration and foraging habits (Furness & Camphuysen, 1997). In this project, we use two "case study" experiments to test the utility of a custom ToxChip PCR array developed for an Arctic seabird species (Black guillemot). Based on known contamination at multiple sites in the Arctic, I hypothesize that the chemistry profiles of birds collected from distinct areas and exposed, primarily, to two classes of contaminants (PCBs and PACs) will affect gene expression profiles in these birds and demonstrate contaminant exposure trends. Furthermore, I hypothesize that this gene expression evaluation tool can be used for contaminant monitoring in wildlife species.

I predict that:

(1) Gene expression and chemical profiles will be distinct among birds collected on a temporal (between collection years) and spatial (sub-sites) scale.

(2) Birds collected from a diesel spill site will have distinct chemistry and gene expression profiles compared to birds collected from a non-spill site in the same year.

In Chapter 2, the aim was to determine if the ToxChip tool could be used on archived samples to determine biological pathways most impacted by PCB exposure at variably contaminated sites. I investigated whether chemistry and gene expression profiles would differ between sub-sites or collection years and if gene expression profiles could be used to differentiate chemical burdens among variably contaminated sub-sites and pre-/post-remediation. Chemistry profiles were more distinct based on sub-site than on year. Samples from the most contaminated site (Beach) had the highest PCB burdens before and after the remediation project, while samples from the lowest concentrated sub-site (Reference) had the lowest PCB burdens. High levels of PCB contamination were found in several samples collected from the Reference site in 2007. This indicates that despite the sediment remediation project at the Beach site, there is still some PCB contamination present in wildlife at the site furthest from the source after the remediation project was complete. Differences in chemistry profiles were found on a temporal and spatial scale. Therefore, we employed a ToxChip PCR array analysis to determine if chemistry influenced gene expression trends. In contrast with the chemistry data, gene expression profiles were more distinct based on collection year than sub-site of sample origin. This distinction is likely due to degraded RNA quality for the samples collected from 1999 (before remediation).

In Chapter 3, the aim was to determine distinction in chemistry and gene expression profiles between birds collected after a diesel spill and birds collected from a non-spill site in the same year. I investigated whether birds from the spill site would have higher PAC burdens and an increased number of altered genes compared to birds collected from the non-spill site. I also explored if distinct chemistry profiles at both sites would allow for gene expression profile identification based on PAC source. Finally, I used a predictive model to test if chemical concentration could be identified based on gene expression. While diesel-related PACs influenced the spill site chemistry profiles, it did not have as much influence on sample categorization as expected. This likely indicates that birds from the non-spill site were exposed to diesel-related PACs as well, along with other PACs and sources. Gene expression profiles were distinct between the non-spill site and the spill site. Using PLS regression, we found that gene expression could be used to determine chemical concentration for several PACs.

4.2 Discussion of Results and Future Considerations

4.2.1 Overall hypothesis: Chemistry profiles will affect gene expression profiles and show contaminant exposure trends.

The goal of this project was to use two "case study" projects to validate the use of a transcriptomics tool to determine effects from contaminant exposure and facilitate monitoring in an Arctic avian species. In both experimental chapters, chemistry profiles followed predictable trends. For Chapter 2, the highest PCB burdens were found at the Beach (most contaminated) site, regardless of collection year. Gene expression profiles were more indicative of the sub-site where a sample was collected compared to collection year. Results of Chapter 3 showed that diesel-related PACs were important for the

identification of samples collected from the spill site. This chapter also demonstrated that the non-spill site had notable amounts of PAC contamination. We also found that predicting chemical concentration using gene expression was possible for certain genes, and some genes were more successful for predicting individual chemicals. Based on the results from Chapters 2 and 3, the ToxChip tool can be used to determine transcriptomic effects of contaminant exposure, using fresh tissue. Chapter 2 showed the limitations of using older liver tissue in gene expression analysis. Using capillary electrophoresis, we were able to determine that RNA integrity based on RIS values, particularly for samples collected in 1999, were sub-optimal for the amplification of qPCR gene targets. Chapter 3 proved that genes related to thyroid hormone pathways, oxidative stress and xenobiotic metabolism were altered the most following exposure to PACs.

4.2.2 Limitations of using older tissue samples and time constraints

Poor RNA quality was a significant contributor to the inability of the ToxChip to be used for samples from Chapter 2. Liver samples were used to extract RNA, which was then used on the ToxChip. RNA aliquots run on a capillary electrophoresis machine (QIAxcel) showed that some samples, particularly those collected in 1999, were degraded and therefore sub-optimal for qPCR amplification. Using a tissue storage reagent such as RNAlater could be employed in the future to ensure RNA stability and protection against degradation. To ascertain the storage length of tissue for subsequent qPCR, tissue aliquots could be stored at -80°C and tested every 6 months on a ToxChip. This would allow for the identification of degradation and help distinguish acceptable tissue storage time frames.

Due to the advances in analytical chemistry techniques in the early 2000s, inconsistencies arise when attempting to compare PCB concentrations from samples collected in different years. For Chapter 2, samples were collected 8 years apart and the analytical techniques used were more sensitive in 2007. It is likely that information was lost for samples collected in 1999 when techniques were less sensitive. For future studies, it would be worthwhile to ensure that the analytic method parameters remained the same for all collection years, regardless of advancements made in the time between collections. This might require selecting a smaller number of target congeners/analytes to ensure consistency among sampling years.

Pairing biomarker data with gene expression data would have contributed greatly to understanding the full scope of contaminant effects on birds following exposure. A study using the same cohort of nestlings found biomarker responses which were dose- and sex-dependent (Kuzyk et al., 2003). Namely, the study found enlarged livers in females, elevated EROD and reduced retinol and retinyl palmitate levels in nestlings from the most contaminated site. We could have used these biomarker data along with gene expression profiles to formulate trends and patterns relating to more apical adverse outcomes (e.g., whole organismal effects). This analysis was beyond the scope of this project but would have enhanced the investigation of contaminant effects in a significant way.

4.2.3 Recommendations to improve transcriptomics for contaminant monitoring

The need for invasive and lethal tissue sampling is a considerable constraint of the ToxChip in the context of this thesis. While the ToxChip reduces the number of animals used considerably compared to traditional toxicity testing, it nevertheless requires

euthanizing birds. Other researchers have used artificially incubated eggs as an alternative testing strategy that relies on vertebrate developmental stages that are considered an alternative to animal use and could represent a proxy for adult or nestling collection (Xia et al., 2020). Liver is the main detoxifying organ and can provide a snapshot in time of contaminant burden. While the ToxChip is currently calibrated for use with hepatic tissue, it is possible to consider non-lethal tissue selection, such as blood or preen oil, for future applications. Using non-lethal tissue for the ToxChip would allow for multi-year monitoring of the same cohort to track potential changes in exposure and effect metrics over time. While non-lethal tissue sampling or early-life stage testing should be considered for future ToxChip use, the use of whole birds was justified for this application. The dissections of birds collected for Chapter 3 were completed as part of a training program conducted at the Nunatsiavut Research Centre. This work is part of a broad collaborative partnership with the Nunatsiavut Government to ensure natural resources in their communities are safe to consume and protected from increasing economic development. Additionally, this work contributes to the increasing push to bridge traditional and scientific knowledge in order to enhance the future of contaminant research.

While the ToxChip is useful for fast and easily repeatable experiments, the number of genes included on the ToxChip limits the amount of information generated. While the size of the Black guillemot transcriptome is still unknown, the chicken genome contains approximately 20,000 to 23,000 genes (ICGSC, 2004) and is expected to be similar in size to the guillemots. The current ToxChip contains only 29 target genes (with 3 control genes) for Black guillemot. Therefore, we are no doubt missing out on

potentially impacted genes following exposure to contaminants. Shifting to newer technologies such as whole transcriptome analysis (RNASeq) would help ensure that important gene-contaminant interactions are not overlooked. Programs such as Seq2Fun (https://www.seq2fun.ca/; Liu et al., 2021) or the Annotation-free pipeline analysis option for FastBMD (https://www.fastbmd.ca/; Ewald et al. 2021) allow for the interpretation of whole transcriptome data for non-model species and thus, represent a means to address the challenge of poor annotation of wildlife species' transcriptomes. Another ToxChip is currently in development for the Common eider, a species vulnerable to contaminant exposure via ingestion through benthic feeding strategies. Recent work found high levels of PACs in Common eider eggs collected after the 2020 diesel spill in Postville (Smith et al., unpublished). This provides evidence that individual species metrics, such as feeding strategies and egg laying time, can impact effects of contaminant exposure. The ToxChip remains useful as a tool to be employed following contaminant exposure to attain gene expression profile information quickly, and this PCR array can be run by virtually any lab with a qPCR machine.

Transcriptomic tools such as the ToxChip can be useful when monitoring for specific classes of contaminant mixtures or following acute exposure to a known contaminant class. In most applications, tissues are sent for analysis of a small suite of contaminants. This means that the full extent of the contaminant burden remains unknown. For this reason, observed transcriptomic effects using this tool must be considered conservative and interpreted with caution when discussing chemical impacts outside of the suite that are measured.

4.3 Overall Conclusion Statements

The overall aim of this thesis was to understand chemistry and gene expression patterns in Black guillemots following exposure to contaminants. Another goal of this thesis was to determine if a transcriptomics tool (ToxChip) was suitable for contaminant monitoring in wildlife species. The first "case study" used archived nestling liver tissue to determine chemistry and gene expression profiles in birds collected on different spatial and temporal scales. Tissue chemistry was a powerful indicator of collection site for both experimental chapters, a finding that lends support to ongoing monitoring programs that utilize avian tissues and eggs for contaminant analysis. Gene expression profiles were useful in determining pathways impacted by contaminant exposure. We were able to determine chemical concentrations using gene expression with relative success for several chemicals, using several genes. These results can act as a catalyst for exploring whether gene expression can be used to determine contaminant burden concentrations without the need for chemical analysis in tissues.

Overall, the ToxChip proved that it has merit for contaminant monitoring practices, given the samples are not degraded. Future work could include the development of ToxChips for additional species of interest, refinement for non-lethal tissue sampling and collection of baseline gene expression data from relatively non-contaminated sites for future comparisons. This thesis provides a potential alternative to traditional toxicity testing and a new tool for contaminant monitoring in the Arctic.

4.4 References

Ewald, J., Soufan, O., Xia, J., & Basu, N. (2021). FastBMD: An online tool for rapid benchmark dose-response analysis of transcriptomics data. *Bioinformatics (Oxford, England)*, *37*(7), 1035–1036. https://doi.org/10.1093/bioinformatics/btaa700

Folt, C. L., Chen, C. Y., Moore, M. V., & Burnaford, J. (1999). Synergism and antagonism among multiple stressors. *Limnology and Oceanography*, *44*(3part2), 864–877. https://doi.org/10.4319/lo.1999.44.3_part_2.0864

Furness, R. W., & Camphuysen, K. (C. J.). (1997). Seabirds as monitors of the marine environment. *ICES Journal of Marine Science*, *54*(4), 726–737. https://doi.org/10.1006/jmsc.1997.0243

International Chicken Genome Sequencing Consortium. (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature*, *432*(7018), 695–716. https://doi.org/10.1038/nature03154

Kuzyk, Z. Z., Burgess, N., Stow, J., & Fox, G. (2003). Biological Effects of Marine PCB Contamination on Black Guillemot Nestlings at Saglek, Labrador: Liver Biomarkers. *Ecotoxicology (London, England)*, *12*, 183–197. https://doi.org/10.1023/A:1022550709962

Letcher, R. J., Bustnes, J. O., Dietz, R., Jenssen, B. M., Jørgensen, E. H., Sonne, C., Verreault, J., Vijayan, M. M., & Gabrielsen, G. W. (2010). Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish. *The Science of the Total Environment*, *408*(15), 2995–3043. https://doi.org/10.1016/j.scitotenv.2009.10.038

Liu, P., Ewald, J., Galvez, J. H., Head, J., Crump, D., Bourque, G., Basu, N., & Xia, J. (2021). Ultrafast functional profiling of RNA-seq data for nonmodel organisms. *Genome Research*, *31*(4), 713–720. https://doi.org/10.1101/gr.269894.120

Xia, P., Crump, D., Chiu, S., Chan, H. M., & O'Brien, J. M. (2020). Toxicogenomic Assessment of Complex Chemical Signatures in Double-Crested Cormorant Embryos from Variably Contaminated Great Lakes Sites. *Environmental Science & Technology*, *54*(12), 7504–7512. https://doi.org/10.1021/acs.est.0c02725

Zahaby, Y., Xia, P., Crump, D., Provencher, J. F., Thomas, P. J., Pauli, B., Braune, B. M., Franckowiak, R. P., Gendron, M., Savard, G., Sarma, S. N., Mallory, M. L., & O'Brien, J. (2021). ToxChip PCR Arrays for Two Arctic-Breeding Seabirds: Applications for Regional Environmental Assessments. *Environmental Science & Technology*, *55*(11), 7521–7530. https://doi.org/10.1021/acs.est.1c00229

Appendix

Table S1.1: List of genes on the Black guillemot PCR ToxChip array

Gene	Primary Pathway	Secondary Pathway
RGN	calcium homeostasis	cellular processes
IGFBP1	growth	
CYP1a5	xenobiotic metabolism	
ALAS1	xenobiotic metabolism	
IL16	immune function	
LBFABP	lipid homeostasis	
ACSL5	lipid homeostasis	
CYP3a7	xenobiotic metabolism	thyroid hormone pathway
TTR	thyroid hormone pathway	
THRSP	thyroid hormone pathway	
IGF1	thyroid hormone pathway	
UGT1a9	xenobiotic metabolism	
SULT1e1	xenobiotic metabolism	
CDKN1A	cellular processes	
POLK	DNA repair	
APOB	lipid homeostasis	
GPX3	phase I & II metabolism	
GSTM3	oxidative stress	
LSS	lipid homeostasis	
MGMT	DNA repair	DNA signaling
FOXa1	transcription	
PDK4	lipid homeostasis	metabolism
PAH	metabolism	
FRZB	cellular processes	
SLCO1a2	lipid homeostasis	
ALDH1a1	metabolism	
MT4	oxidative stress	
NCOA3	endocrine signaling	
MDM2	tumor regulation	translation
RPL4	structure (housekeeping)	
EEF1a1	signal transduction (housekeeping)	
NTC	No-template control	

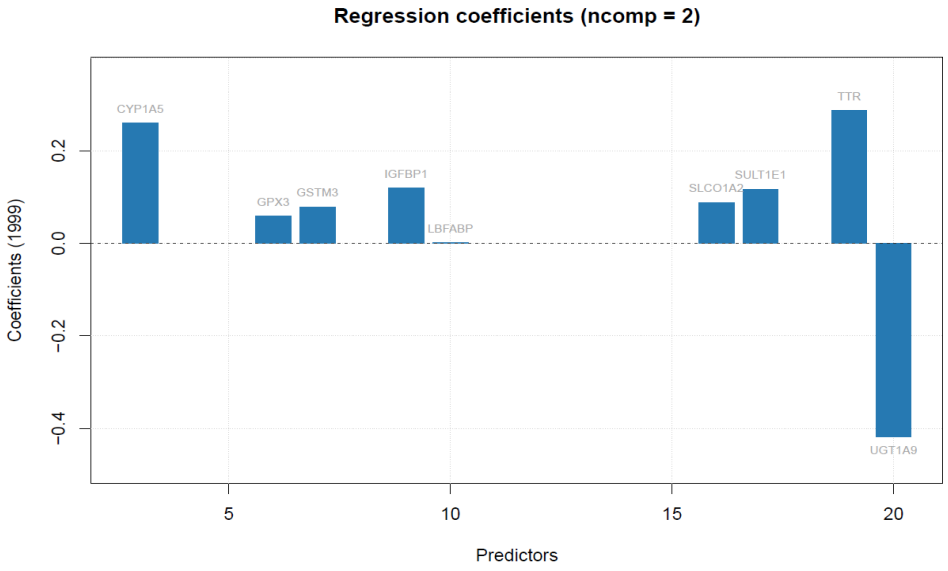


Figure S2.1: Regression coefficients for VIP genes used for sub-site prediction based on gene expression.

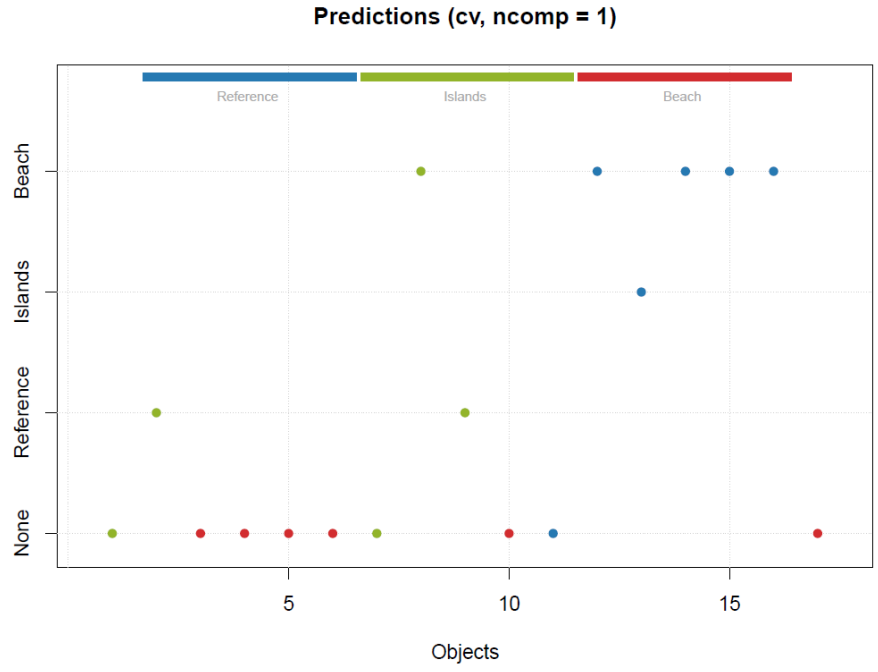


Figure S2.1: PLSDA shows predictions of sample collection site based on gene expression data for 1999. The algorithm was not able to predict any sites correctly for this year.

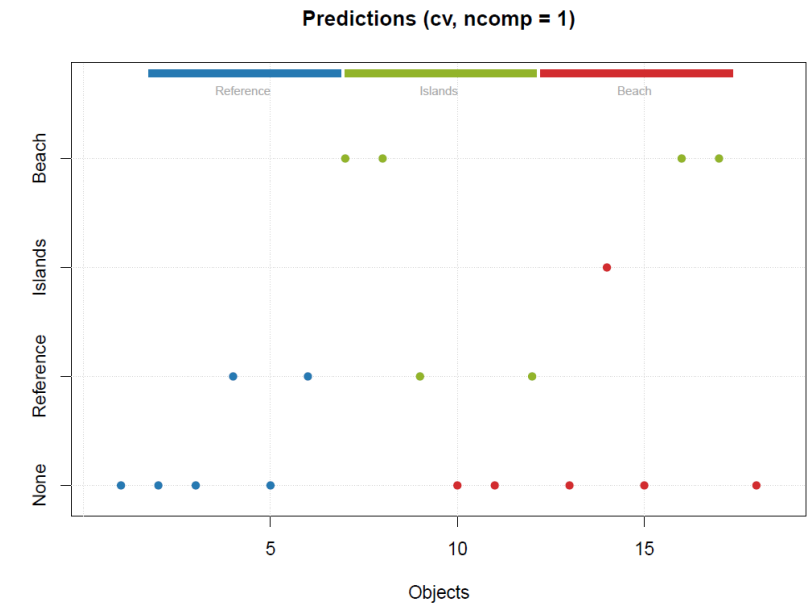


Figure S2.2: PLSDA shows predictions of sample collection site based on gene expression data for 2007. The algorithm was not able to predict any Beach or Island sites correctly for this year.

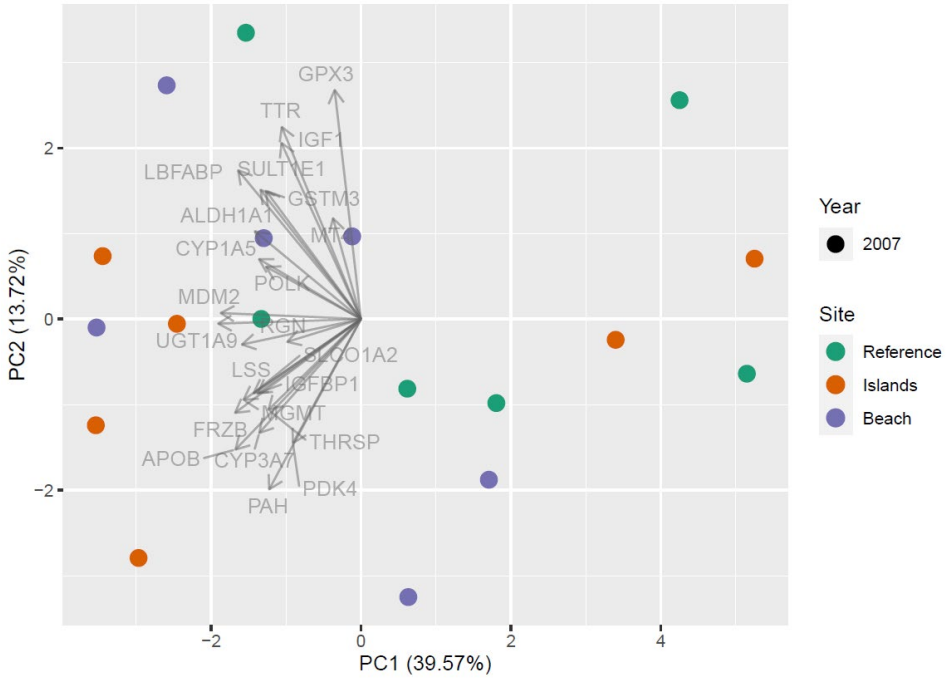


Table S2.1: RNA integrity Score (RIS) values for hepatic liver samples from Saglek, NL (Chapter 2). The RIS value was measured using a QIAxcel capillary electrophoresis machine. RIS values ≥ 5 are acceptable for qPCR amplification and indicate acceptable quality RNA. N/A values indicate the inability of the QIAxcel to calculate a RIS value for that sample.

Sample	Year	Site	RIS value
SGL_99_01	1999	Islands	4.9
SGL_99_03	1999	Islands	4.4
SGL_99_05	1999	Beach	4.5
SGL_99_06	1999	Beach	5.5
SGL_99_07	1999	Beach	N/A
SGL_99_08	1999	Beach	5.3
SGL_99_10	1999	Islands	3.1
SGL_99_11	1999	Islands	3.4
SGL_99_14	1999	Islands	3.0
SGL_99_17	1999	Beach	4.9
SGL_99_19	1999	Reference	3.9
SGL_99_22	1999	Reference	3.8
SGL_99_24	1999	Reference	N/A
SGL_99_25	1999	Reference	3.9
SGL_99_27	1999	Reference	5.3
SGL_99_28	1999	Reference	4.0
SGL_99_29	1999	Islands	6.7
SGL_99_30	1999	Beach	4.4
SGL_07_01	2007	Reference	7.5
SGL_07_02	2007	Reference	7.8
SGL_07_05	2007	Reference	7.1
SGL_07_06	2007	Reference	5.9
SGL_07_07	2007	Reference	7.0
SGL_07_08	2007	Reference	4.8
SGL_07_17	2007	Islands	8.1
SGL_07_18	2007	Islands	7.6
SGL_07_20	2007	Islands	7.4

SGL_07_21	2007	Beach	6.7
SGL_07_22	2007	Beach	7.6
SGL_07_27	2007	Islands	7.2
SGL_07_28	2007	Beach	7.0
SGL_07_29	2007	Beach	7.1
SGL_07_30	2007	Beach	7.5
SGL_07_33	2007	Islands	7.7
SGL_07_34	2007	Islands	7.5
SGL_07_35	2007	Beach	7.1

Table S2.2: Raw PCB chemistry data for samples collected from Saglek Bay, NL during A) 1999 and B) 2007. Only 9 co-eluting congeners were included in the analysis due to an abundance of non-detects in many of the samples. Geometric means and ranges of all PCB congeners measured has been reported by Brown et al., 2009.

A)

Sample	Group	Sample Size	Units	PCB 153	PCB 180	PCB 138/163/164	PCB 187/182	PCB 170/190	ΣPCB
SGL_99_05	Beach	1.03 g (wet)	ng/g (wet weight)	8.80	10.00	11.00	8.90	13.00	51.70
SGL_99_06	Beach	1.01 g (wet)	ng/g (wet weight)	7.90	6.40	9.50	5.60	8.10	37.50
SGL_99_07	Beach	1.09 g (wet)	ng/g (wet weight)	12.00	10.00	15.00	9.00	13.00	59.00
SGL_99_08	Beach	1.09 g (wet)	ng/g (wet weight)	6.90	9.00	8.20	8.10	11.00	43.20
SGL_99_17	Beach	1.18 g (wet)	ng/g (wet weight)	8.78	9.47	10.56	8.53	11.99	49.33
SGL_99_30	Beach	1.08 g (wet)	ng/g (wet weight)	8.20	8.01	9.86	7.21	10.14	43.42
SGL_99_10	Islands	1.03 g (wet)	ng/g (wet weight)	0.46	0.38	0.55	0.34	0.50	2.22
SGL_99_11	Islands	0.991 g (wet)	ng/g (wet weight)	0.52	0.44	0.62	0.39	0.57	2.53
SGL_99_14	Islands	1.01 g (wet)	ng/g (wet weight)	0.48	0.45	0.57	0.40	0.59	2.49
SGL_99_01	Islands	1.01 g (wet)	ng/g (wet weight)	1.60	1.90	1.90	1.60	2.40	9.40
SGL_99_03	Islands	1.31 g (wet)	ng/g (wet weight)	1.60	1.70	1.90	1.50	2.10	8.80
SGL_99_29	Islands	1.17 g (wet)	ng/g (wet weight)	1.50	1.40	1.80	1.20	1.80	7.70
SGL_99_19	Reference	1.13 g (wet)	ng/g (wet weight)	0.54	0.66	0.66	0.57	0.84	3.27
SGL_99_22	Reference	1.16 g (wet)	ng/g (wet weight)	0.77	0.81	0.94	0.69	1.00	4.21
SGL_99_24	Reference	1.07 g (wet)	ng/g (wet weight)	0.97	1.50	1.20	1.40	2.00	7.07
SGL_99_25	Reference	1.25 g (wet)	ng/g (wet weight)	0.50	0.60	0.61	0.52	0.77	3.00
SGL_99_27	Reference	1.11 g (wet)	ng/g (wet weight)	1.00	1.80	1.30	1.70	2.50	8.30
SGL_99_28	Reference	1.13 g (wet)	ng/g (wet weight)	0.35	0.48	0.41	0.42	0.62	2.28

B)

Sample	Group	Sample Size	Units	PCB 153	PCB 180	PCB 138/163/164	PCB 182/187	PCB 170/190	ΣPCB
SGL_07_21	Beach	4.99 g (wet)	ng/g (wet weight)	92.60	89.10	65.50	43.90	37.70	328.80
SGL_07_22	Beach	3.57 g (wet)	ng/g (wet weight)	194.00	168.00	133.00	83.50	76.20	654.70
SGL_07_28	Beach	5.06 g (wet)	ng/g (wet weight)	11.10	7.47	7.81	4.85	3.66	34.89
SGL_07_29	Beach	5.08 g (wet)	ng/g (wet weight)	43.60	33.00	29.50	15.80	14.30	136.20
SGL_07_30	Beach	5.01 g (wet)	ng/g (wet weight)	12.00	8.54	8.30	4.97	3.85	37.66
SGL_07_35	Beach	5.02 g (wet)	ng/g (wet weight)	166.00	131.00	116.00	85.50	56.80	555.30
SGL_07_17	Islands	4.99 g (wet)	ng/g (wet weight)	3.57	2.87	2.48	1.49	1.22	11.63
SGL_07_18	Islands	5.10 g (wet)	ng/g (wet weight)	4.67	3.29	3.11	2.03	1.54	14.64
SGL_07_20	Islands	5.01 g (wet)	ng/g (wet weight)	11.70	15.40	7.13	6.78	6.34	47.35
SGL_07_27	Islands	5.01 g (wet)	ng/g (wet weight)	4.54	3.06	2.69	1.20	1.20	12.69
SGL_07_33	Islands	4.58 g (wet)	ng/g (wet weight)	3.00	1.64	2.05	1.58	0.69	8.96
SGL_07_34	Islands	4.99 g (wet)	ng/g (wet weight)	2.52	1.56	1.69	1.41	0.69	7.87
SGL_07_01	Reference	5.22 g (wet)	ng/g (wet weight)	0.92	0.38	0.60	0.34	0.15	2.39
SGL_07_02	Reference	5.08 g (wet)	ng/g (wet weight)	0.92	0.43	0.66	0.48	0.22	2.71
SGL_07_05	Reference	4.08 g (wet)	ng/g (wet weight)	0.59	0.29	0.41	0.24	0.13	1.66
SGL_07_06	Reference	5.07 g (wet)	ng/g (wet weight)	1.85	0.80	1.35	0.85	0.34	5.19
SGL_07_07	Reference	5.06 g (wet)	ng/g (wet weight)	1.25	0.61	0.98	0.66	0.24	3.74
SGL_07_08	Reference	5.00 g (wet)	ng/g (wet weight)	0.90	0.38	0.61	0.36	0.16	2.41

Chapter 3

Table S3.1: RNA integrity Score (RIS) values for hepatic liver samples from Nain & Postville, NL (Chapter 3). The RIS value was measured using a QIAxcel capillary electrophoresis machine. RIS values ≥ 5 are acceptable for qPCR amplification and indicate acceptable quality RNA.

Sample	Year	Site	RIS value
NG_20_01	2020	Nain, NL	6.8
NG_20_02	2020	Nain, NL	6.9
NG_20_03	2020	Nain, NL	6.0
NG_20_04	2020	Nain, NL	7.5
NG_20_05	2020	Nain, NL	6.1
NG_20_06	2020	Nain, NL	7.2
NG_20_07	2020	Nain, NL	6.3
NG_20_10	2020	Nain, NL	5.0
NG_20_11	2020	Nain, NL	6.4
NG_20_12	2020	Nain, NL	7.1
NG_20_20	2020	Nain, NL	6.0
NG_20_21	2020	Nain, NL	5.5
NG_20_24	2020	Nain, NL	6.7
NG_20_25	2020	Nain, NL	7.3
PG_20_07	2020	Postville, NL	6.1

PG_20_08	2020	Postville, NL	7.3
PG_20_09	2020	Postville, NL	6.7
PG_20_10	2020	Postville, NL	7.5
PG_20_11	2020	Postville, NL	6.4
PG_20_12	2020	Postville, NL	6.0
PG_20_13	2020	Postville, NL	7.2
PG_20_14	2020	Postville, NL	7.1
PG_20_15	2020	Postville, NL	6.7
PG_20_19	2020	Postville, NL	6.7
PG_20_20	2020	Postville, NL	5.9
PG_20_21	2020	Postville, NL	6.1
PG_20_23	2020	Postville, NL	6.7
PG_20_24	2020	Postville, NL	6.1

Table S3.2: Raw PAC chemistry data for samples collected from Nain and Postville in 2020.

Units		Limit of Detection	Bird ID		22-0755	22-0513	22-0753	22-0752	22-0758	22-0489	22-0496	22-0490	22-0754	22-0514	22-0512	22-0491	22-0511	22-0497
			NWRC ID		W222324-01 B	W221122-01 B	W222322-01 B	W222321-01 B	W222327-01 B	W221098-01 B	221099-01	W222323-01 B	W221123-01 B	W221121-01 B	W221100-01 B	W221120-01 B	W221106-01 B	
			OG ID		N-G-01-20	N-G-02-20	N-G-03-20	N-G-04-20	N-G-05-20	N-G-06-20	N-G-07-20	N-G-10-20	N-G-11-20	N-G-12-20	N-G-20-20	N-G-21-20	N-G-24-20	N-G-25-20
			Species		BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU
			Year		2020	2020	2020	2020	2020	2020	2020	2020	2020	2020	2020	2020	2020	2020
			Location		Nain	Nain	Nain	Nain	Nain	Nain	Nain	Nain	Nain	Nain	Nain	Nain	Nain	Nain
ng/g (lipid weight)	0.1348		Acenapthene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.1128		Acenapthylene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0868		Anthracene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.1180		Benz[a]anthracene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.1098		Benzo[a]pyrene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0591		Benzo[b]fluoranthene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0654		Benzo[g,h,i]perylene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0878		Benzo[k]fluoranthene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.1581		Chrysene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0917		Dibenz[a,h]anthracene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.1050		Fluoranthene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0696		Fluorene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.45	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.2266		Indeno[1,2,3-c,d]pyrene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0555		Naphthalene		6.19	0.00	0.00	6.51	8.33	40.41	10.48	20.15	14.22	0.00	2.22	10.91	0.00	3.80
ng/g (lipid weight)	0.1521		Phenanthrene		0.00	0.00	0.00	0.00	0.00	9.71	3.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0907		Pyrene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0071		C1 Benzo[a]pyrene		1.14	0.00	0.00	0.00	1.24	0.00	0.00	0.00	1.68	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0027		C1 Dibenzothiophene		0.00	0.23	0.00	0.00	0.00	1.07	0.41	0.35	0.00	0.00	0.00	0.86	0.00	0.00
ng/g (lipid weight)	0.0096		C1 Fluorene		0.00	3.12	0.00	1.16	1.05	17.44	0.43	4.13	1.65	5.17	5.84	7.03	5.43	9.97
ng/g (lipid weight)	0.0064		C1 Pyrene		0.00	0.00	0.21	0.26	0.42	0.00	0.33	0.62	0.56	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0071		C2 Benzo[a]pyrene		62.74	6.82	28.58	46.34	13.33	4.95	6.57	6.11	8.50	10.00	9.26	0.00	8.53	4.72
ng/g (lipid weight)	0.0104		C2 Dibenzothiophene		0.00	0.27	0.00	0.00	0.00	1.40	0.64	0.00	0.00	0.00	0.42	0.00	0.00	0.00
ng/g (lipid weight)	0.0096		C2 Fluorene		0.00	0.57	0.00	0.72	0.00	12.82	2.44	2.25	1.48	0.00	3.55	4.25	0.00	3.82
ng/g (lipid weight)	0.0349		C2 Pyrene		0.00	0.00	0.00	0.00	0.00	1.23	2.15	0.00	0.00	0.00	0.00	0.00	0.00	1.16
ng/g (lipid weight)	0.0054		C3 Dibenzothiophene		0.00	0.44	0.00	0.00	0.62	2.53	2.34	0.00	0.00	0.49	0.66	1.78	0.36	1.50
ng/g (lipid weight)	0.0096		C3 Fluorene		0.00	3.37	0.00	0.00	0.00	18.28	11.94	0.00	1.21	3.45	0.00	2.52	5.73	8.84
ng/g (lipid weight)	0.0349		C3 Pyrene		0.00	1.05	0.00	0.00	2.37	5.33	4.70	1.73	0.00	0.00	0.00	0.00	6.34	1.56
ng/g (lipid weight)	0.0054		C4 Dibenzothiophene		0.00	0.68	0.00	0.00	1.02	3.97	2.40	0.30	0.00	0.92	0.80	2.02	0.36	1.56
ng/g (lipid weight)	No data		C4 Fluorene		0.00	0.77	0.00	0.00	0.00	36.87	26.12	0.00	0.00	0.00	3.91	11.48	0.00	27.37
ng/g (lipid weight)	0.0349		C4 Pyrene		0.00	1.45	0.00	0.00	1.42	11.71	4.05	8.41	0.00	2.27	4.53	3.35	1.39	6.71
ng/g (lipid weight)	0.0558		Dibenzothiophene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0106	1,7-Dimethylphenanthrene		0.00	0.00	0.00	0.00	0.00	0.00	0.73	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0136	1,8-Dimethylphenanthrene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0114	1-Methylnaphthalene		0.00	1.38	0.00	0.38	0.00	1.40	16.77	4.65	6.94	0.00	3.68	3.03	5.27	5.80	1.21
ng/g (lipid weight)	0.0064	1-Methylphenanthrene		0.00	0.00	0.00	0.00	0.00	1.02	0.21	0.45	0.00	0.00	0.00	0.52	0.33	0.15	0.24
ng/g (lipid weight)	0.0113	2,6-Dimethylphenanthrene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0114	2-Methylnaphthalene		0.00	2.37	0.00	0.51	2.86	0.59	9.12	13.36	0.00	6.42	5.24	10.15	10.38	2.35
ng/g (lipid weight)	0.0066	2-Methylphenanthrene		0.00	0.17	0.00	0.00	0.18	0.00	0.00	32.99	2.19	0.59	1.04	0.00	0.26	1.12	0.53	0.44
ng/g (lipid weight)	0.0147	3,6-Dimethylphenanthrene		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ng/g (lipid weight)	0.0067	3-Methylphenanthrene		0.00	0.11	0.00	0.00	0.00	1.55	0.38	0.72	0.00	0.00	0.00	0.18	0.73	0.34	0.31
ng/g (lipid weight)	0.0059	9/4-Methylphenanthrene		0.00	0.13	0.00	0.00	0.00	1.33	0.31	0.69	0.00	0.00	0.00	0.21	0.82	0.38	0.24
ng/g (lipid weight)	0.0078	C1 Chrysene		0.00	0.70	0.00	0.96	0.28	5.10	1.52	4.57	0.85	0.00	1.91	2.84	2.80	0.48	2.44
ng/g (lipid weight)	0.0019	C2 Chrysene		0.00	0.27	0.13	0.29	0.26	1.61	0.20	0.69	0.15	0.42	0.36	0.70	0.48	0.68
ng/g (lipid weight)	0.0099	C2 Naphthalene		0.00	3.83	0.00	0.00	3.11	41.16	14.71	19.90	0.42	8.99	8.08	15.96	15.71	12.30
ng/g (lipid weight)	0.0130	C2 Phenanthrene		0.00	0.74	0.00	0.00	0.53	3.32	1.70	3.27	0.00	1.85	1.14	1.74	1.21	1.58
ng/g (lipid weight)	0.0019	C3 Chrysene		1.05	0.00	0.36	6.04	0.00	1.20	1.55	0.78	1.53	0.18	0.07	0.84	0.22	1.36
ng/g (lipid weight)	0.0089	C3 Naphthalene		0.00	5.81	0.31	0.00	1.80	45.68	15.14	29.53	1.04	14.22	11.82	22.10	21.56	15.52
ng/g (lipid weight)	0.0202	C3 Phenanthrene		0.00	0.89	0.00	0.00	1.00	7.06	2.52	4.20	0.00	1.97	1.64	3.69	2.78	2.53
ng/g (lipid weight)	0.0019	C4 Chrysene		3.79	0.00	0.37	2.47	2.08	0.00	1.65	0.00	0.70	0.00	0.00	1.14	0.12	0.88
ng/g (lipid weight)	0.0078	C4 Naphthalene (Butyl)		0.00	4.09	0.00	0.48	2.38	25.04	9.73	13.63	0.99	9.18	8.69	11.47	13.28	9.57
ng/g (lipid weight)	0.0278	C4 Phenanthrene		0.00	2.10	0.00	0.00	2.52	18.71	10.16	9.77	0.00	6.09	6.27	12.43	10.48	11.27
	No data	Retene		0.00	0.10	0.00	0.00	0.15	1.09	0.26	0.71	0.03	0.63	0.25	0.59	0.56	0.26
		ΣPAC		74.91	41.45	29.97	66.13	49.33	374.23	152.60	156.72	35.00	75.93	79.92	136.73	108.76	138.87
		ΣPriorityPAHs		6.19	0.00	0.00	6.51	8.33	50.13	13.70	22.60	14.22	0.00	2.22	10.91	0.00	3.80

22-0480	22-0744	22-0743	22-0484	22-0475	22-0470	22-0481	22-0741	22-0485	22-0763	22-0767	22-0766	22-0765	22-0764
W221089-01 B	W222313-01 B	W222312-01 B	W221093-01 B	W221084-01 B	W221079-01 B	W221090-01 B	W222310-01 B	W221094-01 B	W222332-01 B	W222336-01 B	W222335-01 B	W222334-01 B	W222333-01 B
P-G-07-20	P-G-08-20	P-G-09-20	P-G-10-20	P-G-11-20	P-G-12-20	P-G-13-20	P-G-14-20	P-G-15-20	P-G-19-20	P-G-20-20	P-G-21-20	P-G-23-20	P-G-24-20
BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU	BLGU
2020	2020	2020	2020	2020	2020	2020	2020	2020	2020	2020	2020	2020	2020
Postville	Postville	Postville	Postville	Postville	Postville	Postville	Postville	Postville	Postville	Postville	Postville	Postville	Postville
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
13.11	6.30	0.00	32.51	14.03	49.89	24.07	37.85	33.54	52.80	34.11	8.32	21.28	29.72
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.31	2.78	0.59	0.00	0.74	0.00	0.85	2.06	0.00	3.93	1.00	0.00	0.92	1.00
0.40	0.36	0.08	0.51	0.00	0.00	0.19	0.62	0.29	0.00	0.00	0.09	0.09	0.09
8.79	1.73	0.00	8.54	4.95	5.35	10.21	0.51	4.36	0.00	1.01	0.55	1.82	0.36
1.50	1.99	0.00	0.52	2.00	1.68	2.33	0.49	0.17	0.00	0.00	0.00	0.00	0.00
17.12	11.08	0.00	1.88	6.18	5.57	14.87	10.45	2.67	56.22	0.74	5.10	6.85	4.90
2.08	0.47	0.48	1.39	0.00	1.38	1.35	1.85	0.96	0.00	0.00	0.00	0.00	0.00
6.76	0.90	0.00	4.53	2.65	5.59	8.18	1.82	3.19	0.00	0.00	0.86	1.00	0.00
1.73	2.22	0.00	0.69	2.20	1.98	3.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3.81	1.47	0.23	1.26	1.75	1.90	3.19	1.59	0.99	0.00	0.00	0.00	0.00	0.00
23.19	2.20	0.00	10.60	13.39	9.29	23.41	2.43	5.26	0.00	1.46	0.64	1.12	0.00
7.54	4.81	1.01	2.03	7.28	3.10	8.30	5.07	2.16	0.00	0.00	0.00	1.40	0.00
3.68	1.14	0.00	1.75	0.95	1.02	2.98	1.02	0.98	0.00	0.00	0.00	0.00	0.00
11.59	0.00	0.00	20.86	10.11	13.81	20.06	0.00	18.59	0.00	0.00	0.00	0.00	0.00
3.33	2.31	0.00	10.50	5.11	5.75	7.32	0.00	4.41	0.00	0.00	0.00	2.48	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.55	2.00	0.28	9.15	0.62	2.02	6.08	4.19	10.82	1.70	2.06	0.00	1.52	2.13
0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.38	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.99	4.13	0.54	17.02	0.89	2.80	9.04	8.62	19.14	2.82	3.66	0.35	3.24	4.04
0.00	0.42	0.00	1.21	0.00	0.38	0.22	0.53	0.78	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.82	0.00	0.45	0.26	0.42	0.60	0.00	0.00	0.00	0.00	0.00
0.00	0.21	0.00	0.70	0.00	0.15	0.00	0.00	0.56	0.00	0.00	0.00	0.00	0.00
3.35	0.00	0.00	3.29	1.10	1.76	2.50	0.00	2.31	0.00	0.52	0.00	1.15	0.00
0.41	0.00	0.05	0.78	0.31	0.41	0.28	0.00	0.33	0.00	0.12	0.00	0.22	0.19
3.16	3.67	0.48	21.90	1.26	2.63	12.11	8.36	25.21	2.48	2.10	0.00	2.08	2.32
0.99	0.94	0.00	2.32	0.00	1.74	1.04	0.92	2.12	0.00	0.00	0.00	0.00	0.33
0.00	0.00	0.00	0.68	0.00	0.00	0.00	0.00	0.40	0.91	0.00	0.00	0.00	0.00
6.91	3.03	0.45	20.31	2.71	2.93	10.18	4.50	19.02	0.00	1.22	0.60	1.62	1.37
0.00	1.27	0.00	2.85	2.57	0.00	5.26	0.00	2.65	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	1.48	0.24	0.00	0.00	0.00	0.68	0.00	0.00	1.60	0.00	0.18
4.13	1.92	0.24	11.67	2.55	3.22	5.13	1.83	8.63	0.00	0.81	0.00	1.46	0.53
3.15	0.00	0.00	7.96	1.60	1.83	3.29	0.00	7.02	0.00	0.00	0.00	0.00	0.00
0.36	0.18	0.00	0.42	0.07	0.05	0.30	0.05	0.44	0.00	0.00	0.00	0.05	0.02
131.97	57.52	4.44	200.63	85.26	126.69	186.16	95.16	179.00	120.87	48.81	18.12	48.29	47.18
13.11	6.30	0.00	32.51	14.03	49.89	24.07	37.85	33.54	52.80	34.11	8.32	21.28	29.72