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Immunomodulation by metals and environmental pollutants in seabirds breeding on Svalbard

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Environmental Toxicology and Chemistry
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Abstract

This master thesis investigated the levels of arsenic, cadmium, chromium, copper, mercury, lead, selenium and zinc in plasma and feathers of black-legged kittiwakes (*Rissa tridcatyla*) (n = 17), as well as feathers of Brünnich's guillemots (*Uria lomvia*) (n = 13) sampled in July 2017. Secondly, this thesis aimed to determine the immunological impact pollutants may be having in the kittiwake. Samples were collected from adult birds during chick rearing period at breeding colonies (Blomstrandhalvøya and Ossian-Sarsfjellet) located in Kongsfjorden, Svalbard. Of the non-essential metals, arsenic was the most concentrated metal in plasma of kittiwakes, while mercury was the most concentrated in the feathers of kittiwakes and guillemots. Kittiwakes had significantly higher concentrations of arsenic, cadmium and mercury than guillemots, while guillemots had significantly higher concentrations of copper, lead and zinc. Immunological analysis was conducted on plasma sampled from kittiwakes in Svalbard in 2014 and 2015 (n = 17). The kittiwakes from 2014, 2015 and 2017 were all screened for avian influenza virus, and n = 4 of the kittiwakes, all sampled in 2015, were infected. microRNAs are small non-coding RNAs that have been found to be implicated in the development and function of the immune system at an increasing level. Variation in expression of a specific microRNA, miR-155, was predicted by linear models. One linear model, including both years, predicted that variation could be explained by sex of the birds (n = 5 males, n = 12 females), avian influenza virus infection of the birds (n = 4 infected, n = 13 non-infected), levels of p,p'-DDE and levels of sumPCBs. A second model, including only 2015 kittiwakes (n = 10), predicted that variation in miR-155 expression could be explained by avian influenza virus infection of the birds and levels of Se, Pb and As. Both models indicate that environmental pollution might be implicated with immunomodulation and disease prevalence in the kittiwakes. The difference between sexes indicate that this effect could be sex dependent, which needs to be investigated further. To the knowledge of the author, this is the first study to observe modulation of miR-155 by pollutants in wild animals, and it would be interesting to do further research on this microRNA with other pollutants and wild species.

Sammendrag

Denne masteroppgaven undersøkte nivå av arsenikk, kadmium, krom, kobber, kvikksølv, bly, selen og zink i plasma og fjær hos krykkje (*Rissa tridactyla*) (n =17), samt fjær hos polarlomvi (*Uria lomvia*) (n = 13). I tillegg siktet denne masteroppgaven mot å fastslå den immunologiske påvirkningen miljøgifter kan ha på krykkjene. Det ble samlet inn prøver fra voksne fugler i løpet av hekkesesongen ved kolonier (Blomstrandhalvøya og Ossian-Sarsfjellet) lokalisert i Kongsfjorden, Svalbard. Av de ikke-essensielle metallene var arsenikk det mest konsentrerte metallet i plasma hos krykkje, mens kvikksølv var det mest konsentrerte metallet i fjærene til krykkje og polarlomvi. Krykkje hadde signifikant høyere fjærkonsentrasjoner av arsenikk, kadmium og kvikksølv sammenlignet med polarlmovi, mens polarlomvi hadde signifikant høyere fjærkonsentrasjoner av kobber, bly og zink. Immunologiske analyser ble utført på plasma samlet inn fra krykkje på Svalbard i 2014 (n = 7) og 2015 (n = 10). Krykkje fra 2014, 2015 og 2017 ble alle undersøkt for fugleinfluensavirus, og n = 4, alle fra 2015, fikk påvist viruset. Uttrykk av et spesifikt mikroRNA, miR-155, ble prediktert ved hjelp av lineære modeller. mikroRNA er små, ikke-kodende RNA som i økende grad har vist seg å være involvert i utvikling og funksjon av immunsystemet. En lineær modell, som inkluderte begge årene, predikterte at variasjon i miR-155 uttrykk kunne forklares av fuglenes kjønn (n = 5 hanner, n = 12 hunner), infeksjon av fugleinfluensavirus (n = 4 infisert, n = 13 ikke-infisert), nivå av p,p'-DDE og nivå av PCB. En annen lineær modell, som bare inkluderte krykkjer fra 2015, predikerte at variasjon i miR-155 uttrykk kunne forklares av infeksjon av fugleinfluensa hos fuglene samt nivå av metallene selen, bly og arsenikk. Begge modellene indikerer at miljøgifter kan være involvert i modulering av immunforsvaret og utbredelse av sykdom hos krykkje. Forskjellen mellom kjønnene indikerer at denne effekten kan være kjønnsavhengig, noe som må undersøkes videre.

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Silje Strand Lundgren

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Abbreviations

Ab	Antibody
AIV	Avian influenza virus
ALAD	δ-aminolevulinic acid dehydratase
AMAP	Arctic Monitoring and Assessment Programme
As	Arsenic
BIC	B-cell Integration Cluster
Cd	Cadmium
cDNA	Complementary deoxyribonucleic acid
Cq	Quantitative cycle
Cr	Chromium
Cu	Copper
ELISA	Enzyme-linked immunosorbent assay
DDE	[ethylene, 1,1-dichloro-2,2-bis(p-chloropheynyl)]
DDT	1,1,1-trichloro-2,2-bis(p-chlorphenyl)ethane
GCF	Genomics Core Facility
H1N1	Strain of Avian Influenza Virus
HAH	Halogenated aromatic hydrocarbons
HCB	Hexachlorobenzene
Hg	Mercury
HNO_3	Nitric acid
ICP-MS	Inductively coupled plasma mass spectrometry
IDL	Instrumental detection limit
Ig	Immunoglobulin
IFN	Interferon
IKK	I Kappa B alpha kinase
IUCN	International Union for Conservation of Nature
LOD	Limit of detection
MCMC	Markov Chain Monte Carlo
MeHg	Methylmercury
NK	Natural killer
NF_kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Pb	Lead
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxins
Poly I:C	Polyinosinic:polycytidylic acid
POP	Persistent Organic Pollutant
qPCR	Quantitative polymerase chain reaction
RBCs	Red blood cells
RNA	Ribonucleic acid
RT	Reverse transcription
SD	Standard deviation
Se	Selenium
SNORD68	Small nuclear RNA, C/D box 68
SOCS1	Suppressor of cytokine signaling 1
SYBR green	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
Zn	Zinc
miRNA	microRNA
miR-155	microRNA-155
WHO	World Health Organization

1 Introduction

1.1 The Arctic

The circumpolar Arctic includes waters and land within the political boundaries of Canada, Finland, Greenland (Denmark), Iceland, Norway, Sweden, Russia and Alaska (USA) (Letcher et al., 2010). Organisms living in the Arctic region must tolerate extreme climatic conditions, such as large variations in light and temperature, extensive snow and ice cover, permafrost, short summers, short growing seasons and limited precipitation. All these factors have an impact on the productivity, species diversity, wildlife behavior and food chain characteristics of Arctic ecosystems (AMAP, 1998). The Norwegian Arctic includes Svalbard, an archipelago of 63 000 km^2 and the Jan Mayen island, which covers 377 km^2. Svalbard is a cluster of various islands (Spitsbergen, Nordaustlandet, Barentsøya, Bjørnøya and more), as well as rocks and shears, situated between 74° and 81° northern latitude and 10° and 35° eastern latitude, in the Barents Sea region. The climate on Svalbard is affected by frequent low-pressure passages and warm Atlantic Ocean water, making this area milder than other areas at the same latitude (NPI, 2018). The marine ecosystem in the Barents Sea region is considered highly productive and supports various wildlife species, of which seabirds are numerous (Borgå et al., 2005).

Increasingly, Arctic ecosystems faces various challenging environmental factors such as global warming, changes in climate variability, long-range transport of pollutants and reduced stratospheric ozone (Ørbek et al., 2007). The Arctic is warming at a much faster pace compared to other regions of the globe, and the Barents area (Fig 1.1), which the Svalbard archipelago is a part of, is considered a "hot-spot" even within the Arctic context (AMAP, 2017). Warmer temperatures have several consequences for the Arctic: melting of polar ice caps and mountain glaciers (UNEP/ AMAP, 2011), reduced sea-ice extent (MacDonald et al., 2005), increased air-water exchange where ocean is no longer covered by ice (UNEP/AMAP, 2011) and alterations in transportation, distribution and behavior of pollutants reaching the Arctic (Ma et al., 2016, UNEP/AMAP, 2011). Increase in sea temperatures and reductions in sea ice can impact the entire food web of the marine ecosystem in the Arctic. The changes can influence the foraging and breeding ecology of most marine birds and mammals, and are associated with increase in abundance of several temperate fish, seabird and marine mammal species (Dsecamps et al., 2017). In addition, climate change can have an impact on infectious diseases in Arctic species. Increasing environmental temperatures, changes in biodiversity, introduction of domestic animals and invasive species and increasing levels of persistent environmental contaminants

will all be contributing factors in the influence of climate change on infectious diseases (Bradley et al., 2005).

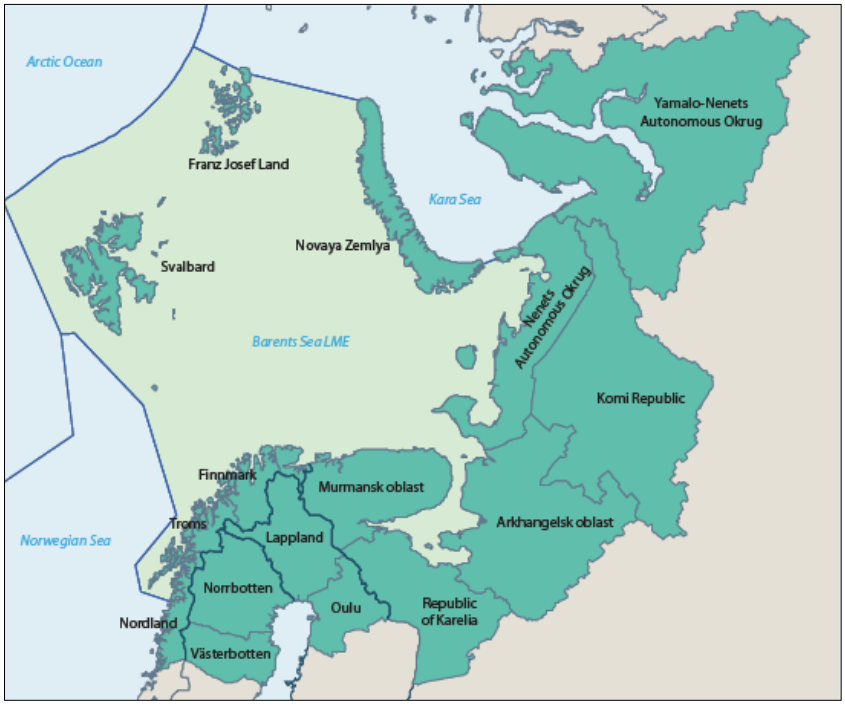

Fig 1.1 Illustration of the area that encompasses the Barents area (AMAP, 2017).

1.2 Contaminants in the Arctic

Svalbard is considered an important sink for various anthropogenic chemicals. These compounds are transported from distant sites of production and use to the Arctic via the atmosphere, water currents, sea-ice drift and the Great Arctic rivers (Burkow & Kallenborn, 2000; Verreault et al., 2010). After transportation, the contaminants reach Arctic soils, snow, ice, water and biota in different forms and phases. Transportation and distribution of these pollutants are highly affected by climate change and variability. This involves complex interactions with temperature, winds, precipitation, runoff patterns, snow and ice, organic carbon cycling, ocean circulation and human activities (Ørbek et al., 2007; MacDonald et al., 2005). The Arctic Monitoring and Assessment Programme (AMAP) has since 1991 documented the presence of various compounds in the Arctic in abiotic media, as well as freshwater, terrestrial and marine biota across the circumpolar area (AMAP, 2016). The target pollutants of AMAP include persistent organic pollutants (POPs), heavy metals, radioactivity, acidifying substances, and petroleum hydrocarbons. AMAP also addresses effects of climate change, ozone depletion, and ultraviolet-B radiation (AMAP, 2005).

1.2.1 Persistent organic pollutants (POPs)

POPs are organic compounds resistant to environmental degradation through chemical, biological and photolytic processes (Stockholm Convention Secreteriat, 2017). They compromise various pesticides (e.g. aldrin, lindane, 1,1,1-trichloro-2,2-bis(p-chlorphenyl)ethane (DDT)), industrial chemicals (e.g. polychlorinated biphenyls (PCBs)), and combustion by-products (e.g. polychlorinated dibenzo-*p*-dioxins (PCDDs)) (AMAP, 2016). These chemicals can remain intact in the environment for long periods, are capable of long-range transport, accumulate in tissue of living organisms and biomagnify at higher trophic levels. They also have potentially significant impacts on human health as well as the environment (Stockholm Convetion Secreteriat, 2017). AMAP has monitored POPs in the Arctic environment and biota for several years, and while most legacy POPs show declining levels in the Arctic environment (AMAP, 2016), the presence of novel contaminants is increasing in the region (Bytningsvik, 2014). Legacy POPs are compounds considered banned or regulated, and present day contamination of these chemicals are for the most part a "legacy" of past releases (Rigét et al., 2010). Many of the emerging POPs are replacements for phased-out chemicals (e.g. brominated flame retardants and pesticides), but some are emitted from increased industrial industry and human settlements in northern areas (Bytningsvik, 2014). Atmospheric long-range transport and distribution of POPs can be explained by the "global fractionation hypothesis" (Wania & Mackay, 1995;1996) (Fig. 1.2). This hypothesis explains atmospheric transport as a complex phenomenon that depends on the physical-chemical properties of the transported contaminant, such as solubility, vapor pressure and molecule size. These properties determine the distance and deposition of the substance via rain, fog, snow in the water column, sediment or snow. Semi-volatile compounds are distributed between airborne particles and gaseous phase depending on temperature. They can be temporarily deposited in seawater and soil via precipitation, and evaporate again into the atmosphere during favorable warm weather conditions. This remobilization is called the "grasshopper effect" (AMAP, 2004) (Fig 1.2).

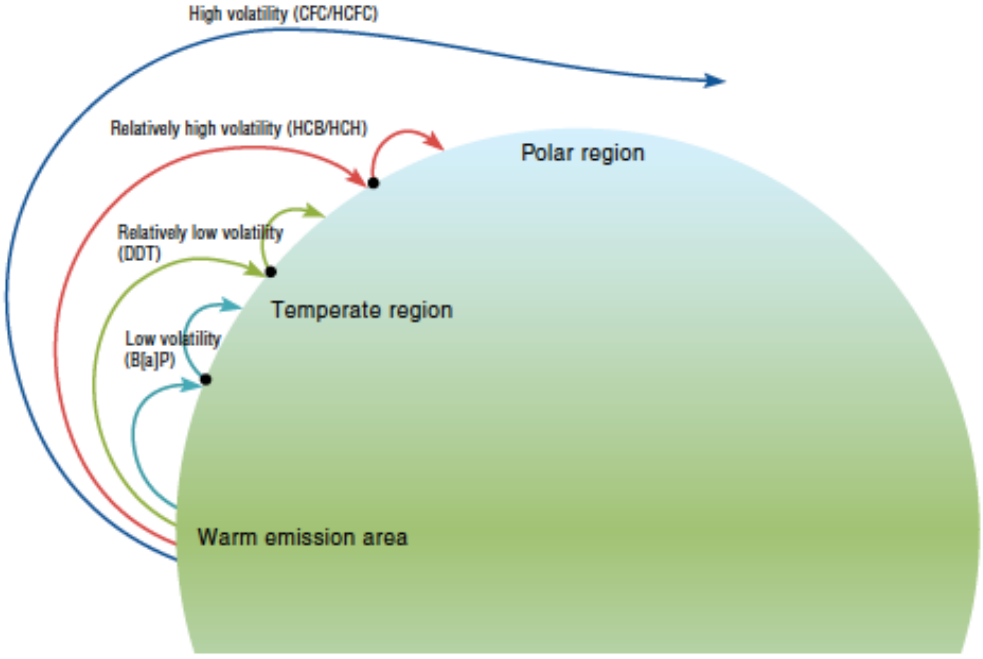

Fig 1.2 Schematic representation of the global fractionation hypothesis and the revolatilization or "grasshopper" effect. From Wania & Mackay (1996).

POPs are of a concern for humans and the environment since they are highly persistent, bioaccumulative and toxic (Noyes et al., 2009). Arctic marine organisms are particularly vulnerable as they are dependent on large lipid storage for metabolism in a harsh environment with varying sources of energy throughout the year and several POPs are lipophilic. Their lipophilicity and persistency allow them to accumulate in lipid-rich tissue, which means they can also be transferred up the food chain (Hop et al., 2002). Comparing with other Arctic areas, mammals and seabirds from the Barents Sea region have some of the highest levels of organic contaminants, with particularly high abundance of PCBs (Borgå et al., 2005). PCBs, as well as other POPs, are suggested to cause adverse effects in thyroid hormone system, sex steroid homeostasis, vitamin status, organ morphology, immune system and behavior in Arctic wildlife and fish (Letcher et al., 2010).

1.2.2 Metals

Metals and metal compounds occur naturally in all ecosystems and move between atmosphere, hydrosphere, lithosphere and biosphere (Bargagli, 2000). A portion of the introduction of metals to the Arctic environment stems from natural sources, such as rock weathering, but the rest originates from various anthropogenic sources (Zaborska et al., 2017). The anthropogenic activities, such as industrial processes, agricultural practices, transportation and waste disposal,

redistribute the metals, increasing or decreasing their levels (AMAP, 2005). In the Arctic, the most important anthropogenic sources of metals are long-range atmospheric transport from industry, coal burning and transportation (Pacyna et al., 1984). Air masses, ocean currents and drifting sea ice with trapped contaminants all transport metals emitted in Europe and Russia to the Arctic (Zaborska et al., 2017).

The first assessment of the Arctic environment conducted by AMAP in 1997 concluded that metals are a potential risk to the ecosystems and biotic communities found in the Arctic (AMAP, 2005). Deposition of metals on ocean surfaces allow them to enter the aquatic food chain and be distributed among tissues or secreted. With each step of the food chain, there is potential for bioamplification (Lewis & Furness, 1991; Burger & Gochfeld, 2009). Mercury (Hg) and other metals have been detected in various Arctic biota and environmental samples across the region. Hg, as well as lead (Pb) and cadmium (Cd) have all been detected in Arctic biota at concentrations above Health Canada and WHO guidelines for human consumption (Campbell et al., 2005). Marine mammals and seabirds can accumulate large amounts of metals in their liver since they occupy the highest trophic positions in the marine food web and have a long lifespan (Ikemoto, 2004). Uptake, accumulation and biomagnification of metals in birds are influenced by exposure pathways, the species of the metals and their bioavailability (Burger et al., 2008). In addition, there are numerous host factors, such as trophic status, location, foraging behavior, nutrition, body condition, gender, size, genetic variability and age, also playing an important part (Stewart et al., 1997; Burger et al., 2003; Burger et al., 2008).

Exposure to metals is potentially detrimental, especially metal compounds that do not have any physiological role in the metabolism of cells (Florea & Büsselberg, 2006). The toxicokinetics and toxicodynamics of metals depend on the metal, the form of the metal or metal compound and the organism's ability to regulate and/or store the metal. These processes can be highly dynamic and directly influence the expression of metal toxicity (Fairbrother et al., 2007). Ingestion of metals could possibly modify metabolism of other essential metals, such as zinc (Zn), copper (Cu), iron (Fe) and selenium (Se) (Abdulla & Chmielnicka, 1989). Metal toxicokinetics and elimination in animals are affected by factors such as age, gender, nutritional status, seasonal and yearly variation, geographical variation and trophic level (Burger et al., 2003). Many metals can also form covalent bonds with carbon, which can result in metal-organic compounds. This transformation can alter the metal's mobility, accumulation and toxicity. Metals and metal compounds can interfere with the central nervous system, the hematopoietic system, liver and kidneys (Florea & Büsselberg, 2006). Metals can influence the

cells of the immune system by a variety of mechanisms (MacGillivray & Kollmann, 2014). Depending on concentration, metal speciation, bioavailability, as well as length and timing of exposure (MacGillivray & Kollman, 2014), metals can cause effects such as immunosuppression, immune stimulation, hypersensitivity and autoimmunity (Hultman & Pollard, 2015). Damage caused by metals at molecular and cellular levels can lead to negative effects at higher organizational levels, such as growth inhibition, metal accumulation in internal organs, reduced avian clutch sizes and reduced reproductive success, which could threaten wildlife populations (Eeva et al., 2009).

1.3 The immune system

The immune system protects animals and humans from infectious organisms, such as bacteria, parasites or viruses, as well as their toxic products (Murphy & Weaver, 2017). It is comprised of a complex network of various tissues, cells, soluble circulating proteins and antibodies working in a concerted effort to resist infections (Desforges et al., 2016). When the immune system fails, or is overwhelmed by the number of "nonself" particles, it causes disease and the individual becomes sick (Fairbrother et al., 2004). The development of the avian immune system is primarily based on poultry research and the immune system of birds conforms to the basic immunologic mechanisms identified in mammals, making it reasonable to assume close similarities between poultry and other avian taxa. However, it is important to acknowledge that there is little information about the difference in ontogeny of immune function between altricial, precocial and semi-precocial birds (Fairbrother et al., 2004). Invading pathogens are fought off by the innate and adaptive immune system, two separate, but interconnected functional systems working together to protect the host within minutes and hours after exposure to antigenic stimulation (Desforges et al., 2016). Innate immunity refers to processes of phagocytosis by fixed or circulating macrophages and the inflammatory response. In addition, there are natural killer (NK) cells providing primary surveillance mechanisms against cancers and tumors. Adaptive immunity refers to specific immune responses that are inducible, are specific to particular antigens, and have memory (Fairbrother et al., 2004).

The adaptive immune system can be divided further into two parts: cell-mediated and humoral immunity (Fairbrother et al., 2004). Humoral immunity is characterized by production of antibodies (Abs), glycoproteins with specific receptors that can bind particular pathogens. Abs are secreted by B lymphocytes, which develop in the bursa of Fabricius (Fairbrother et al., 2004). The bursa of Fabricius is a primary lymphoid organ that is unique to birds (Sharma,

1991), while in other vertebrates the B lymphocytes differentiate and develop in the bone marrow (Galloway & Handy, 2003). The bursa of Fabricius develops as an outgrowth of the cloacal epithelium and is located at the dorsal end of the hind gut (Sharma, 1991). Different types of Abs develop depending on function, location within the body and time course of infection. Birds have only three Ab classes: immunoglobulin (Ig) M, which is the primary Ab, IgG, which is the secondary response, and IgA, which are mucosal Abs. Abs act by neutralizing viruses, coating bacteria or other particles for phagocytosis, binding to target T lymphocytes or by activating the complement system to enhance lysis of cells (Fairbrother et al., 2004). Cellular immunity acts through the development and proliferation of T lymphocytes, which regulate the function of humoral immunity as well as nonspecific immune responses (Fairbrother et al., 2004). T lymphocytes develop in the thymus, a primary lymphoid organ originating from an epithelial outgrowth of the pharangeal pouches at an early embryonic age. In chickens (*Gallus gallus*), the thymus gland is composed of multiple lobes located along the side of the neck, extending into the thoracic cavity (Sharma, 1991). T lymphocytes may either enhance the immune response as "helper T cells" classified by the CD4+ surface antigens, or suppress the response as "suppressor T cells" with CD8+ surface antigens. Communication between T lymphocytes and macrophages occur through the release of cytokines, soluble proteins such as interferon or prostaglandins (Fairbrother et al., 2004).

1.3.1 Factors influencing the immune system

The immune response to a challenge can be influenced by many factors, both intrinsic (e.g. age, sex, etc.) and extrinsic (e.g. environmental conditions, social interactions, diet or exposure to toxicants). Immunity can be highly regulated by the endocrine system since almost all cells of the immune system have receptors for one or more of the stress hormones, metabolic hormones, sex hormones and other endocrine-signaling molecules. Hormones can directly affect the immune system and are responsible for alterations in immunocompetence that occur due to development and growth, changes in reproductive status, migration, molt, stress and dietary change (MacGillivray & Kollmann, 2014). Sexual dimorphism in relation to immune function is a common pattern found in vertebrates and often females are considered more immunocompetent than males (Nunn et al., 2009). Difference in susceptibility to parasitism and disease has been shown in different bird species (e.g. Møller et al., 1998; Tschirren et al., 2003) showing that males are often more susceptible to parasitism than females. The underlying causes for sexual dimorphism can be explained by the role of immunosuppressive substances (e.g. testosterone) or by differences found in male and female life histories (Nunn et al., 2009).

Recent studies also suggest sexual dimorphism could be due to sex-biased gene expression (Grath & Parch, 2016; Mank, 2017).

The discipline of immunotoxicology originates from the 1970s, following recognition of altered immune function and increased sensitivity to infections and cancers after exposure to environmental chemicals and therapeutic drugs (Germolec et al., 2017). The potential for chemicals to affect the immune system of birds has been studied for various pesticides, organochlorine compounds, petroleum hydrocarbons, heavy metals, organometallics and radiation (Fairbrother et al., 2004). Studies with birds have revealed that developmental exposure to planar halogenated aromatic hydrocarbons (HAHs) can reduce the number of developing T lymphocytes in the thymus and B lymphocytes in the bursa of Fabricius (Andersson et al., 1991; Fox & Grasman, 1999; Grasman & Whitacre, 2001). Association between organochlorines and altered immune function have been found in colonies of herring gulls (*Larus argentatus*), Caspian terns (*Hydroprogne caspia*) (Grasman et al., 1996; Grasman & Fox, 2001) and glaucous gulls (*Larus hyperboreus*) (Sagerup et al., 2001).

Metals such as As, Cd, Pb and both inorganic and organic Hg have shown to suppress the function of all immune cells, including T - and B lymphocytes and monocytes/macrophages (Hultman & Pollard, 2015). Hawley et al (2009) suggest Hg can exert sub-lethal immunosuppression in free-living three swallows (*Tachycineta bicolor*). Nain & Smits (2011) investigated the effect of Pb on immune responses in Japanese quail (*Coturnix coturnix japonica*). They observed no immunotoxicity in the Pb-exposed quail, and saw instead that exposed quail had lower morbidity and better survival than controls, suggesting that subchronic Pb exposure could be immunostimulatory rather than suppressive as earlier studies have shown. Metals that are considered essential for several biological functions (e.g. Zn, Se and Cu) are also important for the immune system to function efficiently (Chaturvedi et al., 2004). Se deficiency has been shown to cause a wide range of abnormalities of the immune system, resulting in reduced immunity to infections, tumors, cardiovascular and other diseases (Ferencik & Ebringer, 2003). Deficiency of Zn can cause increased sensitivity to pathogens, due to decrease in the ability of macrophages to kill intracellular parasites, a decreased production of several cytokines and antigen-mature cells, reduced levels of T helper cells and a decreased activity of NK cells (Ferenick & Ebringer, 2003). While increasing intake of some of these essential metals can be beneficial for immune function, excess amounts can impair immune function as well (Chaturvedi et al., 2004). An example of this is the observed decrease

in immune function in adult mallards (*Anas platyrhynchos*) exposed to Se in drinking water (Fairbrother & Fowles, 1990)

1.3.2 The role of microRNAs in immunology

MicroRNAs (miRNAs) are a family of evolutionary conserved, small (~18-24 bp) non-coding RNAs known to control gene expression at a post-transcriptional level and regulate various cellular processes, such as proliferation, differentiation, apoptosis and metabolism in eukaryotes (Bartel, 2004; Filipowicz et al., 2008). They have been predicted to target and control expression of at least 30% of the entire mammalian genome (Filipowicz et al., 2008). Recently, the number of miRNAs implicated in the development and function of the immune system has increased. miRNAs have been found to regulate cell fate and function of hematopoietic stem cells (stem cells giving rise to blood cells), to influence development of innate immune cells (e.g. macrophages, granulocytes, NK cells) and to regulate development and function of adaptive immune cells (e.g. B- and T lymphocytes) (Mehta & Baltimore, 2016).

miRNA-155 (miR-155) is a miRNA that has been observed to be involved in numerous biological processes, such as hematopoiesis, inflammation and immunity (Faraoni et al., 2009). Its deregulation has been associated with different types of cancer, cardiovascular diseases as well as viral infections (Faraoni et al., 2009). miR-155 is excised from the non-coding B-cell Integration Cluster (BIC), located on chromosome 21. BIC is found to be highly expressed in lymphoid organs and cells among humans, mice and chickens, which implies an evolutionary conserved function (Tam, 2001; Lagos-Quintana et al., 2002). During virus infection regulation of cellular miR-155 expression can determine the disease outcome. Expression of miR-155 can suppress RNA virus replication, while inhibition or deregulation of induced miR-155 facilitates RNA virus replication. This indicates that miR-155 expression limits viral replication (Mehta & Baltimore, 2016).

A targeted study by Waugh et al (2018) investigated deregulation of miR-155 expression in a virus exposed host due to PCB exposure. Chicken embryo fibroblasts were exposed to either 50 ppm of A1250 (PCB mixture), a synthetic virus intermediate (Poly I:C), or a combination of both A1250 and Poly I:C, as well as a control. A significant downregulation was found in both groups exposed to A1250, regardless of being stimulated with Poly I:C or not, when compared to virus stimulated group only (Waugh et al., 2018). Downregulation of miR-155 has been linked to increased viral titres and mortality in different virus models and species (Faraoni

et al., 2009). Waugh et al proposed that increased virus induced mortality seen in animal models when exposed to pollutants could be partly due to deregulation of miR-155 (Fig 1.3).

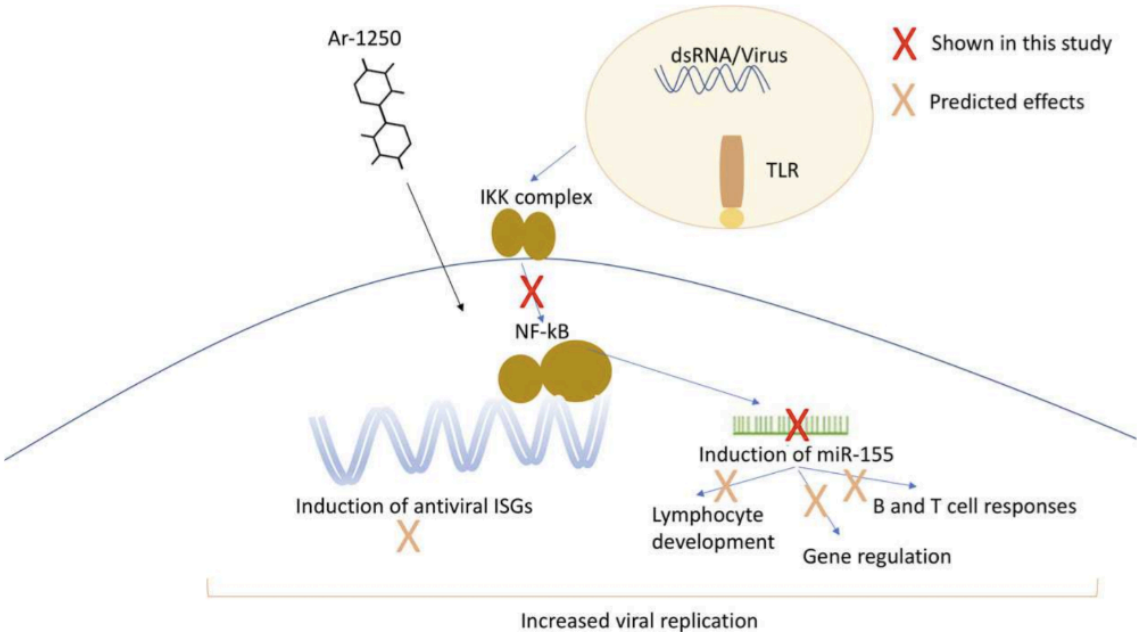

Fig 1.3 Predicted effects of A1250 exposure in virus exposed chicken embryo fibroblasts. Activation of I kappa B alpha kinase (IKK) complex by double stranded RNA/virus induces NF-kappa beta. Induction of NF-kB is downregulated by A1250, which leads to downregulation of miR-155. Since miR-155 controls several components of the immune response to a viral infection, impairment of these responses could lead to increased viral replication and/or pathogenesis (Waugh et al., 2018).

Due to the role of miR-155 in immunology and the fact that environmental contaminants, such as PCB, has shown to influence its expression, it is likely that this miRNA can be used as a biomarker of immunomodulation by environmental contaminants. Biomarkers can be used for evaluation of biological hazard of toxic compounds and assessment of environmental health (Fossi, 1994). It is important that biomarkers are quantitative, sensitive, non-invasive, specific, easy to measure, relate to the biochemical mechanism and work at doses that are realistic (Timbrell, 1998). The most important and overarching concept of biomarkers, regardless of endpoint(s), is that basic scientific knowledge of how pollutants interact with the essential cellular machinery can be used to develop tests that assess early cellular responses to pollutant exposures and provide a scientific link between compound exposure, early cellular effects and a degree of prediction on possible long-term health consequences for risk assessment purposes (Fowler, 2012).

1.4 Aims and objectives of study

This master thesis used seabirds used as study species due to their widespread distribution and because they are common, numerous and long-lived. In addition, they can be considered good bioindicators of environmental contamination as they are often on top of the food chain (Burger & Gochfeld, 2009). Wild birds also play a key role in the spread of diseases, either by serving as reservoirs or facilitating transfer of pathogens through short- and long-distance movements (Van Hemert et al., 2014). Charadriiformes (gulls, terns, shorebirds) are, for example, considered the major natural reservoir for avian influenza virus (AIV) (Munster et al., 2007). Further, it is particularly interesting to investigate the impact of increasing occurrence of infectious diseases in the Arctic, since there are large information gaps concerning disease in Arctic regions. In addition, several resident Arctic organisms can be considered immunologically naïve hosts, lacking previous experience with recently introduced pathogens, which mean they might be especially vulnerable (Van Hemert et al., 2014).

The thesis had two main aims; 1) to investigate levels of metals and POPs in seabirds breeding on Svalbard and 2) to investigate possible immunomodulation by environmental contaminants in kittiwakes breeding on Svalbard based on expression of miR-155.

The first aim of the thesis was to measure contaminants in back feathers and blood of black-legged kittiwakes (*Rissa tridactyla*), as well as back feathers of Brünnich's guillemots (*Uria lomvia*). For kittiwakes, contaminant levels were measured in blood at two different time periods, to investigate if there was an increase of circulating pollutants as the birds lose body mass during the breeding season. The body mass of kittiwakes can decrease almost 20% from pre-breeding to late chick rearing, and loss of mass leads to a subsequent release of lipids, which can redistribute lipophilic contaminants (Henriksen et al., 1996). Levels of metals were expected to be higher in feathers than plasma due to sequestering of metals in feathers. Levels of metals in feathers of kittiwakes and guillemots were expected to differ since levels of metals vary widely among different seabird species (e.g. Wenzel & Gabrielsen, 1995) depending on differences in feeding ecology, intensity and timing of exposure in foraging areas, as well as the bird's physiological and biochemical characteristrics (Savinov et al., 2003).

The second aim of the thesis used miR-155 as a biomarker of immunomodulation by environmental contaminants since it has been shown to be deregulated in chicken embryo fibroblasts exposed to a PCB mixture (Waugh et al., 2018), and its deregulation has been linked to increased viral titres and mortality in different virus models and species (Faraoni et al., 2009).

miR-155 expression was investigated in plasma samples of kittiwakes breeding on Svalbard. miR-155 expression was expected to vary depending on levels of different POPs and metals, as well as depending on the sex of the birds and whether they were infected with AIV.

2 Material and methods

2.1 Study species

2.1.1 Black-legged kittiwake

The kittiwake is a medium-sized gull (Fig 2.1). Adults are approximately 41 cm long and weigh around 330-450 grams. They are the most numerous species of gull in the world, with a circumpolar distribution, breeding in the arctic and boreal zone in the northern hemisphere. The kittiwake is one of the most common breeding species in all parts of the Svalbard archipelago and about 215 colonies are known. The species breed in colonies ranging in size from tens to tens of thousands in bird cliffs along the coast. The colonies can be mixed with other bird colonies, such as the Brünnich's guillemot (Strøm, 2017).

Fig 2.1 Small black-legged kittiwake (*Rissa tridactyla*) colony cliff at Blomstrand, in Kongsfjorden, 9 km northeast of Ny-Ålesund, Svalbard. Pictures by Courtney Waugh, NTNU.

According to a report conducted by Fauchald et al. in 2015, the large populations of kittiwake have declined substantially in all Norwegian regions, except on Bjørnøya (Fig 2.2). The population on the Norwegian mainland has according to their analyses declined from about 280 000 pairs in 1980 to 82 000 pairs in 2013. On Svalbard (Bjørnøya and Spitsbergen), the population has declined from about 300 000 pairs to 240 000 pairs from 1988 to 2013. On the Norwegian Redlist kittiwakes are considered endangered, while on the Svalbard Redlist they are considered near-threatened (Fauchald et al., 2015). The International Union for Conservation of Nature (IUCN) Red List of

threatened species lists kittiwakes as vulnerable due to rapid decline over the past three generations, a decline that is likely to be continuing (BirdLife International, 2017).

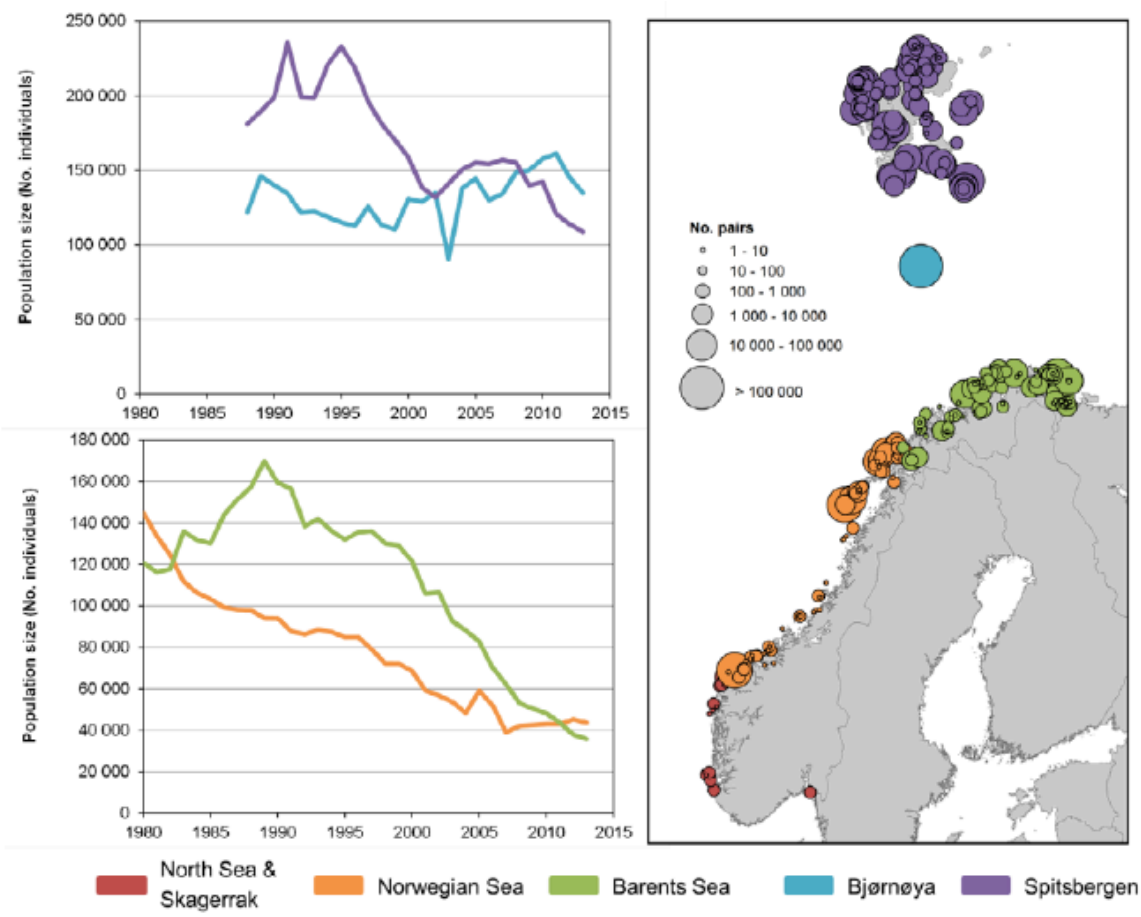

Fig 2.2 The two graphs on the left shows regional dynamics in breeding populations of black-legged kittiwake *(Rissa tridactyla)* at Spitsbergen, Bjørnøya, Barents Sea and Norwegian Sea. The figure on the right shows distribution and size of breeding colonies. Breeding population sizes were calculated by combining monitoring data (time series of counts from specific monitoring sites) with population census data (all-covering counts of breeding individuals in specified areas (breeding locations)). Because so little monitoring data was available before 1980, the present time-series span from 1980 (1988 for Spitsbergen) to 2013 (Fauchald et al., 2015).

Kittiwakes become sexually mature at an age of 4-5 years (Strøm, 2017). Some kittiwakes can arrive in Svalbard already in February, but most birds arrive in April. Egg laying occurs in early June and the eggs are incubated by both sexes for about four weeks. At about six weeks old the young leave the nest. Kittiwakes often forage far out in the open sea or ice-filled waters along glacier faces during breeding season (Isaksen & Bakken, 1995), feeding their chicks a varied diet consisting mainly of different fish species and crustaceans (Strøm, 2017). Warming of the sea due

to climate change has resulted in capelin (*Mallotus villosus*) replacing polar cod (*Boreogadus saida*) in the diet of breeding kittiwakes. This shift in diet does not seem to have any effect on the breeding success of the kittiwakes, but the lower lipid content can influence the exposure to lipophilic pollutants such as POPs (Gasbjerg, 2009). Outside the breeding season kittiwakes have a pelagic way of life. Most leave the Svalbard area in September for wintering areas in the North Atlantic (Isaksen & Bakken, 1995).

2.1.2 Brünnich's guillemot

The Brunnich's guillemot is a large black and white auk (Fig 2.3). The adults are approximately 41 cm long and weigh around 700-1200 grams. They are one of the most numerous seabirds in the northern hemisphere and on Svalbard. Breeding occurs on narrow cliff ledges in dense colonies all over the archipelago and the colonies can range from a few hundred pairs to several hundred thousand pairs. On Svalbard, the guillemots often breed in mixed colonies alongside kittiwakes (Strøm & Descamps, 2017).

Fig 2.3 The Brunnich's guillemot (*Uria lomvia)*. Photo by Odd Harald Selboskar, Norwegian Polar Institute (NPI) (Strøm & Descamps, 2017).

The total breeding population on Svalbard is estimated to be around 850 000 pairs. Since 1995 the annual monitoring programme in Norway has shown a decrease in the size of the breeding population in all monitored colonies (Fig 2.4), a decline that could be linked to alterations in oceanographic conditions in the wintering areas. The red list status of Brunnich's guillemots on Svalbard is near threatened (Strøm & Descamps, 2017). On the IUCN Red List the guillemots are

listed as least concern, mostly due to the species extremely large range, as well as trends indicating increase in population number on an international basis (BirdLife International, 2018).

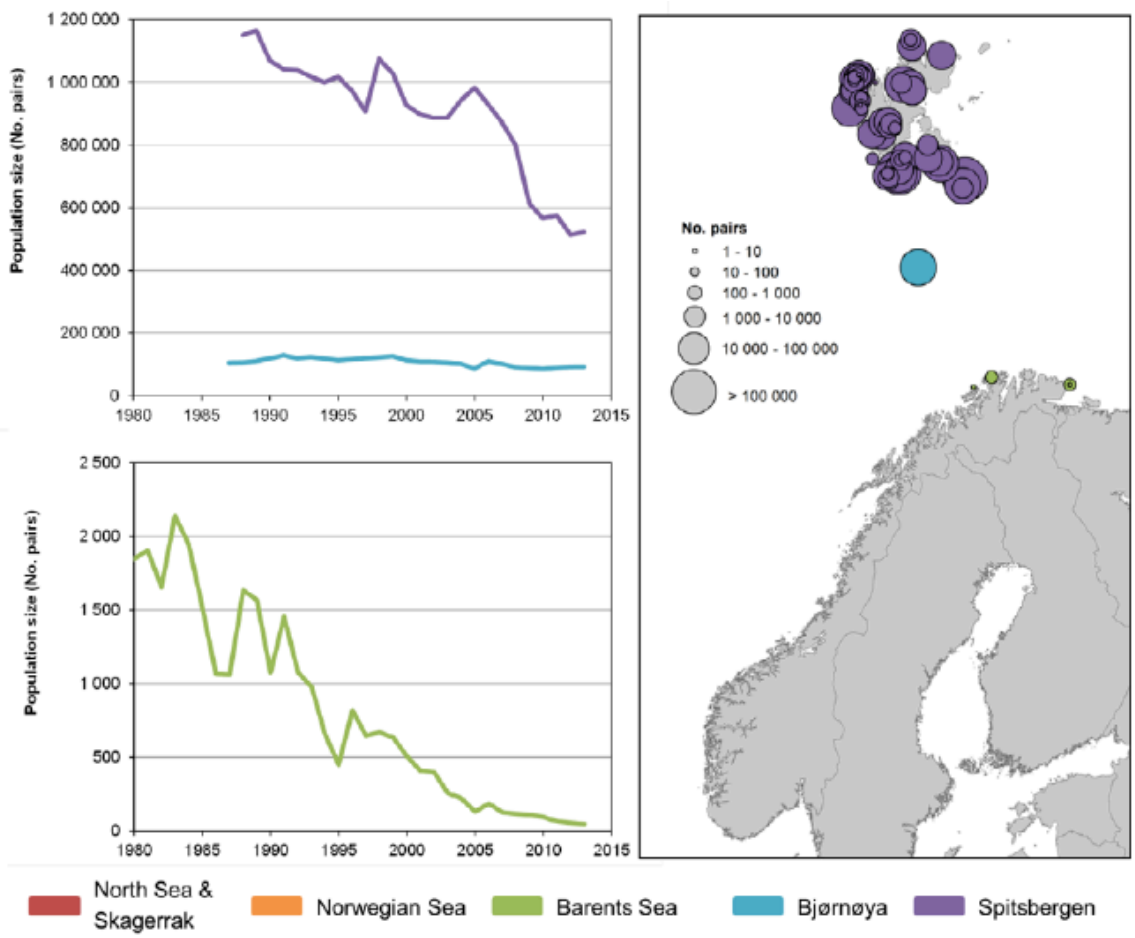

Fig 2.4 The two graphs on the left shows regional dynamics in breeding populations of Brünnich's guillemot *(Uria lomvia)* at Spitsbergen, Bjørnøya and Barents Sea. The figure on the right shows distribution and size of breeding colonies. Breeding population sizes were calculated by combining monitoring data (time series of counts from specific monitoring sites) with population census data (all-covering counts of breeding individuals in specified areas (breeding locations)). Because so little monitoring data was available before 1980, the present time-series span from 1980 (1988 for Spitsbergen) to 2013 (Fauchald et al., 2015).

Egg laying begins in the end of May or early June and they all lay a single egg directly on bare rock or soil. The pear-shape of the egg prevents it from rolling off the ledge. The females lay their eggs about the same time, so that hatching and jumping of the young off the ledges is synchronized. Both sexes incubate the egg, which hatches after about 32 days. The young offspring jump off the breeding ledges before they are fully fledged, at around 20-21 days old. They become independent

6-8 weeks after leaving the nest. The diet of the Brunnich's guillemots consists mainly of fish and crustaceans (Strøm & Descamps, 2017. During breeding season the parents use large feeding ranges at sea (Isaksen & Bakken, 1995). On Spitsbergen, important prey food includes polar cod, blennies (*Blennioidei*) and capelin. The Arctic fox (*Vulpes lagopus*) and the glaucous gull are important predators of eggs, chicks and sometimes adult birds (Strøm & Descamps, 2017).

2.2 Fieldwork

2.2.1 Study location and time

The kittiwake colony sampled for this master thesis was located on Blomstrand (Fig 2.5), a seabird cliff 9 km northeast of Ny-Ålesund in Kongsfjorden, Svalbard, Norway (78°99'N, 12°11'E). The guillemot colony sampled was located on Ossian-Sarsfjellet (Fig 9), a seabird cliff approximately 11 km northeast of Ny-Ålesund in Kongsfjorden (78°94'N, 12°44'E). Kongsfjorden is a glacial fjord system, approximately 20 km long and 4-10 km wide, that is located at the northwestern part of Spitsbergen.

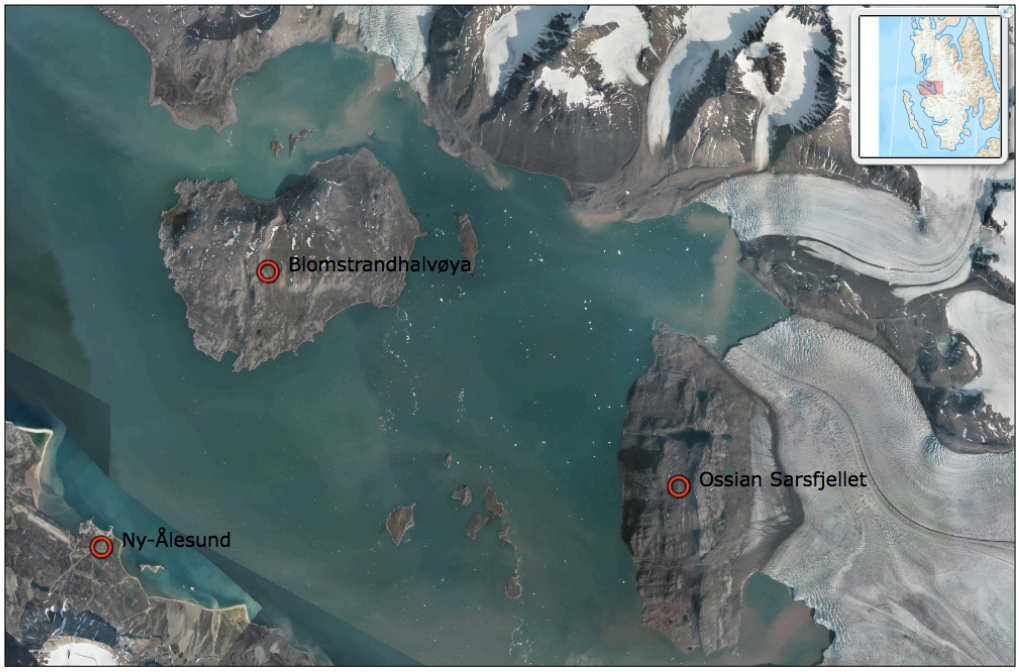

Fig 2.5 Areal map 2 km above ground showing the sampling area Blomstrand (78°99'N, 12°11'E), 9 km northeast of Ny-Ålesund and Ossian-sarsfjellet (78°94'N, 12°44'E), 11 km northeast of Ny-Ålesund. Both in Kongsfjorden, Svalbard, Norway. Taken from http://toposvalbard.npolar.no

For the kittiwakes, the sampling period was two weeks in July 2017, from the 14th until the 24th. The sampling period for the guillemots was three days from the 18th to the 20th of July 2017. In addition to the samples obtained in 2017, some previously collected samples were analyzed in this thesis as well. In July 2014 (Svendsen et al., 2018) and 2015 (Castaño-Ortiz, 2016) kittiwakes were sampled from the same colony, as well as another colony in Kongsfjorden called Krykkjefjellet.

2.2.2 Sampling method for kittiwakes

The kittiwakes were captured on their nests using a telescopic fishing rod with a nylon snare attached on its end (Fig 2.6). The nylon wire was placed around the neck and the bird was brought to the ground. All the birds sampled were in adult breeding plumage.

Fig 2.6 Capturing kittiwakes from their nests using a long fishing rod with a snare attached. Pictures by Megan Lee (NTNU) and Silje Strand Lundgren (NTNU).

Feathers (n = 8-10), swabs (cloacal and laryngeal) and blood (200-1000 µL) was sampled from each individual (n = 17, eight males, nine females) at two different periods in July 2017. Kittiwakes can spontaneously regurgitate their food when handled, so when this happened the food sample was collected. All the samples were collected in plastic bags or plastic tubes, which were marked with area, bird number and date. The first sampling period was during late incubation/early chick hatching (11-15th of July), while the second sampling period was approximately one week after (21st of July). For each period, 15 individual kittiwakes were sampled. 12 of these were recaptured for the second period, and the remaining three were individuals that had not sampled the first time. Recapturing would be more difficult during the incubation period, since they can stay out twelve

hours or longer before returning to the nest for switching. But during rearing of the chicks they stay out for a much shorter period. All samples were to be used for contaminant and immunological analyses.

Feathers were taken from the back of the bird, and one or two feathers were stored separately for DNA analysis. Swabs were taken from the cloacal and laryngeal area of the bird with cotton buds, which were stored in 15-mL CORNING CentriStar™ plastic tubes. Blood samples were taken from the brachial vein using a heparin-treated 2-mL or 1-mL BD Plastipak™ syringe with a 100 Sterican® 30G or BD Microlance™ 25G needle. The heparin prevents the formation of blood clots. The blood was transferred from the syringe into a BD vacutainer, which was stored in a tube to protect them from light, at ambient temperature. The BD vacutainer contains a fluid which allows for separation of white blood cells from the plasma. After each sampling day, feathers and swabs were stored at -20°C. The blood was centrifuged (Medifuge centrifuge from Thermo Scientific) at 2.5 RPM for approximately 15 minutes, and the plasma was transferred to 1.5-mL Nalgene® System 100™ Cryogenic Tubes (Thermo Scientific) and stored at -20°C.

In addition to obtaining samples, biometrics of the captured birds were measured (Appendix I), which included skull length, wing length, tarsus and body weight. Skull length was measured to the nearest 1 mm with a sliding caliper from the back of the head to the tip of the bill. Wing length and tarsus was also measured using the sliding caliper. Body weight was measured with a 500-gram spring balance (± 5 grams). Only body weight was measured for recaptured birds in the second period, to see how much weight they lost during the week of feeding and caring for the chicks.

After all samples and biometrics had been measured during the first sampling period the birds were marked with a red or blue marker on the top of their head and on their neck. The color was used to recognize which bird we had sampled and needed to recapture for the second sampling period. To distinguish between nest partners, they were colored with different colors.

Blood sampling of kittiwakes from 2014 and 2015 was conducted in a similar manner as described above. In total, there were n = 18 blood samples from 2014, 8 which were from Krykkjefjellet and 14 from Blomstrand. From 2015, there were a total of n = 25 blood samples, 11 which were from Krykkjefjellet and 14 from Blomstrand. For this project, 7 and 10 of the blood samples were used from 2014 and 2015, respectively.

2.2.3 Sampling method for guillemots

The guillemots (n = 13) were captured on their nests from above by using a net. Feathers (n = 8-10), swabs (cloacal and laryngeal) and blood was sampled. Only a small amount of blood could be extracted from just two guillemots, so blood samples were not included in this project. All samples were to be used for contaminant and immunological analyses. When returning from the field the feathers and swabs were immediately stored at -20°C. The blood samples were centrifuged and transferred to 1.5-mL cryogenic tubes and stored at -20°C as well. Guillemots with or without ticks were distinguished and the ticks were sampled and stored in a small cryotube with ethanol. In addition to the samples taken, biometrics were measured similarly to the kittiwakes. This included skull length, wing length, tarsus length and body weight.

2.3 Contaminant analysis

2.3.1 Feathers

Feather samples from both kittiwakes (n = 30) and guillemots (n = 13) were prepared for analysis of trace metals using ICP-MS (inductively coupled plasma mass spectrometry). Before conducting the analysis, the feathers had to be cleaned in a series of washings involving acetone, nitric acid (HNO_3) and milli-Q-water using a laboratory shaker (laboshake heavy-load shaker from Gerhardt), to remove external contamination. Another important step before ICP-MS is acid digestion of the samples using microwave digestion system (Milestone UltraCLAVE, Leutkirch, Germany). Feathers were weighed and transferred to Ultraclave vials, that had been rinsed with distilled water. Feather weight per vial ranged from 27-88 mg. Based on their weight 3 mL of 50% nitric acid v/v was added to each vial. Then the vials were placed in the reaction chamber of the UltraClave. The chamber is pressurized with inert gas (N_2) and then heated by microwaves. Pressure of the chamber is around 50 bar, and the highest temperature the machine reaches is around 260°C. The machine runs for about 2.5 hours. After digestion, the liquid in the vials were transferred to a different tube and diluted until total sample weighed between 28-32 grams. This was further transferred to a 15-mL tube by first flushing 15 mL of the liquid in the tube and then adding the remaining liquid to the tube. After all samples had been digested and diluted they were ready for analysis with ICP-MS. ICP-MS was conducted by Syverin Lierhagen at The Department of Chemistry (NTNU) in Trondheim, Norway.

2.3.2 Plasma

Plasma samples from the kittiwakes (n = 28) were also prepared for metal analysis using ICP-MS. No cleaning was necessary, as with the feathers. But acid digestion was conducted in a similar way as with the feathers, with a few differences. The plasma samples weighed between 195-1030 mg, which meant a different amount of HNO_3 had to be added before UltraClave. 1.5 mL of 65% HNO_3 v/v was added to the plasma samples. After the plasma samples were diluted to 14-16 grams, which was added directly to the 15-mL tubes. After all samples had been digested and diluted they were ready for analysis with ICP-MS.

Originally, plasma was also supposed to be analyzed for various POPs. Unfortunately, the amount of blood sampled from the kittiwakes was too small to be able to conduct any contaminant analysis of organochlorine compounds.

2.4 Immunological analysis

2.4.1 Quantitative polymerase chain reaction (qPCR)

The plasma from kittiwakes sampled during the summer of 2014 (n = 7, two females, five males) (Svendsen et al., 2018) and 2015 (n = 10, all females) (Castaño-Ortiz, 2016) were also investigated. This plasma was used to conduct an immune assay investigating expression of miRNA-155. First RNA was isolated from the plasma using RNeasy Mini Kit (Qiagen, Oslo, Norway). Standard operating procedure from the manufacturer was used. The first step of the procedure involved homogenization of 50 µL of plasma with TRIzol reagent and chloroform. Afterwards ethanol and two different buffers were added in various steps to remove everything except for the RNA from the sample. Lastly, the extracted RNA was eluted into RNase-free water and a Nanodrop spectrophotometer was used to investigate concentration (ng/µL) and quality of RNA in each sample. 1 µl of sample was added to the instrument. Samples with RNA below 2 ng/µl or A60/A80 ratio different from 1,8-2 was discarded before cDNA synthesis.

Reverse transcription (RT), for synthesis of cDNA from the RNA samples, was performed using the miScript II RT kit (Quiagen, Oslo, Norway). This kit contains miScript RT Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer and 5x miScript HiFlex Buffer. For all samples 4 µL of Hispec Buffer, 2 µL of Nucleics mix and 2 µL of RT mix was added. Volume of water and RNA depended on RNA concentration and was added until total volume of 20 µL. After the mixture was

incubated at 37°C for 60 minutes and then at 95°C for 5 minutes. Afterwards the samples were frozen at -20°C before qPCR.

qPCR was conducted using diluted cDNA (1:5 with distilled water) and miScript SYBR Green PCR kit (Quiagen, Oslo, Norway). This kit contains QuantiTect SYBR Green PCR Master Mix and the miScript Universal Primer. In addition, two primer assays for miRNA were used; miRNA-155 and SNORD68 (reference gene). The SYBR Green PCR Master Mix, Universal Primer and Primary Assays were added to one master mix to avoid pipetting errors. In total, there were 24 cDNA samples to be analyzed, which equals 48 PCR reactions, since we wanted technical duplicates in case of any errors. There were some biological duplicates as well. In addition, there were two blank wells with only master mix. First 12 µL of master mix were added to fifty wells on a 96-well plate. Then 6 µL of diluted sample were added in duplicates to 48 wells. The plate was then covered with a plastic film and centrifuged for 1 minute at 2000 rpm. The plate was run in a LightCycler® 96 Instrument with specific conditions and parameters set. The running conditions for the miRNA assay were 15 min at 95°C, three step cycling at 15 s at 94°C, 30 s at 55°C and 30 s at 70°C for 45 cycles. Two plates were analyzed in the LightCycler, one with miRNA-155 as the primer assay, and one with SNORD68 as the primer assay. Following amplification and collection of fluorescence data, melt curve analysis was performed to exclude the possibility of non-specific amplification. All reactions bore similar PCR amplification efficiencies. No *Cq* values were detected for SNORD68, indicating that this reference gene was perhaps not an adequate gene for use in kittiwakes.

2.4.2 Cloacal microbiome

The cloacal microbiomes of the kittiwakes were investigating using the cloacal swabs (n = 26) taken during field work July 2017. To investigate bacterial DNA from the cloacal swabs, QIAmp® DNA Microbiome kit (Quiagen, Oslo, Norway) was used. Standard operating procedure from the manufacturer was used. The host cells were lysed and degraded using Benzonase®, a chemically engineered endonuclease. Benzonase attacks and degrades all forms of DNA and RNA. The bacterial cells are kept intact due differential lysis of human and animal host cells based on differences in physiology of host and bacterial cells. After incubation and centrifugation, the DNA was isolated completely. DNA concentration (ng/µL) was estimated using Nanodrop spectrophotometer by adding just 1 µL per sample. 39 µL of isolated bacterial DNA was sent to

the Genomics Core Facility (GCF), Norwegian University of Science and Technology (NTNU) for genome sequencing. The facility provides DNA sequencing service based on state-of-the-art "high throughput sequencing" technology (illumina HiSeq4000 and NS500 instruments). GCF is funded by the Faculty of Medicine and Health Sciences at NTNU and Central Norway Regional Health Authority. Unfortunately, the facility found no bacterial DNA in the samples, which indicates technical or human errors occurring during the extraction process.

2.4.3 ELISA investigating avian influenza virus (AIV) infection

Infection of AIV of the kittiwakes were investigated by performing an enzyme-linked immunosorbent assay (ELISA) with an influenza A Ab test kit (IDEXX). Kittiwakes from 2014, 2015 and 2017 were all screened for AIV infection. Influenza A is a zoonosis of significant concern due to its global prevalence, numerous subtypes and reassortant nature (the genome consists of parts derived from the genomes of two or more different viruses). It presents with a wide range of pathogenic profiles, from almost no clinical signs to high mortality, depending on the affected species and which subtype is involved. Standard operating procedure from the manufacturer was used. The general principle of the assay was to measure the relative level of antibody to influenza A in animal serum. The assay was performed on 96-well plates that were coated with AIV antigen. Upon incubation with test sample in the coated wells, AIV specific antibodies formed a complex with the coated antigens. After unbound material was washed away, an anti-influenza A nucleoprotein monoclonal antibody enzyme conjugate was added to the wells. If AIV antibodies were present in the sample, the anti-influenza A nucleoprotein conjugate was blocked from binding to the antigen. After unbound conjugate was washed away, an enzyme substrate was added. Subsequent color development was inversely proportional to the amount of anti-influenza A antibodies in the test samples. The ELISA was performed by Megan Lee at the Department of Biology (NTNU) in Trondheim, Norway.

2.5 Data analysis

2.5.1 Contaminant data

Analysis of data was performed in Microsoft Excel ® 2017 and R (version 3.4.3) (R Core team, 2017). The limit of detection (LOD) for all metals measured by ICP-MS was calculated in Excel,

and is shown for selected metals in Table 2.1. LOD was defined as the highest value between instrument detection limits (IDL) and 3x SD of the mean of the blank measurements.

Table 2.1 Limit of detection (LOD) concentrations for arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), selenium (Se) and zinc (Zn) in plasma (ng/g ww) and feathers (ng/g dw). Calculated in Microsoft Excel ® 2017.

	LODs	
Metals	*Plasma (ng/g ww)*	*Feathers (ng/g dw)*
As	0.85	17.44
Cd	0.07	1.4
Cr	0.17	3.49
Cu	1.02	20.93
Hg	0.46	13.95
Pb	0.07	1.4
Se	1.7	34.9
Zn	0.85	17.44

Since there was no reason to separate between metals detected in early and late sampled feathers from kittiwakes, the concentrations detected by ICP-MS were pooled together. After ensuring concentrations of the selected metals were all above LOD, a dataset for plasma and feather concentrations of kittiwakes and feather concentrations of guillemots were imported into R. Minimum, maximum and mean concentrations of trace metals, as well as standard deviation were calculated in R. Selected metal concentrations in plasma were illustrated with boxplots. Bottom and top of boxplots are always the first and third quantiles, the band inside the box is always the second quantile (the median). The whiskers show the minimum and maximum of the data. Plasma concentration of selected metals at early and late sampling were compared by performing a paired sample t-test in R. Feather concentrations of kittiwakes and guillemots were compared by performing a two-sample Welch's t-test assuming unequal variance in R. Pearson's correlation coefficient was used to measure linear correlation between trace metals measured in kittiwake plasma and feathers at early and late sampling, as well as guillemot feathers. Scatterplots of correlations can be found in Appendix III.

2.5.2 Immunological data

Analysis of data was performed using R (version 3.4.3) (R Core Team, 2017), rejecting the null-hypothesis at $\alpha = 0.05$. First, the specialized package *MCMC.qpcr* (Matz et al., 2013) was used. MCMC is essentially a simulation technique to obtain the distribution of each parameter in a model.

This analysis transformed raw *Cq* values (Appendix IV) from qPCR into molecule counts, a method explained further in Matz et al. (2013). The counts are obtained using a formula relying on knowledge of amplification efficiency (E, the factor of amplification per cycle) and the *Cq* of a single target molecule (*Cq1*):

$$Count = E^{(Cq1-Cq)}$$

(Eq 2.1)

This method has been validated by Matz et al (2013), and has also been used in a target study by Waugh et al. (2018). The Markov Chain Monte Carlo chain was run for 13 000 iterations. qPCR was performed with technical duplicates; therefore, the molecule counts were averaged. As mentioned, no *Cq* values were detected for the reference gene, SNORD68, but Matz et al (2013) has proven that even in the complete absence of control genes the method provides sensible and validated answers.

A dataset with average molecule count of miR-155, biometrics of the birds (Appendix I) and contaminant levels of the birds (Appendix II) were imported into R. All model data and residuals were explored for influential outliers, normality and homoscedasticity (Zuur et al., 2010). To model the average miR-155 molecule count as a function of the covariates, a linear model was used (Eq 2.2). The *drop1* method in R, based on the AIC criterion, provided the best fit model, which included four fixed covariates; p,p'-DDE levels [ethylene, 1,1-dichloro-2,2-bis(p-chloropheynyl)] (continuous), sumPCB levels (continuous), sex of the birds (categorical with two levels) and AIV infection (categorical with two levels).

$$ln(miR-155) \sim p,p' - DDE\ levels + sumPCB\ levels + Sex\ of\ birds + AIV\ infection$$

(Eq 2.2)

Model assumptions were verified by plotting residuals versus fitted values, versus each covariate in the model and versus each covariate not in the model. Residuals for temporal and spatial dependency were assessed. P-values for the fixed covariates in the model (Eq 2.2) were 0.014 for p,p'-DDE levels, 0.01 for sumPCB levels, 0.024 for sex of birds and 0.009 for AIV infection. Adjusted R-squared for model was 0.55, while p-value was 0.008. The R packages *lattice* and *ggplot2* were used to make graphs to illustrate the linear model (Eq. 2). Boxplots were used to illustrate categorical covariates relative to the average miR-155 molecule count. Scatterplots were used to illustrate the continuous covariates relative to the average miR-155 molecule count.

A different dataset containing only kittiwakes sampled in 2015 (n=10, all females) was imported into R. This dataset included the same covariates as the one above, but with concentrations of various metals detected in RBCs (red blood cells) as well. Since the kittiwakes sampled in 2014 were not investigated for metals they were not included in this dataset. All model data and residuals were explored for influential outliers, normality and homoscedasticity (Zuur et al., 2010). Including metal data to the dataset yielded a different linear model than the previous one. Fixed covariates used to model miR-155 as a function were AIV infection (categorical with two levels), Se concentration (continuous), Pb concentration (continuous) and As concentration (continuous) (Eq 2.3).

$$ln(miR - 155) \sim AIV\ infection + Se\ + Pb\ + As$$

(Equation 2.3)

Model assumptions were verified by plotting residuals versus fitted values, versus each covariate in the model and versus each covariate not in the model. Residuals for temporal and spatial dependency were assessed. P-values for the fixed covariates in the model (Eq 2.3) were 0.002 for AIV infection, 0.003 for Se concentration, 0.006 for Pb concentration and 0.00006 for As concentration. Adjusted R-squared for model was 0.78, while p-value was 0.017. The R packages *lattice* and *ggplot2* were used to make scatterplots to illustrate the linear model (Eq. 3).

3 Results

3.1 Metal concentrations

3.1.1 Plasma concentrations in kittiwakes

The studied metals (As, Cd, Cr, Cu, Hg, Pb, Se, Zn) were detected above LOD for all samples. Concentrations of the investigated metals in plasma are summarized in Table 3.1.

Table 3.1 Statistics for concentration (ng/g ww) of arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), selenium (Se) and zinc (Zn) in plasma of kittiwake (*Rissa tridactyla*) at early (n=15) and late sampling (n=13).

	Early sampling (N=15)					***Late sampling (N=13)***				
Metals	*Min*	*Max*	*Median*	*Mean*	*SD*	*Min*	*Max*	*Median*	*Mean*	*SD*
As	7.76	113.70	63.17	59.98	33.55	16.57	113.70	39.99	47.85	28.37
Cd	0.20	0.69	0.36	0.38	0.16	0.15	0.64	0.32	0.33	0.14
Cr	4.36	11.75	6.32	6.66	1.69	4.36	13.19	7.01	7.45	2.14
Cu	63.26	268.47	117.00	141.64	66.35	38.56	212.03	81.16	96.93	45.21
Hg	1.64	9.93	3.57	3.85	2.35	1.76	5.37	3.98	3.88	1.18
Pb	1.15	5.21	3.03	3.52	1.23	1.64	6.77	2.59	3.49	1.74
Se	52.76	517.71	192.30	209.12	108.58	45.90	467.55	192.34	198.99	102.05
Zn	2056.70	4394.27	2606.69	2742.31	615.86	1930.29	4394.27	2626.06	2784.16	722.17

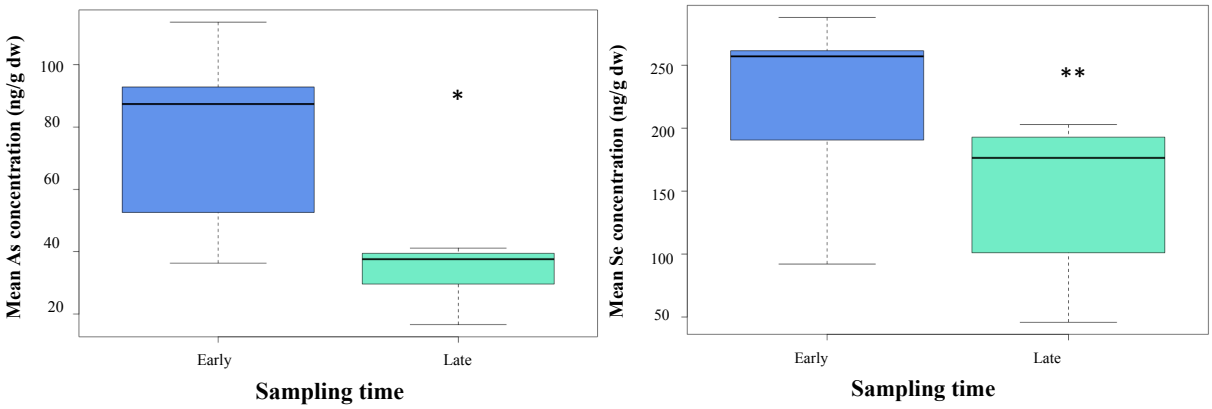

Fig 3.1 Plasma concentrations (ng/g ww) of arsenic (As) (left) and selenium (Se) (right) in male kittiwakes at early (n=7) (blue) and late (n=7) (green) sampling. *p*-value indicated by stars (*) above the boxes, where $p < 0.001$ is indicated by ***, $p < 0.01$ by ** and $p < 0.05$ by *.

Mean concentrations of the metals did not significantly differ between early and late sampling; $p = 0.53$; $p = 0.17$; $p = 0.42$; $p = 0.05$; $p = 0.86$; $p = 0.39$; $p = 0.48$; $p = 0.83$, for As, Cd, Cr, Cu, Hg, Pb, Se and Zn, respectively. When separating between sexes, males at early sampling had significantly higher concentration of As ($p = 0.014$) and Se ($p = 0.001$) compared to males at late sampling (Fig 3.1). Females at late sampling had significantly higher levels of Se ($p = 0.028$) than males at late sampling (Fig 3.2). There was no significant difference between females at early and late sampling.

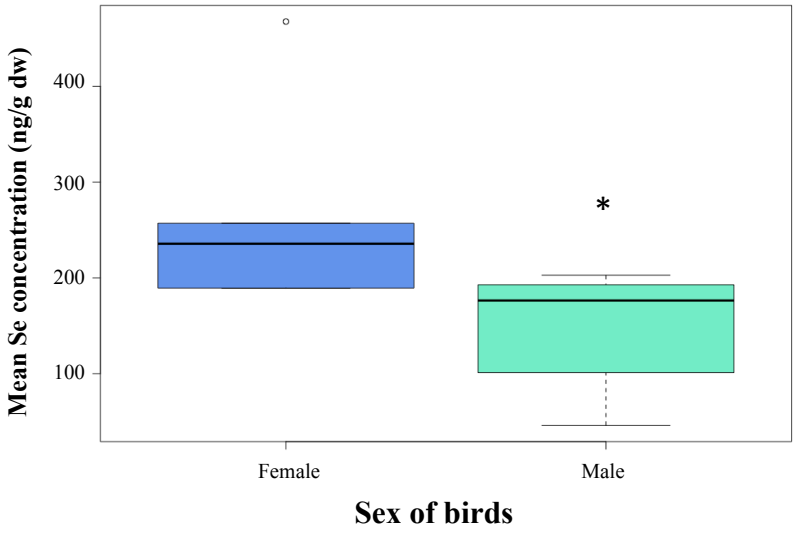

Fig 3.2 Plasma concentrations (ng/g ww) of selenium (Se) in female kittiwakes at late (n=6) (blue) and male kittiwakes at late (n=7) (green) sampling. *p*-value indicated by stars (*) above the boxes, where $p < 0.001$ is indicated by ***, $p < 0.01$ by ** and $p < 0.05$ by *.

3.1.2 Kittiwake feathers

The studied metals (As, Cd, Cr, Cu, Hg, Pb, Se, Zn) were detected above LOD for all samples. Concentrations of the investigated metals in feathers of kittiwakes are summarized in Table 3.2.

Table 3.2 Statistics for feather concentrations (ng/g dw) of arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), selenium (Se) and zinc (Zn) in kittiwake (*Rissa tridactyla*) (n=17).

Metals	*Min*	*Max*	*Median*	*Mean*	*SD*
As	76.71	144.01	116.22	114.64	18.29
Cd	12.38	47.74	17.02	21.14	9.97
Cr	28.21	1883.62	62.61	206.55	459.46
Cu	5168.44	7499.44	6153.58	6295.96	634.76
Hg	3277.11	7905.57	5158.29	5259.47	1122.27
Pb	27.91	98.41	57.39	56.96	21.79
Se	2561.02	4582.82	3311.99	3439.42	621.06
Zn	25504.32	55073.74	44025.36	42220.01	8874.27

3.1.3 Guillemot feathers

The studied metals (As, Cd, Cr, Cu, Hg, Pb, Se, Zn) were detected above LOD for all samples. Concentrations of the investigated metals in feathers are summarized in Table 3.3.

Table 3.3 Statistics for feather concentrations (ng/g dw) of arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), selenium (Se) and zinc (Zn) in Brünnich's guillemot (*Uria lomvia*) (n=13).

Metals	*Min*	*Max*	*Median*	*Mean*	*SD*
As	40.16	101.85	78.20	71.85	20.96
Cd	7.94	26.14	11.79	14.81	6.44
Cr	31.55	171.23	51.51	61.97	34.61
Cu	13047.57	22447.77	15088.09	16333.48	3041.74
Hg	755.60	1698.48	1084.72	1130.03	278.64
Pb	39.14	96.12	79.36	75.81	17.50
Se	1323.34	6241.81	2268.94	2599.24	1355.66
Zn	57219.72	92317.40	71013.56	72912.55	10606.15

3.1.4 Comparing kittiwake and guillemot feathers

Figure 3.3 compares mean feather concentrations (ng/g dw) of eight selected metals in kittiwakes (n = 16) and guillemots (n = 13). Mean concentrations of As, Cd and Hg were significantly higher (p = 4.94E-06; p = 0.04; p = 1.32E-11) in kittiwakes compared with guillemots. Mean

concentrations of As, Cd and Hg were 1.6, 1.4 and 4.7 times higher in kittiwake feathers. Mean concentrations of Cu, Pb and Zn were significantly higher (p = 3.3E-08; p = 0.01; p = 1.6E-08) in guillemots compared with kittiwakes. Mean concentrations of Cu, Pb and Zn were 2.6, 1.3 and 1.7 times higher in guillemot feathers.

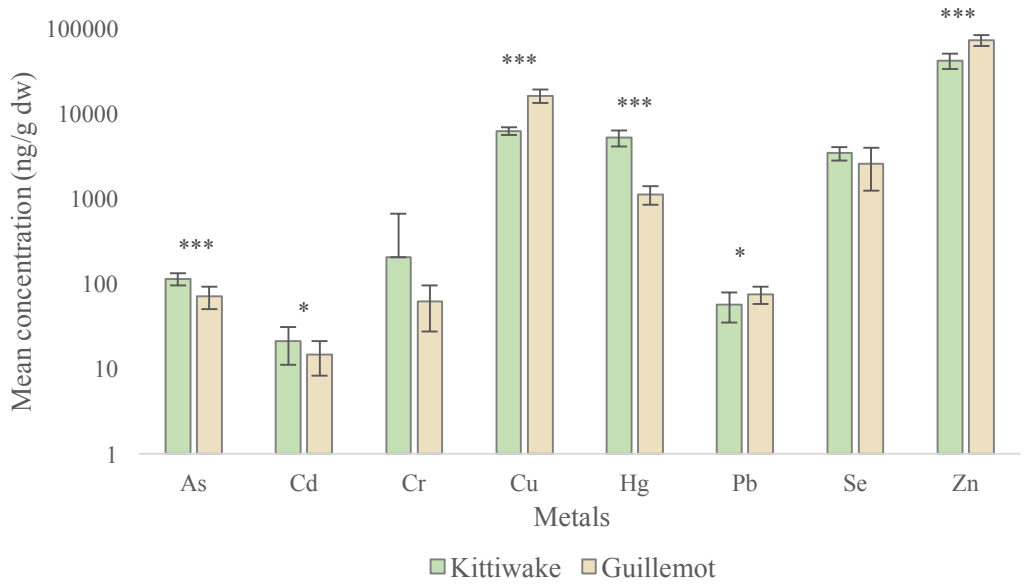

Fig 3.3 Comparison of feather concentration (ng/g dw ± standard deviation) of arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), selenium (Se) and zinc (Zn) in guillemot (*Uria lomvia*) and kittiwake (*Rissa tridactyla*). (Green bars = kittiwake, beige bars = guillemot). Y-axis is log-transformed. *p*-values are indicated by stars (*) above the histograms, where $p < 0.001$ is indicated by ***, $p < 0.01$ by ** and $p < 0.05$ by *.

3.1.5 Correlations

In plasma at early sampling Pb was significantly correlated with Cu (r = 0.86, p = 0.00004) and Cr (r = 0.69, p = 0.004), Hg was significantly correlated with Se (r = 0.62, p = 0.014) and As was significantly correlated with Cr (r = -0.76, p = 0.001). The rest of the metals were not significantly correlated at early sampling. In plasma at late sampling Cu was significantly correlated with Pb (r = 0.63, p = 0.02) and Zn (r = 0.66, p = 0.014). The rest of the metals were not significantly correlated at late sampling. In feathers of guillemots, Zn was significantly correlated with Hg (r = 0.58, p = 0.038) and Cd (r = -0.59, p = 0.035), and Se was significantly correlated with Cu (r = 0.69, p = 0.009). The rest of the metals were not significantly correlated in guillemot feathers. For kittiwake feathers no metals were significantly correlated.

3.2 miR-155 expression in plasma

3.2.1 Kittiwakes from 2014 and 2015

Figure 3.4 shows statistically significant difference ($p = 0.024$) between female (n = 12) and male (n = 5) kittiwakes relative to miRNA-155 molecule count. Mean miR-155 expression is higher in the females compared to the males.

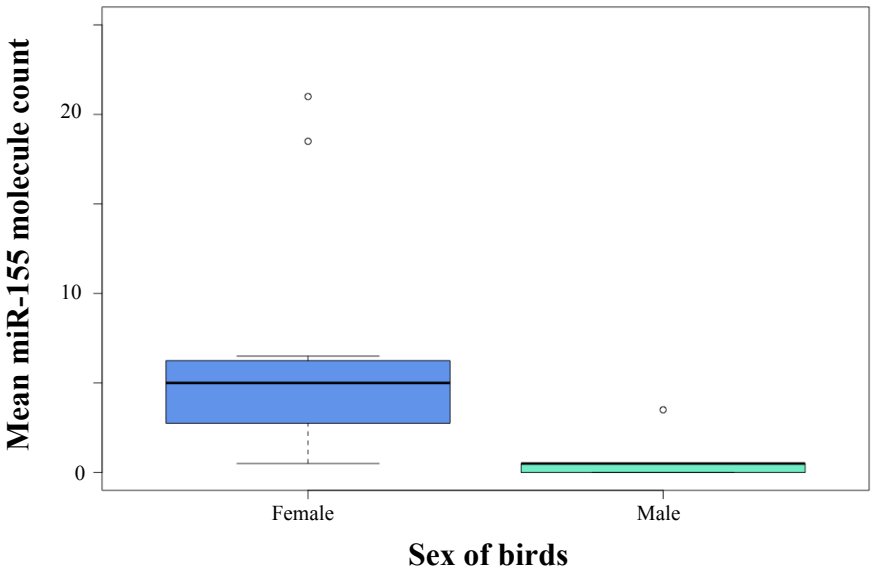

Fig 3.4 Difference in miR-155 molecule count (y-axis) between female (blue box, n=12) and male (green box, n=5) kittiwakes (x-axis). The plots show medians (thick vertical lines in boxes) along with the interquartile range (IQR, box), maximum and minimum values within 1.5 IQR (whiskers) and values outside IQR (outliers).

The ELISA assay revealed that 23% (n = 4) of the kittiwakes were infected with AIV. Figure 3.5 shows statistically significant ($p = 0.009$) difference between AIV-infected (n = 4) and non-infected (n = 13) kittiwakes relative to miRNA-155 molecule count. Individuals infected with AIV have a higher mean miR-155 expression.

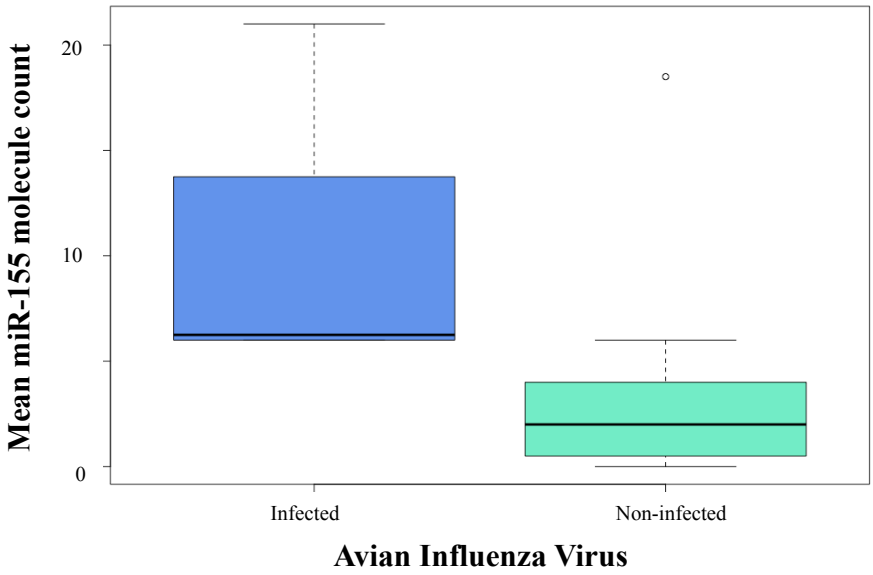

Fig 3.5 Difference in miR-155 molecule count (y-axis) between avian influenza virus (AIV) infected (blue box, n=4) and non-infected (green box, n=13) kittiwakes (x-axis). The plots show medians (thick vertical lines in boxes) along with the interquartile range (IQR, box), maximum and minimum values withing 1.5 IQR (whiskers) and values outside IQR (outliers).

Figure 3.6 shows the levels of p,p'-DDE (pg/g) relative to the mean miR-155 molecule count in the kittiwakes. Kittiwakes with lower levels of p,p'-DDE seem to have a higher expression of miR-155.

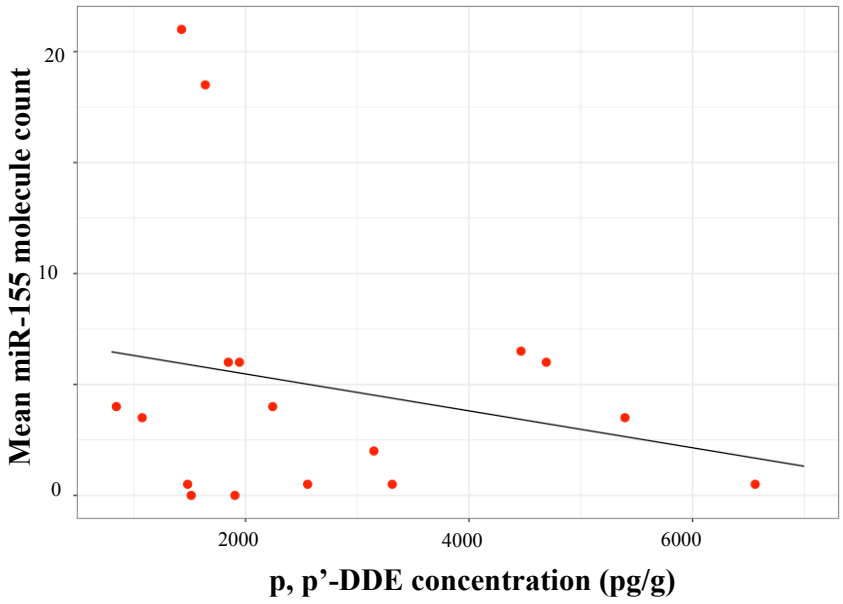

Fig 3.6 Mean miR-155 molecule count relative to concentration of ethylene, 1,1-dichloro-2,2-bis(p-chloropheynyl) (p,p'-DDE) (ng/g) in kittiwakes (n=17). R^2 = 0.051

Figure 3.7 shows the levels of sumPCBs (pg/g) relative to the mean miR-155 molecule count in the kittiwakes. The results show high expression of miR-155 in individuals with both highest and lowest levels of PCBs.

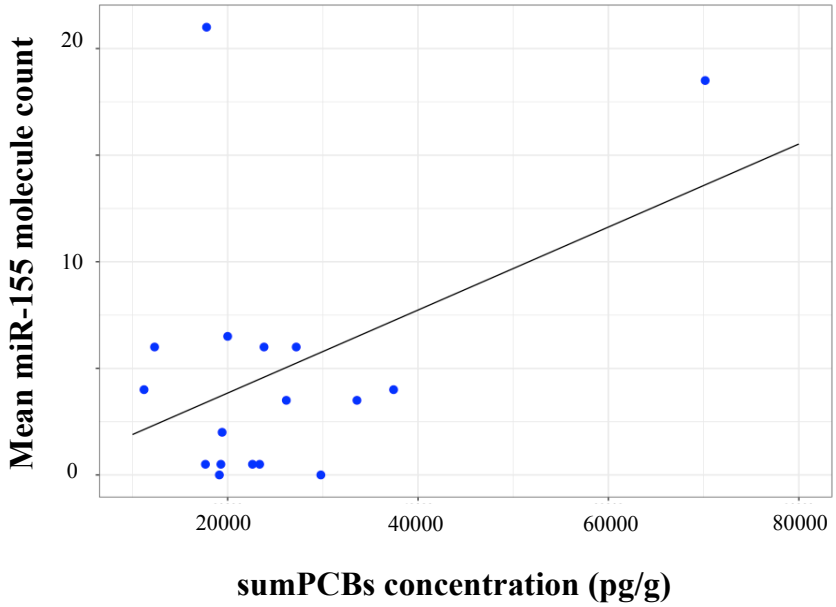

Fig 3.7 Mean miR-155 molecule count relative to concentration of sum of polychlorinated biphenyls (sumPCBs) (pg/g) in kittiwakes (n=17). $R^2 = 0.185$

3.2.2. Kittiwakes from 2015

The ELISA assay revealed that 66% (n = 4) of the kittiwakes were infected with AIV. Figure 3.8 shows the difference between AIV-infected (n = 4) and non-infected (n = 6) kittiwakes relative to miRNA-155 molecule count. Individuals infected with AIV have a higher mean miR-155 expression.

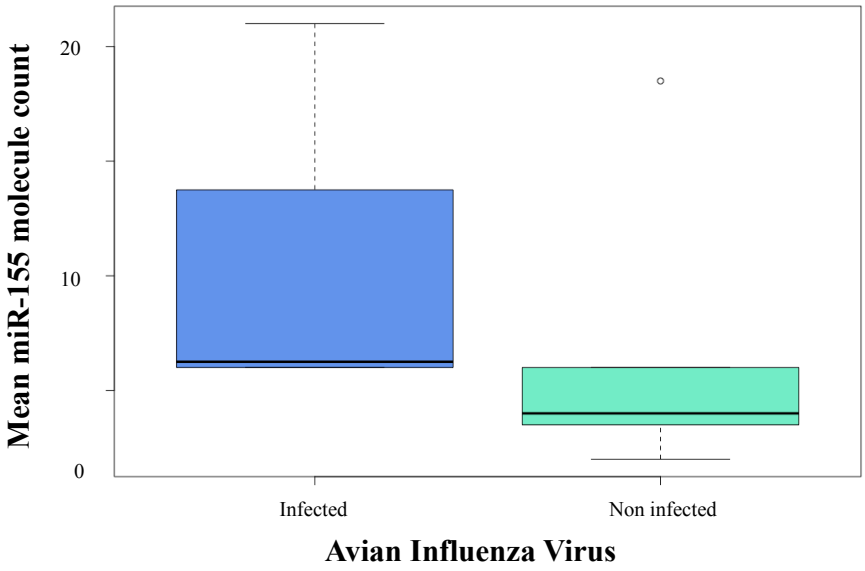

Fig 3.8 Difference in miR-155 molecule count (y-axis) between avian influenza virus (AIV) infected (blue box, n=4) and non-infected (green box, n=6) kittiwakes (x-axis). The plots show medians (thick vertical lines in boxes) along with the interquartile range (IQR, box), maximum and minimum values withing 1.5 IQR (whiskers) and values outside IQR (outliers).

Figure 3.9 shows the levels of Se (ng/g dw) in RBCs relative to the mean miR-155 molecule count in the kittiwakes. Lower levels of Se seem to be related to higher expression of miR-155.

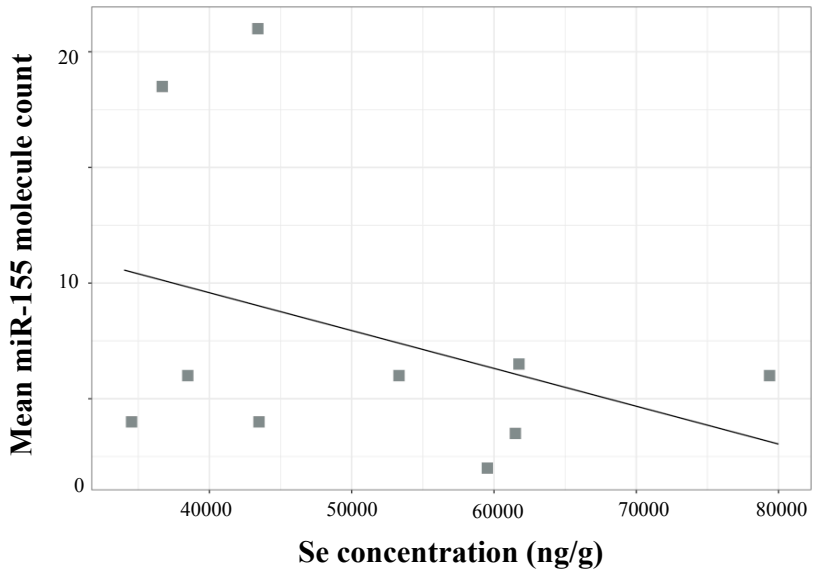

Fig 3.9 Variation in miR-155 molecule count (y-axis) relative to selenium (Se) levels (ng/g) in the kittiwake red blood cells (RBCs) (n=10). $R^2 = 0.13$

Figure 3.10 shows the levels of Pb (ng/g dw) in RBCs relative to the mean miR-155 molecule count in the kittiwakes. Higher levels of Pb seem to be related to higher expression of miR-155.

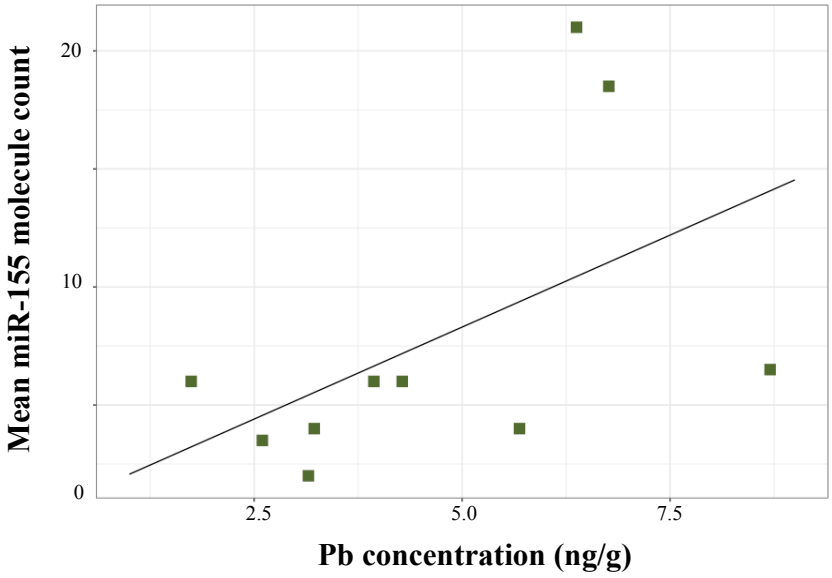

Fig 3.10 Variation in miR-155 molecule count (y-axis) relative to lead (Pb) levels (ng/g) in the kittiwake red blood cells (RBCs) (n=10). $R^2 = 0.271$

Figure 3.11 shows the levels of As (ng/g dw) in RBCs relative to the mean miR-155 molecule count in the kittiwakes. Lower levels of As seem to be related to higher expression of miR-155.

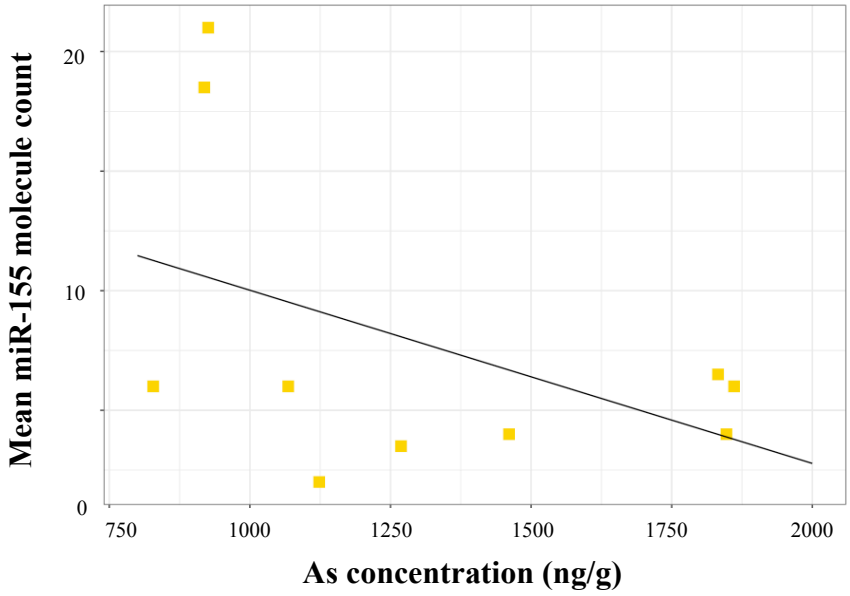

Fig 3.11 Variation in miR-155 molecule count (y-axis) relative to arsenic (As) levels (ng/g) in the kittiwake red blood cells (RBCs) (n=10). $R^2 = 0.29$

4 Discussion

The two main aims of this thesis were to investigate levels of environmental contaminants in seabirds breeding on Svalbard and to relate the contaminant levels to immunomodulation by measuring expression of miR-155. Unfortunately, not enough blood was sampled from kittiwakes or guillemots in 2017 to be able to investigate miR-155 expression in plasma. It would have been interesting to compare miR-155 expression in kittiwakes sampled from the same location during different years, as well as comparing with miR-155 levels in guillemot plasma. Despite this, it is still relevant to compare metal concentrations in kittiwakes and guillemots, to see if there are any significant differences in metal concentrations between the species. Further, it is relevant to investigate expression of miR-155 in the kittiwakes sampled in 2014 and 2015 based on their contaminant levels, especially since some of them were infected with AIV.

4.1 Metal concentrations

4.1.1 Plasma concentrations in kittiwakes

For concentrations of Cu in plasma at early and late sampling the close to significant p value (p = 0.051) suggest a trend towards higher levels of Cu at early sampling compared to late sampling with mean concentrations of 141.64 and 96.93 ng/g ww respectively. This difference can reflect differential intake of Cu-containing foods between early and late sampling. Mussels, snails and crustaceans can have high Cu levels since they have haemocyanin as their blood pigment (Savinov et al., 2003). Cu is considered essential for formation of feathers (Nygård et al., 2001), cell physiology and optimal function and structure of proteins (Janssens et al., 2003). The concentration of Cu is metabolically regulated in seabird tissues (Schneider et al., 1985) and levels can fluctuate, for instance due to co-accumulation with cadmium, as they both bind to the same metallothionein (Bocher et al., 2003). Plasma concentrations of the other selected metals (As, Cd, Cr, Hg, Pb, Se and Zn) were not significantly different between early and late sampling. Originally sampling was divided into two time periods to investigate changes in concentrations of circulating lipophilic compounds as the kittiwakes decrease body mass during breeding season (Henriksen et al., 1996). But, as mentioned in the methods section, not enough blood was sampled to be able to conduct such an analysis.

Plasma concentrations of some metals did become significant between early and late sampling when separating between sexes. Males at early sampling had significantly higher concentrations of As and Se compared to males at late sampling (Fig 3.1). Females at late sampling had significantly higher Se levels than males at late sampling (Fig 3.2). It has been observed that despite sexes having similar diets they might differ in the areas from which they capture food, with males moving further from the colonies to feed (Coulson, 2011), which could explain the difference. However, most studies on Arctic seabird species have shown no or little differences in bioaccumulation of metals between sexes (Borgå et al., 2006), indicting similar feeding habits and metabolism between genders (Jæger et al., 2009).

Bioaccumulation of metals in seabird tissues is influenced by various factors, such as phylogeny, molt pattern, sex, life span and diet (Bocher et al., 2003). For metals such as Cd and Hg diet seems to be one of the factors explaining differences between avian species the most (Bocher et al., 2003). As mentioned, concentrations of essential metals are regulated in the body (Walsh, 1990), but can fluctuate within internal tissues (Bocher et al., 2003). This regulation might be the reason for difference in Se concentrations between early and late sampling observed in this master thesis (Fig 10 & 11), since Se is also considered nutritionally essential in small amounts. Another reason can be fluctuating Hg concentrations influencing Se concentrations, as Hg and Se are thought to interact (e.g. Cuvin-Aralar & Furness, 1991). Levels of Hg has been observed to vary depending on the sex of the bird, usually higher in males than females (Braune & Gaskin, 1987), since females can transfer 20% of their soft tissue MeHg burden to eggs (Lewis et al., 1993). This, however, was not the case for the sampled kittiwake plasma, which might indicate that the females had much higher levels of Hg before egg-laying, and reduced their levels to equal the levels in male plasma by maternal transfer.

The metal results from the kittiwakes in 2017 in plasma are not directly comparable to previous studies in whole blood or red blood cells. In blood, metals are distributed between non-cellular (plasma/serum) and intra-cellular compartments (mostly RBCs). Metals have varying affinity for each compartment, depending on their chemical properties (Schultze et al., 2014). Due to variation in this affinity, metals are usually monitored and measured in whole blood, but there are several experiments investigating levels of metals in RBCs or plasma. RBCs can, for example, be used as a biomarker of high exposure to Cr shortly after exposure has occurred. Any of the Cr inside a RBC is likely to derive from Cr(VI), since Cr(III) is barely able to enter the cell (Landgård &

Costa, 2015). While Pb is mainly present in RBCs, measuring Pb concentration in plasma is more relevant for biomonitoring since lead in plasma is more bioavailable and readily transported to target organs (Skerfving & Bergdahl, 2015).

The metal concentrations in kittiwake plasma are much lower compared with RBC concentrations measured for As, Cd, Hg, Pb, Se and Zn in kittiwakes sampled in 2015 (Castaño-Ortiz, 2016) (Table 4.1). This might indicate more accumulation of metals in RBCs compared with plasma. The only exception was the Pb concentrations, which were not that much higher compared to the Pb concentrations in the plasma (4,65 ng/g dw vs 3,51 ng/g ww, respectively). Perhaps Pb is distributed evenly between RBCs and plasma in blood, indicating that the Pb levels in the kittiwakes sampled in 2015 are equal to levels in those sampled in 2017. But if Pb levels are usually much higher in RBCs compared with plasma, this might indicate high levels of Pb in kittiwakes sampled in 2017. The second hypothesis is quite likely, since Pb has a high affinity for binding to ALAD (δ-aminolevulinic acid dehydratase), which is found in RBCs. When ALAD is saturated, more Pb can be found in the plasma (Skerfving & Bergdahl, 2015), which is perhaps the case for the 2017 kittiwakes.

Table 4.1 Comparing levels of arsenic (As), cadmium (Cd), mercury (Hg), lead (Pb), selenium (Se) and zinc (Zn) in red blood cells (RBCs) of kittiwakes sampled in 2015 (Castaño-Ortiz, 2016) with the same metals in plasma of kittiwakes sampled in 2017.

Metals	**2015** ng/g dw (RBCs)	**2017** ng/g ww (plasma)	
		Early sampling	*Late sampling*
As	1313.45	59.98	47.85
Cd	10.15	0.38	0.31
Hg	347.25	3.85	3.98
Pb	4.647	3.52	3.49
Se	51204.9	209.12	198.99
Zn	8023.78	2741.31	2784.16

When comparing non-essential metals detected in the kittiwakes sampled in 2015, As is the metal at highest concentrations in both plasma and RBCs, followed by Hg. Considering As is rapidly cleared from the blood within a few hours of exposure (Mandal et al., 2003), these levels indicate continuous exposure of As to the kittiwakes at their breeding location. Savinov et al (2003) suggests there could be a local source of As contamination in the Barents Sea area, as high levels have also been found in bottom sediments and in fish (Loring et al., 1995; Savinov et al., 2003). When comparing essential metals detected in the kittiwakes sampled in 2015, the trend for Zn and Se in plasma is the opposite: Se is detected at much lower concentrations than Zn in plasma, while

in RBCs the trend is the opposite. Perhaps this is an indication of Zn being more distributed in plasma than RBCs, while Se is more distributed in RBCs.

4.1.2 Feather concentrations in kittiwakes and guillemots

Feathers can be considered the most advantageous non-destructive matrices for biomonitoring since they are easy to collect, store and transport. In addition, feathers can be sampled repeatedly from a living bird, without seriously affecting the fitness of the bird (Abbasi et al., 2014). During feather growth, metals accumulate in feathers in proportion with the blood concentrations (Eens et al., 1999) and therefore, feathers can be used as a tool to investigate metal exposure during feather formation weeks or months earlier (Burger et al., 2008). After the feather mature, the vascular connection atrophies, which leaves the levels of metals in the feather as a record of blood levels at the time of its formation, and the metal concentration inside the feathers remain nearly constant (Braune & Gaskin, 1987). Due to the energetic cost of molting, new feather formation occurs outside of the breeding season, usually on the species wintering ground (Burger & Gochfeld, 1991). Therefore, it is most likely that feathers sampled in July 2017 represent metal levels kittiwakes and guillemots were exposed to while at their wintering area and does not reflect contamination at the Arctic breeding ground, like the plasma concentrations. Like many seabird species, kittiwakes and guillemots spend most of the year away from their breeding sites. Kittiwakes has been observed to be distributed throughout the North Atlantic outside breeding season, with a major concentration in central and western parts (Fredriksen et al., 2011). It is highly likely that kittiwakes switch to very different food during their pelagic winter distribution, since there are few small fish in the surface waters of the North Atlantic and Pacific oceans during winter (Coulsen, 2011). Recoveries of ringed guillemots indicate that many birds from the Svalbard population winter off mainly in Greenland, as well as Iceland and Newfoundland (Bakken & Mehlum, 2005).

Metals can be deposited from the atmosphere onto the surfaces of feathers, and can further be incorporated into growing feathers from the blood, which could confound the use of feathers to monitor metal pollution via food webs. Cd and Pb, for example, seem to originate from direct atmospheric deposition onto feather surfaces, while ingested Cd and Pb accumulate in kidney and bone respectively, and only enter feathers in small amounts (Furness & Camphuysen, 1997). Therefore, it is likely to assume that Cd and Pb levels measured in the feathers of kittiwakes and

guillemots reflect atmospheric contamination, and not only contamination via the diet. The levels of Cd and Pb are however the lowest of the selected metals measured in both kittiwake and guillemot feathers (Fig 3.3). Other confounding factors are that the plumage of seabirds is regularly flushed by water, which can wash off external contaminants, while at the same time contaminants transported in the water can then become attached to the feathers (Jaspers et al., 2007). It has also been suggested that seabirds can secrete metals together with salt gland secretions (Burger et al., 2000), and thereby spread them on their feathers when they wipe their beaks against themselves.

Levels of metals were significantly higher in kittiwake feathers compared to kittiwake plasma on a wet weight basis, which was expected since many metals are sequestered into feathers as a form of excretion. The feather concentrations of metals are not directly comparable with the levels measured in plasma, since feather concentrations reflect exposure at overwintering areas (Svendsen et al., 2018). Feathers have been considered good biomarkers for pollution in terrestrial and resident bird species, but since kittiwakes are migratory, the feathers sampled are not good biomarkers for pollution at the breeding grounds (Svendsen et al., 2018). Molting is considered an important excretion process of Hg, especially MeHg (Thompson & Furness, 1989). About 50-70% of total Hg burden has been observed to be present in the plumage (Honda et al., 1986). Hg is the non-essential metal of highest concentration measured in the kittiwake feathers, at a mean concentration of 5259.47 ng/g dw (Table 3.2). Hg levels in feathers associated with adverse reproductive effects in birds (e.g. lower clutch and egg size, lower hatching rate, decreased chick survival) are 5000 ng/g (Eisler, 1987), which indicate that the kittiwakes sampled are at risk of reduced reproductive success. Burger et al (2008) investigated As, Cd, Cr, Pb, Mn, Hg and Se in feathers of kittiwakes from Prince William Sound, Alaska. Comparing with the kittiwakes sampled for this master thesis, the mean feather levels of As, Cd, Cr and Pb were higher in the Alaskan kittiwakes, while mean feather levels of Hg and Se were higher in the kittiwakes sampled in Ny-Ålesund. Burger et al (2008) investigated age-related differences in feather concentrations, and even the oldest kittiwakes (over 13 years), which had the highest Hg and Se values, had lower levels compared to the kittiwakes sampled in Ny-Ålesund. Burger & Gochfeld (2000) suggest a concern level of Se in feathers at 1800 ppb (1800 ng/g), which is lower than levels detected in feathers of kittiwakes sampled for this master thesis, as well as the birds investigated by Burger & Gochfeld (2000). Therefore, they suggested that this level of concern was too low for seabirds, or perhaps that there were subtle effects that have not yet been seen.

The levels of metals can vary widely among different seabird species (e.g. Wenzel & Gabrielsen, 1995) depending on the differences in feeding ecology, intensity and timing of exposure in foraging areas, as well as the bird's physiological and biochemical characteristics (Savinov et al., 2003). In terms of differences in diet, the kittiwake is considered a pelagic surface-feeder, while the guillemot is considered a pelagic diver (Norderhaug et al., 1977). For this master thesis, the levels of As, Cd and Hg were significantly higher in kittiwakes compared with guillemots (Fig 13). Diet has been suggested to be one of the main factors that explain differences in Hg and Cd among species (Bocher et al., 2003). Seabirds species with substantial amounts of crustaceans in their diet have been observed to have lower Cd and Hg concentrations than those whose diet predominantly consisted of squid and fish (Stewart et al., 1997). This indicates that the diet of the guillemots perhaps consists of more crustaceans compared with the kittiwakes, since the kittiwake feathers contained 1.4 times more Cd and 4.7 times more Hg than the guillemot feathers. Savinov et al (2003) also found higher levels of Cd and Hg in muscle and liver of kittiwakes compared to guillemots. They suggested that higher levels of Cd in kittiwakes could be due to feeding on invertebrates, such as euphausiids and amphipods, or due to exposure during winter migration, since concentrations of Cd in fish and invertebrates from southern winter grounds of kittiwakes are higher compared to the Barents Sea.

The levels of Cu, Pb and Zn measured in this master thesis were significantly higher in guillemots compared with kittiwakes (Fig 12). Both Cu and Zn are considered essential and their concentrations are therefore metabolically regulated in seabird tissues (Schneider et al., 1985). In feathers, essential metals can fluctuate by interaction of other metals, metabolism, body condition and environmental contamination (Kim & Oh, 2014; Jerez et al., 2011; Deng et al., 2007). Concentrations of Zn and Cu in the internal tissues can fluctuate due to co-accumulation with cadmium, as all three metals can bind to the same metallothionein (Bocher et al., 2003). High levels of Cd have been reported to induce metallothionein synthesis, which leads to greater binding of Zn (Honda et al., 1990). Pb is a non-essential metal that at concentrations of 4 μg/g dw in feathers has been associated with several behavioral, physiological, and nutritional toxicities in wild birds (Burger & Gochfeld, 1994), causing alterations in the nervous system, behavioral deficiencies and growth slowdown (Burger & Gochfeld, 2000). The Pb concentration measured in the guillemot feathers for this master thesis (Table 4) are much lower compared to the Pb toxicity threshold.

Wenzel & Gabrielsen (1995) investigated Hg, Se, Cu, Cd and Zn in body feathers of kittiwakes and guillemots collected at Hornøya (northern Norway). Comparing with the kittiwakes sampled for this master thesis, the mean feather levels of Cu, Cd and Zn were higher in the kittiwakes sampled at Hornøya, while mean feather levels of Hg and Se were higher in Kittiwakes sampled in Ny-Ålesund. Comparing with the guillemots sampled for this project, the mean feather levels of Cd were higher in the guillemots sampled at Hornøya, while mean feather levels of Hg, Se, Cu and Zn were higher in guillemots sampled at Svalbard.

Thompson et al (1992) compared Hg concentrations in body feathers of kittiwakes and guillemots from various sites in the north-east Atlantic (northwest Iceland, Shetland, east Scotland and northeast Norway). Levels were significantly higher in the kittiwakes from northwestern Iceland, at a mean level of 5.5 µg/g fresh weight, which is equal to the mean levels measured in kittiwakes in 2017 from Ny-Ålesund. For the guillemots, the Hg levels were also significantly higher in the birds sampled from northwestern Iceland, at a mean level of 2.1 µg/g fresh weight. The levels measured in the guillemots in 2017 from Ny-Ålesund were lower, equal to the levels measured in northeastern Norway, at 1.3 µg/g fresh weight. Thompson et al (1992) discuss several influential factors that determine the observed patterns of Hg concentration in seabirds, for example the oceanic transport of Hg, together with effects of anthropogenic inputs of Hg to the northeast Atlantic, and the removal of Hg from the water column via biological activity.

Overall there seems to be a pattern of more Hg and Se sequestered in the feathers of kittiwakes and guillemots breeding in Ny-Ålesund compared with e.g. Alaska (Burger et al., 2008) and Northern Norway (Wenzel & Gabrielsen, 1995). It is important to take into consideration that both Wenzel & Gabrielsen (1995) and Thompson et al (1992) investigated these metal levels over 20 years ago, and that the differences observed could be due to temporal changes in Hg distribution over time. The differences could also be due to different geographical distribution of Hg.

4.2 Parameters influencing miR-155 expression in kittiwakes sampled in 2014 and 2015

The linear model explaining variation in miR-155 expression of kittiwakes sampled in 2014 and 2015 (Eq 2.2) included four different fixed covariates from a total of 12 parameters. These covariates were; sex of birds ($p = 0.024$), AIV infection ($p = 0.009$), levels of p,p'-DDE ($p = 0.014$)

and levels of PCB (p = 0.01). Based on p values AIV infection seems to have the most influence on the linear model explaining the variation of miR-155 expression, while sex of birds seems to have the least influence.

4.2.1 Sex-specific differences

The results of this project indicate that there is a sex-specific difference in relation to expression of miR-155 (Fig 3.4). On average the female kittiwakes had significantly higher expression of the miRNA compared to the males (p = 0.024). The difference observed could be due to sex differences in immunocompetence or susceptibility to diseases. Studies investigating this in natural bird populations are unfortunately rare and inconsistent (e.g. Møller et al., 1998; Moreno et al., 2001; Tschirren et al., 2003), but a review by Nunn et al (2009) observed that most studies show higher immunocompetence in females compared to males. The sexual dimorphism observed in many different organisms is thought to arise due to difference in gene expression between sexes, because of natural and/or sexual selection for traits that influence the fitness of the sexes (Ellegren & Parsch, 2007).

The results could also be due to sex-specific differences in miRNA expression, which might arise due to either differential gene dosage (number of gene copies) between the sexes, or through various types of sex-specific regulation. Contrary to mammals, birds do not have a global mechanism of dosage compensation, which means Z-linked coding genes tend to be more highly expressed in males compared to females. Male birds have two Z chromosomes, while female birds have one Z and one W chromosome (Warnefors et al., 2017). However, miR-155 is processed from an exon of a non-coding RNA transcribed from BIC, which is located on chromosome 21 (Lagos-Quintana et al., 2002), and is therefore not linked to either sex chromosome. Different hormones have been found to target miRNA regulation, and in humans the expression of several miRNAs is seemingly regulated by sex hormones (Yang & Wang, 2011). The expression of key factors that are involved in the processing of primary miRNA transcripts into mature miRNAs has also been observed to be sensitive to sex hormones (Nothnick et al., 2010).

The variation in miR-155 expression between female and male kittiwakes can be due to significantly higher concentration of p,p'-DDE in females compared to males (p = 0.026). As will be discussed later, p,p'-DDE is a possible immunomodulator, and it is perhaps because females have higher levels of this contaminant that they also have higher expression of miR-155. Another

possibility is that p,p'-DDE can regulate miRNA expression on a cellular level, and is therefore causing higher miR-155 expression in the individuals with higher contaminant levels. Concerning the other contaminants (e.g. PCBs, HCB) there was no significant differences between sexes.

The difference in miR-155 expression can be due to the small sample size (n = 17) being skewed towards the female sex (f = 12, m = 5). Therefore, future investigations of expression of miR-155 in wild bird populations such as kittiwake should be done with a larger sample size, with an equal number from each sex. This can help determine if there actually is a higher baseline expression in the females compared to males, or if this effect seen in this master thesis is just due to chance.

4.2.2 AIV infection

Results indicate that infection of AIV in the kittiwakes is related to higher expression of miR-155 (Fig 3.5). This suggests that higher expression of miR-155 could be detrimental and facilitates virus replication in the infected kittiwakes. It has been suggested that overexpression of miR-155 could initiate excessive antiviral responses that contribute to viral infection-induced tissue damage (e.g. Pothlichet et al., 2008; Yang et al., 2008). Higher expression of miR-155 in infected birds could also indicate activation of the immune response in these kittiwakes compared to the non-infected individuals, e.g. Lu et al (2008) observed that primary human B lymphocytes infected with Epsein-Barr Virus (EBV) lead to sustained elevation of miR-155. Upregulation of miR-155 has been shown to result in an outcome that is favorable for the host during virus infections (Waugh et al., 2018). Wang et al (2010) have suggested that RNA virus infection upregulates the expression of miR-155, mainly through the RIG-I/JNK/NF-kB-dependent pathway. Via this pathway, induced miR-155 promotes type I IFN (interferon) signaling, which helps suppress viral replication. Induced miR-155 also suppresses SOCS1 (suppressor of cytokine signaling 1), a negative regulator of several immune responses and signal pathways, including type I IFN signaling. Suppression of SOCS1 translation by miR-155 subsequently promotes type I IFN-mediated antiviral response (Wang et al., 2010). In the experiment by Waugh et al (2018) the results indicated that the chicken fibroblasts responded to viral cues similarly to mammalian cells, by upregulating the expression of miR-155. A downregulation of the miRNA has been observed to result in increased viral titres and mortality in different virus models and species (e.g. Lindsay, 2008; Faroni et al., 2009; Lind et al., 2013).

miR-155 can participate in both physiological antiviral immune response and pathological viral infection-induced damage, which indicates that induced expression of miR-155 by viruses could be a double-edged sword. This is probably the reason why miR-155 is mostly moderately induced in macrophages and other antigen presenting cells upon a virus challenge, since a deregulation of miR-155 expression could potentially contribute to pathogenesis of viral infection-induced pathological responses (Wang et al., 2010).

Infected birds (n = 4) had significantly lower concentration of HCB compared to non-infected birds ($p = 0.029$), which is not comparable with Bustnes et al (2004), where they found evidence of decreased immune responses against diphtheria toxoid in female glaucous gulls with high HCB levels. However, HCB was not included in the model explaining variation in miR-155 molecule count. Therefore, it appears that HCB may play a role in viral infection, but does not affect expression of miR-155. Infected birds also had higher mean concentrations of p,p'-DDE (3132 pg/g vs 2579 pg/g) and lower mean concentration of PCBs (22203 pg/g vs 26319 pg/g) compared with the non-infected birds. However, these differences were not statistically significant.

All kittiwakes infected with AIV were female. This does not necessarily mean that female kittiwakes are more susceptible to infection of this virus, again considering the sample size (n = 17) that was highly skewed towards female sex.

4.2.3 Organochlorine compounds

The results indicate an inverse relationship between mean miR-155 expression and p,p'-DDE levels, with lower expression in kittiwakes with higher concentrations of p,p'-DDE (Fig 3.6). p,p'-DDE is an antiandrogenic metabolite of the pesticide DDT (Quinn et al., 2008). Embryonic exposure to p,p'-DDE has been demonstrated to alter the development of the bursa of Fabricius in Japanese quail, which could have adverse effects on B cell maturation, further affecting humoral immunity (Quinn et al., 2006).

The results indicate an inverse relationship between mean miR-155 expression and PCb levels, with higher expression in kittiwakes with lower concentrations of PCBs (Fig 3.7). One of the individuals with the highest expression of miR-155 also had the highest concentrations of PCBs, almost 3 times higher compared to the mean concentration (70164 pg/g vs mean of 25351 pg/g). This might be a possible outlier regarding miR-155 expression, influencing the trend line towards

a positive correlation between the two parameters. The results of the study by Waugh et al (2018) supports this theory, as PCB exposure to chicken embryo fibroblasts showed significant downregulation of miR-155. But, PCB exposure could perhaps also upregulate miR-155, to an extent that it could be detrimental. As mentioned previously, miR-155 is perhaps at its most optimal at a moderate expression, and deregulation (either up- or downregulation) of this optimal expression can have adverse effects.

A field study by Grasman et al (1996) provided strong epidemiological evidence for associations between perinatal exposure to organochlorines (PCBs, p,p'-DDE, dieldrin, Mirex, HCB) and suppression of T-cell mediated immune function in herring gulls and Caspian terns. They found strong evidence that T cell immunity decreased as total PCB and DDE exposure increased. Bustnes et al (2004) observed positive relationships between organochlorines (HCB, oxychlordane, p,p'-DDE, PCBs) and levels of heterophils and lymphocytes in the blood of breeding glaucous gulls. Mean concentrations of DDE and PCB-153 (61.50 ng/g and 113.28 ng/g DDE in female and male gulls, 106.61 and 1237.74 ng/g PCB-153 in female and male gulls) were higher for the glaucous gulls compared to the mean concentrations of p,p'-DDE and sumPCBs detected in kittiwakes (2,709 and 23,53 ng/g for p,p'-DDE and sumPCBs, respectively). Sagerup et al (2001) observed that intensity of parasitic nematodes increased with organochlorine levels in glaucous gulls. All these studies indicate that both PCBs and p,p'-DDE are possible immunomodulators, but they do not necessarily exert their adverse effects on immune functions regulated by miR-155 expression. However, Krauskopf et al (2017) investigated regulation of miRNA expression by different POPs (e.g. PCBs, DDE, HCB) and observed alterations in the miRNA machinery upon environmental exposure to POPs in a population-based study on humans. Several of the miRNAs are related to different types of cancer and involved in relevant signaling pathways and can therefore potentially contribute to biomarker-based environmental health risk assessment. miR-155 was not one of the miRNAs investigated in this study, but it seems likely that POPs can regulate the expression of miR-155 as well, since they seem to have an impact on several other miRNAs.

4.3 Metals influencing miR-155 expression in kittiwakes sampled in 2015

The linear model explaining variation in miR-155 expression of kittiwakes sampled in 2015 (Eq 2.3) included four different fixed covariates from a total of 16 parameters. These covariates were;

AIV infection and levels of Se, Pb and As. Three out of four covariates were different for this model compared to the first linear model (Eq 2.2), which included kittiwakes sampled in 2014. Based on *p* values As seems to have the most influence on the linear model explaining the variation of miR-155 expression, while Pb seems to have the least influence. Birds infected with AIV (n = 4) had higher mean concentrations of Se, Pb and As (55750 vs 48175 ng/g, 5.190 vs 4.28 ng/g, 1421.8 vs 1241 ng/g) compared with non-infected birds (n = 6). But these differences were not significant ($p > 0.05$).

4.3.1 AIV infection

Results for the second linear model (Eq 2.2) indicate that infection of AIV in the kittiwakes is related to higher expression of miR-155 (Fig 3.8). Birds infected with AIV (n = 4) had higher mean concentrations of Se, Pb and As (55750 vs 48175 ng/g, 5.190 vs 4.28 ng/g, 1421.8 vs 1241 ng/g) compared with non-infected birds (n = 6), but these concentrations were not significantly different ($p > 0.05$). Kozul et al (2009b) observed that chronic As exposure had a significant impact on the response of mice to AIV infection (H1N1) compared to mice not exposed. Exposure to As in combination with infection significantly increased morbidity and viral titres compared with the As-unexposed mice (Kozul et al., 2009b). The mice were exposed to 100 pb As (100 ng/g) via drinking water for five weeks, while the mean As concentration in the RBCs of the infected kittiwakes were about 14 times higher. The disease signs associated with AIV infection in avian species varies significantly with the strain of the virus, and most AIV strains are completely asymptomatic in infected birds (WHO, 2002). Since there were no visible signs of disease in the infected kittiwakes, it seems like they are infected with a strain causing them no visible harm, unlike the mice. It also suggests that the kittiwakes are perhaps more immunocompetent than the mice, with regards to AIV infection.

4.3.2 Se

According to the results there seems to be higher mean miR-155 molecule count in the kittiwakes with lower levels of Se (Fig 3.9). Se intakes has a relatively narrow margin between deficiency and toxicity (WHO, 1996), with toxic effects being related to level of exposure and Se status (Fairweather-Trait et al., 2010). Studies with experimental and farm animals show that Se deficiency influences both cell-mediated and humoral components of the immune system

(Fairweather-Trait et al., 2010), and has also been suggested to play a role in viral diseases (e.g. Beck et al., 2003). Most likely the kittiwakes sampled here do not suffer from Se deficiency, considering the high mean values measured in RBCs (51204.9 ng/g dw). Perhaps these values are high enough cause toxic effects in the birds. Adams et al (2009) derived Se toxicity thresholds for birds based on field and laboratory data. The derived teratogenicity EC10 values for Se were estimated to range from 16 to 24 mg/kg dry weight depending on species groups and laboratory and field data. This indicates that mean value of over 50 000 ng/g or 50 mg/kg measured in the kittiwakes could be high enough to potentially have toxic effects in the kittiwakes. Subchronic effects of sodium selenite and selenomethionine on several immune functions has been observed in mallards (*Anas platyrhynchos*) (Fairborther & Fowles., 1990). The mallards were exposed via drinking water to 0, 0.5 and 3.5 mg/L Se as sodium selenite and 2.2 mg/L as selenomethionine, levels which are much higher than those measured in kittiwake RBCs. Most aquatic birds are likely exposed to Se as L-selenomethionine from ingested insects and fish (Spallholz & Hoffman, 2002).

4.3.4 Pb

According to the results there seems to be higher mean miR-155 molecule count in the kittiwakes with higher levels of Pb (Fig 3.10). Various studies have demonstrated that Pb exposure will dampen the immune response to bacterial infections. Pb has also been observed to interfere with interactions between macrophages and T cells and to reduce macrophage functions such as phagocytosis, cell adhesion and nitric oxide production, while increasing antioxidant activity. These responses can be due to the ability of Pb to affect the expression of genes associated with the innate immunity (Hultman & Pollard, 2015). Trust et al. (1990) found that ingested Pb impaired the antibody production in mallards, but the highest Pb blood levels during the experiment were almost 7 ppm (7000 ng/g), which is almost 2000 times higher compared to the mean Pb levels measured in kittiwake RBCs (4.647 ng/g).

4.3.3. As

According to the results there seems to be higher mean miR-155 molecule count in the kittiwakes with lower levels of As (Fig 3.11). As has been identified as a potent immunomodulatory agent in many experimental models and epidemiological studies (Kozul et al., 2009b). As can have

significant effects on many aspects of the immune system in experimental systems, e.g. Nayak et al (2007) observed that As exposure, at concentrations deemed safe for drinking water (2-10 ppb As), suppressed the overall innate immune function in zebrafish (*Danio rerio*). Chronic low-dose exposure to As has also been observed to alter the gene and protein expression of many regulators of the innate immune system in a mouse model of exposure, which may contribute to altered disease risk (Kozul et al., 2009a). Immune function was observed to be impaired in avocet chicks (*Recurvirostra Americana*) from sites with elevated As (Fairbrother et al., 1994). Measured mean As levels in the liver of the chicks ranged from 3.1 to 3.9 ppm dw (3100-3900 ng/g), which is about 3 times higher compared to the mean As levels measured in the kittiwake RBCs (1313.45 ng/g)

4.4 miR-155 as a biomarker of immunomodulation by environmental contaminants

The first model (Eq 2.2) predicting the variation of miR-155 expression in the kittiwakes from 2014 and 2015, indicate that environmental pollution may be implicated with immunomodulation in the kittiwakes. However, it is likely that this effect is sex dependent, which needs to be investigated further. There seems to be no significant effect of concentrations of PCBs or p,p'-DDE on AIV infection in the kittiwakes, but concentration of HCB, which was not included in the model, was significantly lower in the infected birds, indicating an effect on viral infection, but perhaps not on miR-155 expression.

The second model (Eq 2.3) predicting the variation of miR-155 expression in the kittiwakes indicate that metals seems to be more implicated with immunmodulation compared with organochlorine compounds, at least for the kittiwakes from 2015. There was no significant effect of concentrations of Se, Pb and As on AIV infection in the kittiwakes, but the concentrations were higher in the infected birds.

It remains unclear what level of miR-155 expression is beneficial against AIV infection in the kittiwakes, at least in this case. As the kittiwakes sampled were not showing any sign of disease, perhaps these results just show the normal range of miR-155 during immune response to AIV. Therefore, it is important to investigate this miRNA further, to find its normal range within kittiwake, and perhaps other seabirds to see if there are differences between avian species. It is

also important to clarify if there is a sex specific difference in miR-155. Due to the low sample size, and there being more female kittiwakes than males, the difference between sexes relative to miR-155 seen in this master thesis is biased and needs to be investigated further. The relationship between miR-155, immunology and exposure to environmental contaminants should also be investigated further, so that perhaps miR-155 can be used as a biomarker of immunomodulation by contaminants wildlife are exposed to.

5 Conclusions

The metal concentrations in kittiwake plasma reflect exposure at breeding site, and when separating between sexes, concentrations of some metals were significantly different between early and late sampling. This could indicate that despite having similar diets, the sexes might differ in the areas from which they capture food. The metal concentration in kittiwake and guillemot feathers reflect exposure at overwintering location. Higher levels in feathers compared to plasma might be due to higher exposure at wintering site, but could also be due to more sequestering of metals in feathers than plasma, especially for Hg. Differences in metal concentrations between seabird species has been observed previously, and levels of As, Cd, Hg and were higher in kittiwake feathers, while levels of Cu, Pb and Zn were higher in guillemot feathers. Comparing with similar studies investigating metal levels in kittiwake and guillemot feathers show that the concentrations found for Hg and Se are higher in the kittiwakes from this master thesis, perhaps due to temporal and geographical differences in distribution of Hg and Se. The results of the immunological component of this master thesis indicate that expression of miR-155 could be a valuable tool in investigation of immunomodulation by environmental contaminants in seabirds. To the knowledge of the authors, this is the first time that miR-155 modulation due to environmental pollutants has been shown in a wild species. In addition, there seems to be a link between AIV infection, miR-155 expression and certain contaminants. The observed sex-specific difference in miR-155 expression also needs to be investigated more further.

For future studies the kittiwake and guillemot colony on Blomstrand should be investigated further, and with a larger sample size for both species. It would have been interesting to compare miR-155 expression found in kittiwakes sampled in 2014 and 2015 with kittiwakes and guillemots sampled in 2017, especially to see if there are any differences between the species. Investigating miR-155 expression along with other immune variables related to immune function (e.g. differential and total white blood cell counts, PHA skin test, etc) in seabirds could be valuable as well. More research on effect of environmental contaminants and metals on miRNA expression in wild birds would also be relevant.

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Appendix I: Biometrics

Kittiwakes sampled in 2017 (n = 17)

General biometrics and sex of kittiwakes (n = 17) sampled in Kongsfjorden, Ny-Ålesund in 2017. Early body mass was measured for the birds between 11-15th of July, while late body mass was measured 21st of July. Area of sampling is not shown since all kittiwakes were sampled from the same colony at Blomstrandhalvøya.

ID	Sex	Head (mm)	Tarsus (mm)	Wing length (mm)	Early body mass (g)	Late body mass (g)
1	F	89,2	33,9	312	365	*N/A*
2	M	95	36	326	410	405
3	F	89	33,5	315	355	370
4	F	91	34	295	350	360
5	M	90	35	310	430	*N/A*
6	F	86	33,5	300	360	320
7	M	91,5	36	310	410	420
8	F	92	34,5	310	385	360
9	F	89	34,5	310	385	350
10	F	86	33,5	295	335	320
11	M	94	33	305	430	410
12	M	93	35	300	430	425
13	M	94	35	310	405	400
14	F	87	34	305	410	385
15	F	*N/A*	34,5	*N/A*	380	*N/A*
16	M	94	36	295	450	410
17	M	92,5	36	305	*N/A*	415

Kittiwakes sampled in 2015

General biometrics and area of sampling for kittiwakes (n = 10) sampled in Kongsfjorden, Ny-Ålesund in 2015. Sex of the birds is not shown since all sampled kittiwakes were female.

ID	Area	Skull length (mm)	Tarsus (mm)	Wing length (mm)	Body mass (g)
8	Krykkjefjellet	91,5	34	322	380
9	Krykkjefjellet	88,6	34,2	319	375
10	Krykkjefjellet	90,2	34,2	315	380
11	Krykkjefjellet	92	32,1	319	390
12	Blomstrand	88	33,6	319	375
13	Blomstrand	89,6	32,9	320	360
14	Blomstrand	92,2	33,1	315	385
15	Krykkjefjellet	91,8	34,1	326	395
16	Krykkjefjellet	88,1	33	322	380
17	Blomstrand	88,2	33,8	320	375

Kittiwakes sampled in 2014

General biometrics, as well as sex and area of sampling for kittiwakes (n = 7) sampled in 2014 in Kongsfjorden, Ny-Ålesund in 2014.

ID	Sex	Area	Skull length (mm)	Tarsus (mm)	Wing length (mm)	Body mass (g)
1	male	Krykkjefjellet	96,45	36,78	326	432,5
2	female	Krykkjefjellet	87,64	32,31	308	360
3	male	Krykkjefjellet	95,18	34,7	320	420
4	male	Krykkjefjellet	95,06	36,66	326	425
5	female	Krykkjefjellet	90,3	35,41	312	365
6	male	Krykkjefjellet	91,19	35,52	318	410
7	male	Krykkjefjellet	96,83	37,12	326	405

Appendix II: Contamination data

Levels of selected metals in kittiwake plasma from 2017

Individual concentrations (µg/g ww) of selected metals in kittiwake plasma (n = 14) at early sampling in 2017.

ID	As	Cd	Hg	Pb	Se	Cr	Cu	Zn
1	0,00776	0,00030	0,00303	0,00223	0,13846	0,00829	0,06428	2,89600
2	0,04199	0,00069	0,00357	0,00251	0,25699	0,00604	0,10078	2,06332
3	0,04199	0,00069	0,00357	0,00251	0,13846	0,00604	0,10078	2,06332
4	0,04930	0,00042	0,00511	0,00420	0,21894	0,00740	0,09985	2,60669
5	0,11370	0,00039	0,00506	0,00203	0,26565	0,00436	0,12165	2,70708
6	0,10465	0,00024	0,00376	0,00189	0,19230	0,00497	0,11215	2,44016
7	0,06317	0,00042	0,00431	0,00303	0,28783	0,00632	0,10677	2,93942
8	0,07032	0,00038	0,00367	0,00190	0,17466	0,00651	0,06326	3,35119
9	0,01229	N/A	0,00241	0,00993	0,05276	0,01175	0,26847	2,42260
10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
11	0,09264	0,00033	0,00337	0,00317	0,23079	0,00612	0,13379	4,39427
12	0,08740	0,00025	0,00396	0,00546	0,25733	0,00600	0,25619	2,38641
13	0,03631	0,00020	0,00345	0,00733	0,09207	0,00699	0,18845	3,18581
14	0,02120	0,00051	0,00521	0,00521	0,51771	0,00694	0,21938	3,13471
15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
16	0,09308	0,00027	0,00115	0,00470	0,15020	0,00568	0,14714	2,48698
17	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

* N/A = not available, <LOD = below limit of detection

** Cd levels measured in bird no. 9 was below LOD (0.00007 µg/g)

*** N/A for 10, 15 and 17 was due to no blood sampled from these birds

Individual concentrations (µg/g dw) of selected metals in kittiwake plasma (n = 14) at late sampling in 2017.

ID	As	Cd	Hg	Pb	Se	Cr	Cu	Zn
1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	0,01657	0,00032	0,00176	0,00560	0,20278	0,00701	0,12252	3,05062
3	0,11370	0,00039	0,00506	0,00203	0,25699	0,00436	0,12165	2,70708
4	0,04407	0,00021	0,00422	0,00221	0,18932	0,00782	0,05852	2,93777
5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6	0,03759	0,00029	0,00508	0,00259	0,19234	0,01319	0,07685	2,29494
7	0,04318	0,00022	0,00481	0,00223	0,18906	0,00648	0,05833	1,93957
8	0,03999	0,00039	0,00438	0,00502	0,19323	0,00788	0,08116	2,31222
9	0,09264	0,00033	0,00337	0,00317	0,23079	0,00612	0,13379	4,39427
10	0,02214	0,00051	0,00335	0,00222	0,24045	0,00670	0,05629	1,93029
11	0,04116	0,00018	0,00374	0,00164	0,17635	0,00547	0,09500	2,81747
12	0,02436	0,00022	0,00537	0,00192	0,14199	0,00798	0,03856	2,62606

13	0,03484	0,00015	0,00198	0,00557	0,04590	0,00820	0,07942	2,09497
14	0,07289	0,00041	0,00336	0,00433	0,46755	0,00645	0,11623	3,58861
15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
16	0,03894	0,00064	<LOD	0,00677	0,06014	0,00914	0,21203	3,50015
17	0,06394	0,00026	0,00120	0,00164	0,16259	0,00651	N/A	N/A

* N/A = not available, <LOD = below limit of detection

** Hg levels measured in bird no. 16 was below LOD (0.00046 µg/g)

*** N/A for 1, 5 and 15 was due to no blood sampled from these birds.

Levels of selected metals in kittiwake feathers from 2017

Individual concentrations (µg/g dw) of selected metals in kittiwake feathers (n = 16) at early sampling in 2017.

ID	As	Cd	Hg	Pb	Se	Cu	Cr	Zn
1	0,1416	0,0124	3,2771	0,0333	2,5610	6,5186	0,0570	25,5043
2	0,1267	0,0168	7,6637	0,0688	4,5091	6,8159	0,0532	44,6838
3	0,1649	0,0239	6,7301	0,0638	4,1918	8,7225	0,1226	44,6509
4	0,0836	0,0203	5,0381	0,0549	3,6274	6,7442	3,6933	37,0150
5	0,1264	0,0144	5,0052	0,0679	2,8220	6,0484	0,7014	32,9804
6	0,0996	0,0339	3,3623	0,1049	2,6771	5,4388	0,0570	53,5706
7	0,1192	0,0120	5,5016	0,0484	2,7271	7,4929	0,0675	27,8562
8	0,1685	0,0240	4,6553	0,0421	3,1237	6,2075	0,0173	40,2836
9	0,1148	0,0090	5,0449	0,0517	2,8543	6,2357	0,0517	33,3543
10	0,1180	0,0101	5,3164	0,0295	3,0242	5,8649	0,0435	45,6247
11	0,0889	0,0133	4,0376	0,0616	4,0547	5,9261	0,0702	34,1692
12	0,0968	0,0211	4,2717	0,0284	4,4402	6,7919	0,0289	59,8917
13	0,1125	0,0325	4,3274	0,0145	3,6321	5,6125	0,1037	26,4569
14	0,1173	0,0495	4,8821	0,0699	2,7769	5,3042	0,0344	51,3207
15	0,1162	0,0202	4,9753	0,0984	3,6318	5,8866	0,0643	45,8061
16	0,1379	0,0190	7,0790	0,0467	3,4410	5,6215	0,0866	53,9287
17	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

* N/A = not available

** N/A for 17 was due to no feathers sampled from this bird

Individual concentrations (µg/g dw) of selected metals in kittiwake feathers (n = 14) at late sampling 2017.

ID	As	Cd	Hg	Pb	Se	Cu	Cr	Zn
1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	0,0927	0,0132	8,1474	0,0964	4,1985	6,3657	0,1641	43,3670
3	0,0577	0,0102	5,0498	0,0510	3,7329	5,9259	0,0380	35,3904
4	0,1050	0,0112	5,2785	0,0845	3,6150	7,7504	0,0739	26,5317
5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6	0,1569	0,0343	4,6919	0,0431	2,6809	7,3207	0,0934	45,0741
7	0,0833	0,0134	4,8767	0,0344	2,7050	5,9388	0,0437	38,3160

8	0,1196	0,0189	4,4831	0,1243	2,8225	5,4901	0,0391	60,6177
9	0,1041	0,0180	6,3817	0,0251	3,1537	5,1821	0,0663	49,7537
10	0,0920	0,0153	6,0795	0,0622	3,4676	6,4423	0,0574	55,8566
11	0,0645	0,0399	3,8401	0,0822	4,4232	5,8130	0,0770	42,8476
12	0,1616	0,0256	5,0196	0,0340	4,7255	8,2070	0,0671	50,2558
13	0,1330	0,0413	4,7244	0,0429	3,4670	6,1948	0,0579	35,7386
14	0,1374	0,0460	7,2688	0,0643	3,7170	5,0327	0,0414	48,7537
15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
16	0,0996	0,0140	6,5175	0,0521	3,1830	5,8744	0,0386	52,5486
17	0,0865	0,0192	6,0186	0,0279	3,9697	6,4195	0,0447	44,5205

* N/A = not available

** N/A for 1, 5 and 15 was due to no feathers sampled from this bird

Levels of selected metals in guillemot feathers from 2017

Individual concentrations (µg/g dw) of selected metals in guillemot feathers (n = 13) sampled in 2017.

ID	As	Cd	Hg	Pb	Se	Cr	Cu	Zn
1	0,0510	0,0079	1,2045	0,0885	1,4700	0,0466	13,8130	75,2310
2	0,0480	0,0128	1,0847	0,0918	1,9058	0,0427	14,8167	64,6463
3	0,1019	0,0240	0,7556	0,0794	2,7429	0,0515	15,4388	66,8430
5	0,0918	0,0158	0,9995	0,0511	2,2689	0,0759	21,4333	66,8030
6	0,0842	0,0261	0,9571	0,0594	2,0500	0,0577	14,9437	63,9130
7	0,0472	0,0204	1,2989	0,0955	2,6245	0,1712	17,4779	71,0136
9	0,0618	0,0086	1,3926	0,0735	1,5404	0,0562	13,2226	64,2654
8	0,0621	0,0118	0,9290	0,0710	1,4236	0,0480	16,8885	73,2116
10	0,0903	0,0097	1,2552	0,0961	1,3233	0,0658	13,0476	89,0190
11	0,0782	0,0104	0,8108	0,0391	2,8991	0,0476	15,0881	79,9252
12	0,0402	0,0234	0,8714	0,0708	3,3599	0,0507	14,4037	57,2197
13	0,0835	0,0117	1,6985	0,0820	3,9399	0,0602	19,3136	92,3174
14	0,0940	0,0099	1,4325	0,0872	6,2418	0,0315	22,4478	83,4551

Levels of selected metals in kittiwake RBCs from 2015

Individual concentrations (ng/g dw) of selected metals in kittiwake red blood cells (n = 10) sampled in 2015.

ID	Se	Cd	Hg	Pb	Ca	Zn	As
8	36681,63	7,47	422,84	6,76	6753,10	7968,86	918,91
9	43482,13	11,85	368,59	5,69	7603,62	8376,15	1847,34
10	38476,23	15,92	389,74	3,94	5865,43	9166,75	1860,94
11	53323,03	10,29	329,21	4,28	9878,98	8098,24	828,12
12	59532,73	7,80	414,94	3,15	6181,61	7770,67	1123,24
13	61502,20	14,61	370,35	2,60	9407,76	7897,57	1268,72
14	43400,98	7,32	244,49	6,38	6115,12	7457,94	925,88
15	34526,24	5,38	273,37	3,22	6843,06	7763,01	1460,80

16	61755,23	4,82	363,55	8,70	7152,82	7727,27	1832,34
17	79369,29	16,01	295,41	1,74	7585,00	8011,35	1068,17

Levels of organochlorines in kittiwakes from 2014 and 2015

Individual plasma concentrations of selected organic pollutants detected in kittiwakes sampled in Kongsfjorden, Ny-Ålesund in 2014 (Svendsen, 2018) (sample 1-7) and 2015 (Castaño-Ortiz, 2015) (sample 8-17). All concentrations are in pg/g ww.

Sample	p,p'-DDE	HCB	OxC	sumPCB*	sumPBDEs**
1	1514	1578	1058	19130	217
2	3313	2016	1438	17665	589
3	1905	1789	1187	29791	960
4	2556	2110	1470	22610	721
5	6561	2150	1424	23361	338
6	1482	1008	1185	19290	594
7	1075	1778	1585	26164	595
8	1640	2630	1217	70164	4685
9	2243	2438	1203	11216	6246
10	4692	1502	674	27205	3701
11	1846	1658	914	12324	6449
12	3149	1691	514	19420	3374
13	5396	1200	914	33581	4952
14	1427	1603	811	17786	5722
15	844	5465	2538	37430	8497
16	4465	1033	462	20000	1948
17	1945	1017	315	23821	2351

* sumPCBs (2014) = CB-28, CB-99, CB-105, CB.118, CB-138, CB-153, CB-180, CB-183, CB-187, CB-194

sumPCBs (2015) = CB-99, CB-105, CB-118, CB-138, CB-153, CB-156, CB-170, CB-177, CB-180, CB-183, CB-187, CB-199, CB-194, CB-196/203, CB-206

** sumPBDEs (2014) = BDE-47, BDE-99, BDE-100, BDE-138, BDE-153, BDE-154

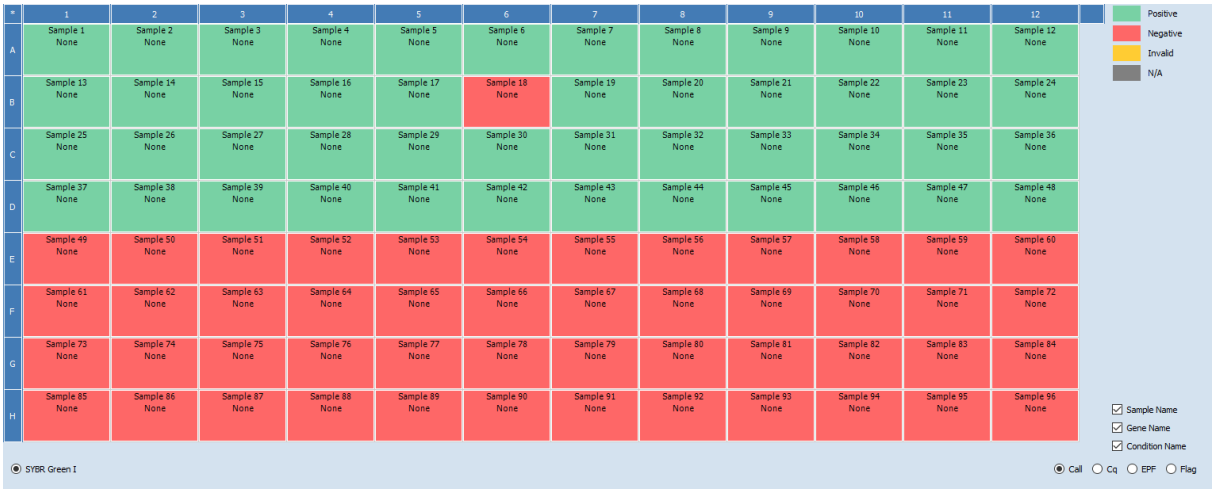
sumPBDEs (2015) = BDE-47, BDE-99, BDE-100, BDE-153

Appendix III: qPCR data

Set up of 96-well plate for qPCR investigating miR-155 expression in plasma of kittiwakes (*Rissa tridactyla*) sampled in 2014 (green) and 2015 (red). Several of the samples from kittiwakes from 2014 turned out to be biological duplicates, so instead of n = 13 samples, the number was reduced to n = 7. 12 µL of mastermix was added to 50 wells (two blanks). 6 µL of diluted sample was added to 48 wells.

1	2	3	4	5	6	7	8	9	10	11	12
13	14	15	16	17	18	19	20	21	22	23	24
B	B										

Heat map of qPCR data investigating miR-155 expression in plasma of kittiwakes sampled in 2014 and 2015. Green indicates the miRNA has been detected, while red indicates no detection. Sample 18 is most likely red due to a pipetting error.



***Cq* (quantification cycle) values of miR-155 in kittiwake plasma.**

Sample	Gene Name	Cq values
Sample 1	miR-155	46.42
Sample 2	miR-155	43.20

Sample 3	miR-155	36.55
Sample 4	miR-155	42.61
Sample 5	miR-155	41.59
Sample 6	miR-155	48.72
Sample 7	miR-155	40.11
Sample 8	miR-155	40.54
Sample 9	miR-155	37.12
Sample 10	miR-155	42.52
Sample 11	miR-155	36.57
Sample 12	miR-155	36.89
Sample 13	miR-155	47.56
Sample 14	miR-155	43.52
Sample 15	miR-155	37.10
Sample 16	miR-155	44.19
Sample 17	miR-155	39.90
Sample 18	miR-155	-
Sample 19	miR-155	39.66
Sample 20	miR-155	41.63
Sample 21	miR-155	37.08
Sample 22	miR-155	44.27
Sample 23	miR-155	36.15
Sample 24	miR-155	36.97
Sample 25	miR-155	37.23
Sample 26	miR-155	34.51
Sample 27	miR-155	36.73
Sample 28	miR-155	31.85
Sample 29	miR-155	35.76
Sample 30	miR-155	34.51
Sample 31	miR-155	33.65
Sample 32	miR-155	36.07
Sample 33	miR-155	33.41
Sample 34	miR-155	38.84
Sample 35	miR-155	38.78
Sample 36	miR-155	35.03
Sample 37	miR-155	36.82
Sample 38	miR-155	34.52
Sample 39	miR-155	36.06
Sample 40	miR-155	31.68
Sample 41	miR-155	35.68
Sample 42	miR-155	34.43
Sample 43	miR-155	33.49
Sample 44	miR-155	36.39
Sample 45	miR-155	33.52
Sample 46	miR-155	36.90
Sample 47	miR-155	39.03
Sample 48	miR-155	35.00