Mercury Bioavailability in Traditional Food and the Effect of Selenium

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Abstract

Methylmercury (MeHg) is a potent neurotoxin capable of crossing the blood-brain barrier causing a profound negative impact on the central nervous system. After its release, Hg may be transported worldwide and eventually deposited in colder Arctic regions. Exposure of Aboriginal communities to MeHg occurs primarily through the consumption of traditional food. Dietary exposure studies are conducted using the total concentration of mercury in the food multiplied by the food consumption rate. This method does not take into account the oral bioavailability of Hg. Therefore, this study determines the bioavailability of Hg in four key traditional foods to provide a better estimation of Hg exposure and to improve the characterization of overall Hg risk to human health. We found that Hg concentrations significantly decreased for all foods after undergoing an in-vitro digestion process. Hg bioaccessibility percentage of ringed seal liver was 32.3%, ringed seal muscle was 69.0%, lake trout muscle was 28.8%, and lastly air-dried beluga muscle was 34.0%. Furthermore, no relationship was observed between bioaccessible Hg concentrations and original Hg concentrations in the raw food. The concentration of MeHg in the bioaccessible fraction was also examined and found to be significantly higher in muscle tissues than in the liver. Bioavailability of the foods was determined using Caco-2 cells. Hg bioavailability percentages were found to be 0.42% for RSL 5.24% for RSM, 7.30% for ADB, and finally, 12.70% for LT. Correlations were found between increased Hg uptake and higher percentages of bioaccessible MeHg as well as lower concentrations of bioaccessible selenium. Lastly, a significant decrease in MeHg uptake after 24 hours was observed when co-incubating with selenium. These results suggest that risk assessments should incorporate bioaccessibility and bioavailability when estimating mercury exposure. Additionally, nutrients such as selenium in traditional food may play a role in reducing mercury uptake in the gut.
Résumé

Le méthylmercure (MeHg) est une puissante neurotoxine capable de croiser la barrière hémato-encéphalique, causant un impact néfaste important sur le système nerveux central. Après qu’il est relâché, le Hg peut se propager à travers le monde et, éventuellement, peut aboutir dans des régions arctiques plus froides. Les communautés autochtones sont principalement exposées au MeHg lorsqu’elles consomment leurs aliments traditionnels. Des études sur l’exposition diététique sont faites en multipliant la concentration totale de mercure d’un aliment par son taux de consommation. Cette méthode ne tient compte de la biodisponibilité orale du Hg. Ceci dit, cette étude détermine la biodisponibilité du Hg dans quatre groupes d’aliments traditionnels afin de permettre une meilleure estimation de l’exposition au Hg, ainsi qu’à améliorer la caractérisation du risque global du Hg sur la santé des humains. Nous avons conclu que la concentration du Hg est grandement diminuée pour tous les aliments après qu’il a subi un processus de digestion in vitro. Les pourcentages observés de la bioaccessibilité du Hg étaient de 32.3% dans le foie du phoque annelé (FPA), de 69.0% dans le muscle du phoque annelé (MPA), de 28.8% dans la truite de lac (TDL) et, dernièrement, de 34.0% dans le béluga séché à l’air (BSA). De plus, aucune relation n’a été observée entre la concentration de bioaccessibilité du Hg et la concentration initiale du Hg dans les aliments. La concentration de MeHg dans l’échantillon bioaccessible a aussi été examinée, et elle a été trouvée significativement plus élevée dans les tissus musculaires que dans le foie. La biodisponibilité des aliments a été déterminée à l’aide de cellules Caco-2. Les pourcentages observés de la biodisponibilité du Hg étaient de 0.42% dans le FPA, de 5.24% dans le MPA, de 7.30% dans le BSA et, dernièrement, de 12.70% dans la TDL. Des corrélations ont été trouvées entre l’augmentation de l’absorption du Hg et des pourcentages plus élevés de MeHg bioaccessible, ainsi que des concentrations plus élevées de sélénium.
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<th>Description</th>
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<tbody>
<tr>
<td>ADB</td>
<td>air-dried beluga</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>Caco</td>
<td>colorectal adenocarcinoma</td>
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<tr>
<td>CH$_3$HgCl</td>
<td>methylmercury chloride</td>
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<tr>
<td>EMEM</td>
<td>Eagle's Minimum Essential Medium</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GI</td>
<td>gastrointestinal tract</td>
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<tr>
<td>GEM</td>
<td>gaseous elemental mercury</td>
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<tr>
<td>GPX</td>
<td>glutathione peroxidase</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
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<tr>
<td>CVAFS</td>
<td>cold vapour atomic fluorescence spectroscopy</td>
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<tr>
<td>HC</td>
<td>Health Canada</td>
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<tr>
<td>HCL</td>
<td>hydrochloric acid</td>
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<tr>
<td>Hg</td>
<td>mercury</td>
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<tr>
<td>Hg$^0$</td>
<td>elemental mercury</td>
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<tr>
<td>HgCl$_2$</td>
<td>mercury chloride</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Plasma Mass Spectrometry</td>
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<tr>
<td>IHS</td>
<td>Inuit health survey</td>
</tr>
<tr>
<td>ISR</td>
<td>Inuvialuit Settlement Region</td>
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<tr>
<td>IVBA</td>
<td>in-vitro bioaccessible</td>
</tr>
<tr>
<td>IVT</td>
<td>in-vitro</td>
</tr>
<tr>
<td>LT</td>
<td>lake trout</td>
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<tr>
<td>MeHg</td>
<td>methylmercury</td>
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<tr>
<td>MeHgCl</td>
<td>methylmercury chloride</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
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<tr>
<td>NG</td>
<td>nanograms</td>
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<tr>
<td>NIHS</td>
<td>Nunavik Inuit Health Survey</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>POPs</td>
<td>persistent organic pollutants</td>
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<tr>
<td>PPM</td>
<td>parts per million</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RSM</td>
<td>ringed seal muscle</td>
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<td>RSL</td>
<td>ringed seal liver</td>
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<tr>
<td>Se</td>
<td>selenium</td>
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<tr>
<td>SeMet</td>
<td>Selenomethionine</td>
</tr>
<tr>
<td>SH</td>
<td>sulphydryl group or thiol group</td>
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<tr>
<td>SRM</td>
<td>standard reference material</td>
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<tr>
<td>TEER</td>
<td>Trans-epithelial electrical resistance</td>
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<td>THg</td>
<td>total mercury</td>
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<tr>
<td>µg</td>
<td>micrograms</td>
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<tr>
<td>µM</td>
<td>micro molar</td>
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<td>WW</td>
<td>wet weight</td>
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Chapter 1: General Introduction

General Overview

Studies in the 80’s conducted in Canada’s Arctic region found high levels of contaminants such as persistent organic pollutants (POPs) and heavy metals such as mercury (El-hayek, 2007). This finding presents a health concern for the 4.6% of Canada’s Aboriginal population who inhabit the North (Simeone, 2008). Inuit populations in Nunavik were found to have Hg blood concentrations in the range of 50.2nmol/L based on the 2004 Nunavik Health Survey (Valera et al., 2009a), while adults living in southern Canadian regions were found to have an average of 4.1nmol/L according to the Canadian Health Measures Survey (Laird & Chan, 2013). The significantly higher levels of blood mercury are specifically detrimental for children and breast-fed infants potentially resulting in cerebral palsy, low birth weight and early sensorimotor dysfunction (Boucher et al., 2016; Jacobson et al., 2015). Exposure of Aboriginal communities to environmental contaminants occurs mainly through the consumption of traditional food. Research conducted in 3 Inuit jurisdictions in Northern Canada (Inuvialuit Settlement Region), attempted to identify the top foods contributing to the intake of Hg for participants of the 2008-2009 Inuit Health Survey (IHS). Despite only being consumed a rate of 32.7g/week, ringed seal liver was found to contain exceptionally high Hg concentrations as it constituted 59% of dietary Hg intake (Laird, James, et al., 2013). In contrast, arctic char, while not containing Hg concentrations as high as ringed seal liver, was frequently consumed at a rate of 378g/week, and was the second highest contributor of dietary Hg intake at 8.4% (Laird, James, et al., 2013). Animals including beluga whale, narwhal, and caribou were also included in list of top ten largest sources of dietary Hg intake within the Inuvialuit Settlement Region (ISR) (Laird, James, et al., 2013).
Epidemiological studies on susceptibility to mercury toxicity have found that despite being exposed to similar chronic low-dose mercury concentrations, the subsequent neurotoxic effects in different regions have varied (Chapman & Chan, 2000). One of the possible reasons for this finding is the source of exposure (i.e. the type of animal) and the specific body part consumed. As we will discuss in this study, each animal accumulates mercury in various forms and concentrations in different parts of its body.

Furthermore, food from different regions will vary in its nutritional content. Recently, there has been a growing consensus of the toxicity of mercury being linked with its high binding affinity with selenium, disrupting selenoenzyme and selenoprotein activity (Farina et al., 2011; Franco et al., 2009). Therefore it follows that mercury neurotoxicity may be related to the Hg:Se molar ratio in the tissues (Berry & Ralston, 2008) and excess of free selenium can potentially play a role in maintaining antioxidant and redox control functions that can be inhibited by MeHg binding (N. V C Ralston & Raymond, 2010).

There are numerous other factors that contribute to the overall risks involved in the consumption of mercury-contaminated food. Several studies have looked into elements such as the preparation method (steaming, grilling) (He & Wang, 2011) or the effects of co-consumption with phytochemical rich foods (Shim et al., 2009) on the bioaccessibility of mercury. In this thesis, we aim to study the bioavailability of mercury in key traditional foods in the Inuit diet. We will also investigate the potential effects of selenium on the absorption of mercury using an in-vitro Caco-2 cell model.
Rationale

Past human health risk assessments have often made the incorrect assumption that all mercury ingested through the consumption of fish and marine mammals is in the dangerous form of MeHg. Additionally, dietary exposure is usually conducted using the total concentration of mercury in the food multiplied by the food consumption rate. However, this estimation does not take into account the oral bioavailability of mercury (He & Wang, 2011). Bioaccessibility is defined as the fraction of a contaminant that remains in soluble form after consumption and digestion. Several studies have looked into the factors (steaming, grilling, and co-consumption with phytochemical rich foods) that may have an impact on the bioaccessibility. Furthermore, after digestion, the fraction of bioaccessible Hg that is absorbed through the gastrointestinal tract and into the bloodstream is referred to as the bioavailable fraction. Therefore, to fully calculate the risks involved in the consumption of Hg contaminated food, it is important to incorporate both factors of bioaccessibility and bioavailability into the estimation.

Looking at a traditional diet as a whole can reveal benefits that may outweigh the mercury risks (Gagné et al., 2012). Selenium is one of the most beneficial nutrients consistently found in the traditional Inuit diet (Laird et al., 2013). Nervous systems have been found to utilize selenium-dependant enzymes to protect the brain from cellular damage which may be caused by neurotoxic contaminants such as mercury. Therefore, it follows that a selenium-rich diet may offer some protective effects against mercury toxicity (IPY, 2008). The Nunavik Inuit Health Survey (NIHS), a study using 25 different types of traditional food in Nunavik, Northern Quebec found high levels of selenium (≥1.0 μg/g) in 20% of the food types researched, while the remaining 80% contained selenium concentrations that were considered “a good source” according to literature reference values (0.20–0.50 μg/g) (M Lemire et al., 2014).
According to data from the International Polar Year Inuit Health Survey conducted in three Inuit jurisdictions in Northern Canada, three of the four traditional foods chosen for this study including ringed seal liver, ringed seal muscle, and air-dried beluga whale muscle were the major sources of Hg contributing to approximately 65% of the dietary Hg intake, and 25% of the selenium intake from the traditional food diet (Laird et al., 2009). We also chose to study lake trout which is often suggested to be an alternative traditional food to lower total Hg exposure. The purpose of this study is to determine the bioaccessibility and bioavailability of four key traditional foods of the traditional Inuit diet to provide a better estimation of Hg exposure and improve the characterization of risk of Hg exposure to human health. Furthermore, we will investigate what impact, if any, selenium may have on the uptake of Hg using an in vitro Caco-2 cell model.

**Objectives and Hypothesis**

**Objectives**

1. To determine the concentration of total mercury (THg) remaining in four selected food samples after undergoing an *in-vitro* digestion process, i.e. the bioaccessible fraction.

2. To determine the fraction of mercury in the four food samples that is absorbed across the gastrointestinal tract over a 24h period using an in vitro Caco-2 cell model, i.e. the bioavailable fraction.

3. To determine the effects of selenium co-incubation on the absorption of mercury.

**Hypotheses**

1. Bioaccessible mercury concentrations will be dependent on original Hg concentration in the food.
2. The uptake of mercury in the gut will show a positive correlation with the concentration of bioaccessible MeHg in the food digest.

3. Selenium co-incubation will significantly decrease the total uptake of mercury after 24h.
Chapter 2: Literature Review – Overview on Mercury and Neurotoxic Effects

Mercury – Sources of Release

Mercury (Hg) is a dense heavy metal that exists in the environment in various forms: Elemental Mercury (Hg\(^0\)), inorganic mercury (e.g. Mercuric ion (Hg\(^{2+}\))), and organic mercury (e.g. Methyl mercury (MeHg)) (Health Canada, 2007). As an element, it can be found in the Earth’s crust and can be released into the environment via natural sources such as volcanoes, forest fires and fossil fuels (UNEP Chemicals Branch, 2008). However, mercury is primarily released into the atmosphere through the incidental pathway. This process occurs when naturally found mercury in coal, rocks and oil is released into the atmosphere through manufacturing and factory processes that do not involve the use of mercury. The largest source of incidental mercury release is as a by-product of fossil fuel combustion accounting for 46% of total mercury emission from anthropogenic sources (AMAP, 2011). Other sources include artisanal and small-scale gold production, cement production, and emissions from ferrous and non-ferrous metal industries (AMAP, 2011). Currently Asian countries account for approximately 65% of global Hg emissions followed by North America and Europe at 8.3% and 7.9%, respectively (AMAP, 2011).

The Mercury Cycle

The large majority of the 5000 tonnes of mercury in the atmosphere is found in the form of elemental mercury (Environmental Investigation Agency, 2003). It is released from both anthropogenic sources (commercial products, industrial processes, etc.) and natural sources such as volcanic eruptions and rock decay. As one of the world’s leaders in industrial advancement, China is currently leading the world as the biggest contributor to atmospheric Hg release.
When Hg is released into the atmosphere, it can undergo several biochemical transformations into different species based on the space it occupies in the environment (W. C. Li & Tse, 2014). Mercury is released into the atmosphere in the form of gaseous elemental mercury (Hg$^0$). Elemental mercury can reside within the atmosphere for long periods of time (between 6 months and 2 years) and can travel worldwide via long-range transport. Depending on the distance of deposition from the source of emission, it can be classified as local deposition, regional deposition or as is the case with Hg deposited in the Arctic region; it can enter the global atmospheric mercury pool. Oxidized forms of mercury (ex. Hg$^{2+}$) are more reactive and can be deposited at local points closer to the origin of emission as it is $10^5$ times more water soluble than elemental mercury (Bullock Jr, 2000).

Through cycles of chemical reactions, gaseous elemental mercury in the atmosphere can be converted to a more reactive form of Hg which have shorter atmosphere lifespans, and are deposited to terrestrial and marine sources (Steffen et al., 2015). Furthermore, the deposited Hg species can also be transformed back into elemental mercury and re-enter the atmosphere using processes such as photolytic reduction (W. C. Li & Tse, 2014).

Once Hg is deposited in the form of inorganic mercury in lakes and oceans, it undergoes a biogeochemical transformation process into the toxic form of MeHg. The most currently accepted theory for this process is by means of microbial transformation. Two processes are thought to compete for the inorganic mercury substrate in water; methylation of Hg$^{2+}$ into MeHg, and the reduction of Hg$^{2+}$ to elemental mercury (Hg$^0$). However, the details of these two processes are not entirely conclusive. In more mild climates, the methylation of mercury has been found to occur via sulfate and iron-reducing microorganisms (Fleming et al., 2006).
However, sulfate-reducing bacteria have also been found in colder environments and are likely to participate in mercury methylation (Chételat et al., 2014).

Methylation of inorganic mercury into organic mercury is a key step in mercury toxicity for humans and wildlife. Inorganic mercury is dangerous in the rare circumstance of exposure to very high concentrations for a short amount of time (acute exposure). However, organic mercury will accumulate in the body over long periods of time which may eventually lead to toxicity even in smaller concentrations (AMAP, 2011).

**Forms of Mercury**

In its elemental state, mercury is the only element that is liquid at room temperature. Liquid mercury can be used to extract pure gold from silver as it forms amalgam with the two elements. As such, miners will often be exposed to high concentrations of elemental mercury through inhalation, its most bioaccessible pathway (Park & Zheng, 2012). Historically, inorganic mercury has been used in skin ointments, antiseptic preservatives and infamously, to treat high quality fur used in hats leading to what was known as “the mad hatter’s” disease (Park & Zheng, 2012). Inorganic mercury (Hg\(^{2+}\)) can be ingested through the gastrointestinal tract at a rate of approximately 7 to 15% (World Health Organization, 2003) where it will predominantly accumulate in the kidneys. However, inorganic mercury is not lipid soluble which means that it will not cross the blood-brain barrier and negatively impact the central nervous system. Acute oral exposures to inorganic mercury at high concentrations may cause corrosion to the chest cavity, impaired kidney function and damage to the gastrointestinal tract. Chronic exposure to inorganic mercury is much rarer (Park & Zheng, 2012).

Elemental mercury (Hg\(^0\)) is absorbed in negligible rates through the gut at and is much more rapidly up taken by means of inhalation through the respiratory pathway at a rate of
approximately 80%. Through this pathway, elemental mercury can reach the central nervous system and cause harmful neurotoxic damage (World Health Organization, 2003).

**Methylmercury**

**Sources of Exposure**

MeHg is composed of a methyl group (CH$_3$-) bound to a mercury ion (Hg$^+$) and it is considered one of the most dangerous forms of mercury. MeHg is a neurotoxin capable of readily crossing the blood-brain barrier and negatively impacting the central nervous system. The lipid solubility of MeHg also supports its bioaccumulation in lipid compartments such as fatty tissue and the brain (Guzzi & La Porta, 2008). Additionally, research shows that the primary method of MeHg uptake through the intestinal epithelium is via the transcellular route by means of passive diffusion through the double lipid bilayer (Marta Vázquez et al., 2014). MeHg will biomagnify along marine food webs starting in organisms at the base of the food chain to marine mammals and higher level predators at the top of the food chain. This process means that environmental exposure and particularly human exposure may be substantially detrimental (Seixas et al., 2014). Particularly, some Indigenous communities relying on a traditional diet as their main source of nutrition may be exposed to fish and marine mammals with mercury concentrations that are in excess of 1 ppm (M Lemire et al., 2014). However, total concentrations of mercury in different species of fish and marine mammals have been known to differ by 100-fold (Mergler et al., 2007). Additionally, the Health Canada food consumption recommended guideline for total mercury concentrations is 0.5ppm for fish species and 1.0ppm for organisms that are higher up on the food chain. In a study within the region of Nunavik, Northern Quebec, most of the organisms sampled showed low levels of MeHg (<0.2ppm) with the exception of organisms that are higher up on the food chain such as beluga whale, ringed seal and lake trout which have been found to have very high concentrations of MeHg (>1.0ppm.)
Despite this finding, the study also looked at blood mercury concentrations of a sample population of 702 individuals and found that over half of the women of child-bearing age in that region showed blood Hg levels above the guidance values (M Lemire et al., 2014).

**Effects of Mercury**

Organic mercury compounds, specifically, MeHg have been widely researched due to their potential for accumulation in the central nervous system and subsequent neurotoxic effects. In 1956, MeHg became well known when after consuming fish and shellfish found in the Minamata bay, residents of Minamata, Japan began showing signs of extreme illness and even death (Harada, 1978). Neurologic symptoms such as paralysis, sensory disturbances in the distal part of extremities, deafness, speech impairment, and mental disorders were all experienced by residents of the Minamata bay and were collectively referred to as the Minamata disease (Ceccatelli et al., 2013). This incident served as the first instance in which MeHg was recognized as a neurotoxin with the central nervous system and the brain as its primary target (McAlpine & Araki, 1958). More mercury outbreaks have occurred over time in countries such as Sweden, Iraq, Pakistan and Ghana (T. W. Clarkson, 2002). In more recent times, due to more strict environmental policies and waste by-product clean up, the frequency of large scale contamination incidents have decreased. However, communities that have been found to exhibit high levels of fish consumption may still be at risk of chronic mercury exposure. Children are considered to be at a higher risk for the toxic effects of mercury exposure than adults due to their developing systems, lower body weight and lower neurologic effect threshold (Health Canada, 2004) Ha et al., 2016). Data has consistently shown that MeHg is transferred to the fetus through the placenta during gestation. In fact, fetal cord MeHg blood concentration has been found to be higher than the corresponding maternal concentration at a ratio of approximately 1.7 (Stern, 2005). At the population level, studies from a variety of regions in the world showed evidence of
poorer neurologic status and delayed development as well as inferior performance on language, memory and attention tests in newborns exposed to MeHg in utero (Mergler et al., 2007; Myers et al., 2003). Additionally, a study by Jacobson et al. (2015) found that “children with cord mercury concentration of ≥7.5µg/L were four times as likely to have an IQ score of <80, which is the clinical cut off for intellectual disability”. A Nunavik study has also shown that the subtle effects of chronic mercury exposure may extend to adults as well. In a sample of 732 Inuit participants over the age of 18, the study found that mercury exposure was correlated with a corresponding increase in blood pressure and pulse pressure (Valera et al., 2009).

The Nunavik Child Development Study (NCDS) conducted behavioral evaluations of 11-year old children using in-class questionnaires which analyzed problem-solving skills and behavioral patterns (AMAP, 2009). Tasks such as the Santa Ana Form Board, the NES-3 finger tapping test, and the Stanford-binet copying subtest were used to assess motor function. Umbilical cord samples as well as blood samples from children were used to determine prenatal exposure to Hg (Boucher et al., 2016). Results showed that cord blood Hg, as well as current blood Hg were associated with poorer performance on two of the three motor function tests (Boucher et al., 2016). Furthermore, umbilical cord Hg concentrations were also related to attention problems such as ADHD (Boucher et al., 2012).

**Mechanisms of Transport**

Communities that rely on a traditional diet of predatory fish and marine mammals are particularly at risk for chronic MeHg exposure. Direct consumption of mercury contaminated food is the dominant method by which humans are exposed to MeHg (Farina et al., 2011). Of all the different forms of mercury, past research has found that MeHg has the highest rate of absorption in the gut at 90-95% uptake (Nielsen, 1992). However, this value was based on two
major studies conducted in 1969 and 1971 with limitations including a small sample size, the use of methylmercury bound to nitrate rather than the commonly found sulfur-bonds, and lastly the lack of a food matrix (Aberg et al., 1969; Miettinen et al., 1971). Furthermore, mercury exposure in both studies was acute rather than the more relevant chronic exposure. Recently, Bradley et al., 2017 reviewed 20 different studies reporting on bioavailability (bioaccessibility or absorption) and found that the mean absorption rate of MeHg is closer to 12-79%. These results challenge the long-held belief that MeHg is completely absorbed into the bloodstream after consumption. Post-ingestion of MeHg, it will be distributed to the entire body, within as little as a few hours, as a result of its lipid solubility (T. Clarkson, 1972). The mechanisms by which mercury is transported across cellular membranes and organs to exert its toxic effects are still being studied today. However, previous studies have attempted to identify the transport of MeHg using a variety of different in-vitro models and cell lines.

MeHg has been found to have a particularly high affinity for sulfhydryl-containing molecules (ex. GSH, cysteine) in various forms in the body forming non-enzymatic bonds with sulphur. When bound to these molecules, MeHg uses “molecular mimicry” to behave as homologs of various vital amino acids and other biomolecules, gaining access to membrane transporters that actively transport these molecules (Zalups & Ahmad, 2005). Past studies have shown that the kidney is the primary site of Hg$^{2+}$ uptake and accumulation. Specifically, it was found that organic ion transporter protein (OAT1) is actively involved in the uptake of the organic form of mercury (Zalups, 1995). Using canine kidney cells (MDCK) transfected with the human isoform of the membrane transporter OAT1, it was found that MeHg bound to N-acetylcysteine (NAC) is a transportable substrate of OAT1 (Zalups & Ahmad, 2005). The rates of survival of MDCK cells transfected with OAT1 compared to control MDCK cells when
exposed to toxic concentrations of CH$_3$Hg-NAC were significantly lower. The study also found substrate specific data that supported the notion that more than one transporter was involved in the uptake of MeHg.

To be capable of wide-spread distribution within the body, MeHg would need the ability to utilize abundant membrane transporters such as the amino acid exchanger proteins LAT1 and LAT2. These proteins are expressed in a variety of tissues and are one of the dominant methods of neutral amino acids entering into the brain from blood (Kerper et al., 1992). One of the amino acids taken up by the L-transporters is methionine, which is structurally similar to MeHg bound in a cysteine complex (MeHg-L-cysteine). Using oocytes from *Xenopus laevis* expressing the two LAT carrier proteins found in humans, a study by Simmons-Willis et al. (2002) attempted to determine if MeHg-L-cysteine can be a substrate for amino acid transporters. The apparent affinities ($K_m$) of MeHg-L-cysteine uptake were found to be similar to methionine in LAT1 transporters (98µM and 99µM). However, the $V_{\text{max}}$ values were higher for MeHg-L-cysteine, suggesting that it could be a better substrate for the LAT transporter than the amino acid methionine. Furthermore, a study using hamster ovary cells found that increased expression of LAT1 transporter was correlated with an increase in the rate of uptake of MeHg when cysteine was available (Yin et al., 2008).

**Mechanisms of Toxicity**

Glutathione (GSH) is a ubiquitous thiol compound found in all tissues including the CNS and plays an antioxidant role in the body (ex. detoxification of peroxidase and protecting the cell against oxidative damage) (Dringen, 2000). A recent study using pregnant mice exposed to varying concentrations of MeHg in drinking water found that control mice showed a post-natal increase in GHS levels over time, while in MeHg exposed group, a dose-dependent inhibition of
the aforementioned increase was found. Furthermore, when Hg levels returned to baseline level, GSH levels and activity remained low which suggests that MeHg may cause biochemical alterations to the GSH development system (Stringari et al., 2008).

The mechanism by which MeHg affects the central nervous system resulting in its neurotoxic effects remains relatively unknown. It has been found that extended exposure of Hg can result in the dysregulation of the important excitatory and inhibitory neurotransmitter glutamate, glycine, γ-aminobutyric acid (GABA) (Fitsanakis & Aschner, 2005). Another important brain neurotransmitter is dopamine. The breakdown of dopamine mechanisms was previously found to be related to the onset of neurological disorders such as Parkinson’s disease and Huntington’s disease (Cui et al., 2006). A recent study investigated the effects of MeHg on dopaminergic pathways using a neuronal cell line (MN9D cells.) The exposure of the neuronal cell line to increasing concentrations of MeHg was compared to MPP⁺, a neurodegenerative compound known for disrupting the dopamine pathway (Shao, Yamamoto, et al., 2015). Results showed that exposure of MN9D cells to high concentrations of MeHg resulted in diminished dopamine levels and degenerative effects similar to MPP⁺ (Shao, Figeys, et al., 2015). Further research studying the gene profile of the MN9D cells after exposure to MeHg using real-time PCR Parkinson’s disease arrays found that 19% and 39% of genes were significantly altered by 2.5µM and 5µM MeHg treatments (Shao & Chan, 2015). However, the general consensus in studies seems to be that not one pathway can explain the plethora of effects associated with MeHg induced neurotoxicity.

The Gastrointestinal Tract

The primary function of the gastrointestinal tract (GI) is to allow the selective absorption and transport of water, electrolytes, nutrients and foreign chemicals such as drugs and medicine. To
aid with absorptive functions, the small intestines employ a large complex viscous-elastic gel called the mucosal surface (Barthe et al., 1999). This surface is specialized in many ways to maximize efficiency of uptake. It relies on several factors such as epithelial cell-covered villi which increase surface area by a factor of eight (Carr & Toner, 1984). Another major component of the GI, the lumen, is located within the external environment, and based on the region, can prevent the uptake of various compounds that would be dangerous if absorbed into the bloodstream. Therefore it follows that the gastrointestinal tract must balance the absorption of valuable macronutrients with acting as a barrier to digestive enzymes, ingested compounds, and bacteria (Barthe et al., 1999). Anatomically, the small intestine is divided into 3 structurally similar sections which total approximately 6 metres in length; the duodenum, jejunum, and the ileum (Carr & Toner, 1984). However, it has been found that 90% of absorption occurs within the first metre of the small intestine (Shen, 2009).

The mucosal layer is the principal component of most absorptive and digestive functions in the GI. The most important structure of the mucosa is the innermost layer of epithelial cells. These cells are a heterogeneous population which most commonly exhibit enterocytes (absorptive cells) but may also include goblet, M cells and endocrine cells specific to the site and function. Enterocytes are responsible for the absorption of the majority of nutrients and drugs and can be commonly identified by the presence of an apical striated brush border made of closely packed microvilli which forms the absorptive surface (Carr & Toner, 1984). Also found on the apical membrane of the enterocytes are various receptor mediated transport systems and proteins. For example, fatty acid absorption in the small intestine was found to be facilitated by the receptor CD36 which is expressed in epithelial cells (Drover et al., 2008). Similarly, a study
by (Morgan & Oates, 2002) found that the expression of receptors TfR1, HFE and DMT1 in the enterocyte lining of the intestinal villi were responsible for iron absorption.

**Pathways of uptake**

Molecules can be absorbed across the gastrointestinal tract by various methods which usually depend on the substance being absorbed. The main pathway by which MeHg is absorbed into the bloodstream is not fully confirmed at this point, however, several possibilities exist. Past research into transport pathways have defined a few of the main methods by which molecules may be taken up across a membrane.

*Passive diffusion through the membrane*

A molecule must possess physiochemical properties such as a low molecular weight and lipophilicity in order to be allowed to cross through the lipophilic apical membrane. Absorption rate for this pathway is dependent on concentration and surface area (Barthe et al., 1999).

*Endocytosis*

This pathway is considered the most important for internalization of macromolecules during intestinal development (Keita & Söderholm, 2010). It is the principal mechanism by which milk macromolecules in suckling mammals and vitamin B12 (Vázquez-Carretero et al., 2014) are absorbed.

*Carrier-mediated Transport*

This mechanism of transport requires contact between a substance and a transport designed to shuttle that specific substance by means of surface protein interactions (Barthe et al., 1999). Two possibilities exist in using this method of transport, the energy-utilizing active transport or passive transport. Active transport may be carried out in the presence of an unfavorable concentration gradient, however metabolic energy is required. Using the everted gut
sac model, it was found that this process is utilized by amino acids (proline, histidine, and methionine) to enter the small intestine against their concentration (Wiseman, 1956). Similarly, drugs and antimicrobial agents such as D-Cycloserine have been found to use this energy dependant method in conjunction with an imino acid carrier (Ranaldi et al., 1994). Alternatively, Passive transport uses no energy and utilizes a concentration gradient.

**Selenium**

**Nutritional Significance**

In the past 50 years, Selenium has transitioned from being classified as a poison to being vital for the optimal function of the human body, and in fact selenium deficiency can have profound negative impacts. Generally, all forms of animal life using a nervous system rely on selenium in the form of selenoproteins (ex. Selenocysteine, selenomethionine) to carry out antioxidant pathways. These processes are essential in the brain tissue and help prevent oxidative damage from reactive oxygen species (ROS) (Berry & Ralston, 2008). Several studies have researched the impact that selenium deprivation would have in a variety of animals. Selenium deficiency was found to induce pancreatic atrophy in chickens by decreasing the activity of 25 selenoproteins in the experimental group vs. the control group. Furthermore, the content of Nitric Oxide (NO), which was found to increase oxidative stress at high levels, was also increased in response to selenium deprivation leading to the formation of ROS (Zhao et al., 2014). In another study, selenium deficiency was found to significantly promote esophageal tumorigenesis in male rats through an oxidative stress and DNA destruction pathway (Yang et al., 2013).

In a similar study by (Hill et al., 2003), selenium deprivation led to the lowest levels of Se found in the brain thus far at 43% of original concentration. However, the impact of selenium deficiency led to severely underweight mice, loss of motor function and significantly reduced
fertility. Finally, the reincorporation of selenium into the diet of the mice helped them regain motor function. These studies outline the necessity of selenium as a dietary nutrient and its importance to regulation in the brain.

Studies have also investigated the impact of excess dietary supplementation of selenium outside the range of dietary intake and found that it may lead to energy alteration and endocrine homeostasis in adult fish (Mcphee & Janz, 2014). In humans, the effects of long-term selenium supplementation (200µg/day) were studied and found to potentially affect glucose metabolism and increase the risk for type-2 diabetes (Stranges et al. 2007).

**Mercury-Selenium Antagonism**

One of the hallmarks of methylmercury’s toxicity is its ability to cross the blood-brain barrier and directly affect the central nervous system. As previously discussed, selenium is responsible for the optimal function of a variety of processes in the brain and central nervous system. MeHg enters the brain and is commonly found in the form of MeHg-SR (SR=amino acid containing sulfur), however selenium’s affinity to mercury is much greater than sulfur’s and therefore the SR is group is replaced by a SeR group via ligand exchange forming a MeHg-Selenocysteine complex (Arnold et al., 1986).

The binding of MeHg to selenoenzymes has been theorized to act as a protective mechanism for the neurodegenerative effects of MeHg by means of its sequestration. Studies such as (Falnoga et al., 2006; Friedman et al., 1978) found similar results in both rats and humans that the neurodegenerative effects of MeHg can be suppressed in the presence of an excess concentration of selenium. More recently, a study using male rats showed that a diet containing high MeHg, low Se ratios was correlated with growth impairment of 24% when compared to the control group. However, rats fed a high MeHg, adequate Se diet only showed 8% impairment,
while rats fed a high MeHg, high Se diet showed no evidence of impairment (Nicholas V C Ralston et al., 2007). These results support the theory that selenium, when in molar ratios in excess of 1:1 is able to sequester MeHg while still having free selenium to carry out important enzymatic functions (Raymond & Ralston, 2004).

Similarly, a study conducted on zebra fish larvae found that there was a significant decrease in response rate even at the lowest concentration of supplemented MeHg. While a supplementation of Selenomethionine (SeMet) alone to the zebra fish environment had no impact on behavior, the co-supplementation of both MeHg and SeMet slightly reduced the longer response rate shown by the zebra fish fed a solely MeHg diet (Weber et al., 2008). Lastly, a study using four groups of rats were supplied with four diets different including a control, a MeHg diet, a selenomethionine diet, and a co-exposure of MeHg and SeMet for a period of 11 days (Sakamoto et al., 2013). Post-euthanasia, the MeHg exposed group showed signs of neurodegenerative damage and degradation by means of reactive astrocytosis and shrinkage. However, the control group, the SeMet fed group, as well as the MeHg – SeMet co-exposure group showed no signs of neuronal damage (Sakamoto et al, 2013.) Furthermore, a significantly lower body weight, liver weight, and lower levels of glutathione were all features of the MeHg exposed group, in comparison with the MeHg – SeMet co-exposed group (Sakamoto et al., 2013.) Results of this study provide further evidence of the protective effects of selenium against MeHg neurotoxicity.
Chapter 3: Determination Mercury Bioaccessibility in Food

Introduction

The importance of traditional food to Indigenous communities has been connected to social values, health and intellect (Inuit Tapiriit Kanatami, 2014). Additionally, the health benefits of a seafood diet are numerous and well known. They are an excellent source of proteins, omega-3 fatty acids, vitamins, and among other nutrients, selenium (Kellogg et al., 2010). Moreover, the traditional customs associated with a traditional diet (hunting, preparation and community feasting) encourage an active lifestyle which can lead to a decrease in the likelihood of obesity and other cardiovascular diseases (Kuhnlein & Chan, 2000).

However, a marine mammals and predatory fish based diet can also be a major source of exposure to environmental contaminants such as mercury (Blanchet et al., 2000; Valera et al., 2009b). When assessing the health risks involved in the consumption of a particular food, the usual factors considered are often the total concentration of a contaminant within the food and the consumption rate. However, this method may not always accurately estimate the true bioavailability of a contaminant (He & Wang, 2011). To address the potential risks of toxicity involved in the consumption of a contaminated food source, we must address two major factors; bioaccessibility and bioavailability. In this context, bioaccessibility refers to the fraction of a contaminant that is released from the food matrix during the process of digestion and is solubilized into the gastrointestinal fluids (Laird et al., 2009). Bioavailability, in comparison, refers to the fraction of an ingested contaminant that is absorbed across the intestinal epithelium and enters systemic circulation (Siedlikowski et al., 2016).

The exact mechanism by which the human body eliminates mercury during digestion is not entirely clear. However, several studies have attempted to study the physiological parameters.
behind mercury excretion and bioaccessibility. A study determined the effects of pH, enzyme concentrations, as well as residence time of food on the solubilisation of mercury at the gastrointestinal level (Jadán-Piedra et al., 2016). They found that at the gastric digestion step of *in-vitro* digestion, increasing the pH level from the standard stomach pH of 2 to ≥pH 3 had significantly reduced solubilized mercury by 82%. This could be due to the decreased performance of the stomach enzyme pepsin which functions optimally at a pH of 2, resulting in a decrease of protein hydrolysis, and more Hg in the protein fraction is left undigested (Jadán-Piedra et al., 2016).

*In-vivo* methods of determining bioaccessibility have usually been a slow and expensive process while *in-vitro* methods present a much quicker and reproducible methodology to determine the risk to human health (Van de Wiele et al., 2007). In 2011, He and Wang used an *in-vitro* digestion protocol to determine factors that affected the bioaccessibility of MeHg in several marine fish species. They showed that a variety of cooking methods (steaming, grilling, and frying) as well as the co-consumption of specific phytochemical-rich foods (green tea) significantly reduced mercury bioaccessibility. Costa et al. (2015) reported that the high heat associated with cooking may result in the denaturing of proteins bound to mercury, making them less prone to hydrolysis by proteases and reducing the amount of Hg released from the food matrix.

Similar to MeHg, selenium was also found to be bound to proteins (Selenoproteins) (Afonso et al., 2015). Therefore, selenium can be readily released from the food matrix during digestion by means of protein hydrolysis (Matos et al., 2015). Studies using fish species such as tuna, swordfish, and sardines reported the bioaccessibility of selenium ranging from 50% to 83% (Cabañero et al., 2007). Furthermore, in blue shark, raw samples were found to have
significantly higher bioaccessible selenium fractions at 98% than in samples that had undergone steaming or grilling at 83% (Matos et al., 2015)

The Inuit diet is diverse but the major contributors of Hg in traditional Inuit diet have been identified and reported (Laird, Goncharov, et al., 2013). In 2008, the Inuit Health Survey (IHS) identified ringed seal, arctic char, and beluga whale as the top 3 foods responsible for dietary Hg intake (IPY, 2008). Total Hg (THg) is a value that incorporates all species of mercury in food. However, in contrast to different mercury species such as metallic Hg (Hg⁰) and inorganic Hg, Hg (II), methylmercury (MeHg) is rapidly absorbed in the gut at rates as high as 95% (Nielsen, 1992). MeHg has a high affinity for thiols (sulfur-containing groups) and will often bind to amino acids within the cell such as cysteine as well as proteins containing cysteine forming a strong covalent bond (Kim & Zoh, 2012). The strength of this bond results in a long half-life of approximately 50-70 days (Mergler et al., 2007) making it a fast and efficient bioaccumulator. The effects of MeHg bioaccumulation in marine mammals and fish have been found to pose a health risk to populations which rely on a seafood diet (Laird, Goncharov, et al., 2013; Valera et al., 2013). Furthermore, due to the structural similarities between the MeHg-L-cysteine complex and the amino acid methionine, Aschner & Aschner (1990) theorized that after ingestion, MeHg may use molecular mimicry as a means of crossing the blood-brain barrier and negatively impact the central nervous system. Determination of the MeHg fraction in traditional food is therefore critical in assessing its risks. In 2014, it was observed that the highest concentrations of MeHg were present in air-dried beluga muscle (4.0µg/g), ringed seal liver (2.7µg/g), and lake trout muscle (1.05µg/g) samples collected from Nunavik, northern Quebec (M Lemire et al., 2014). This study also found that with the exception of air-dried beluga muscle which was found to
have remarkably high selenium concentrations (>1.0µg/g), all analyzed food species displayed “good levels” of selenium as categorized by literature (>0.2µg/g) (Mélanie Lemire et al., 2010).

There are very few studies reporting mercury bioaccessibility in traditional food. Laird et al. (2009) used a Simulator of the Intestinal Human Microbial Ecosystem (SHIME) in-vitro digestion method to determine the bioaccessibility of mercury in traditional foods and found that in-vitro Hg bioaccessibility (IVBA%) was independent of total mercury concentrations (THg). In this study, we propose to use an alternative in-vitro digestion protocol to determine the bioaccessible mercury concentrations of four food types representative of a traditional Inuit diet. The chosen food types for this study were ringed seal liver, ringed seal muscle, lake trout muscle, and air-dried beluga muscle. We hypothesized that the bioaccessible THg concentrations in the 4 food samples are dependent on original THg concentration in the raw food.

Different species of mercury are not equal in toxicity (Health Canada, 2007). Therefore, the secondary objective was to determine the fraction of total bioaccessible mercury that is in the neurotoxic, highly absorbed form of MeHg. Previous studies have shown that after exposure to inorganic mercury, the dominant organs of accumulation are the kidneys and liver (Bridges & Zalups, 2010). A study using zebrafish larvae found differential accumulation in organs following exposure to inorganic and organic forms of mercury. Zebrafish exposed to inorganic mercury showed negligible levels of mercury retention in the brain and muscles. However, significant accumulation was found in both the liver and the pronephric ducts. After exposure to the organic form of mercury (CH$_3$HgCl), significant accumulation was found in the muscles of zebrafish, while generally lower concentrations of MeHg were seen in the liver (Korbas et al., 2012). Based on these findings, we hypothesized that ringed seal liver will contain the lowest
concentration of MeHg, while our remaining food samples (muscle tissues) will exhibit high levels of MeHg.

Current research has provided insight into the potential link between MeHg toxicity and the essential nutrient, selenium. Selenium is a key element in the protective enzyme glutathione peroxidase (GPx), which functions to prevent oxidative damage through the elimination of lipid peroxides (Bjørklund, 2015). Zebrafish studies have found that increased levels of selenium in the diet had a significant effect on the reduction of MeHg concentrations in the fish in comparison with a MeHg-only diet (Amlund et al., 2015; Penglase et al., 2014). Furthermore, during a 4-week depuration period, MeHg was eliminated at significantly higher rates when zebrafish were fed a diet containing 5µg/g selenium in comparison with the control diet (Amlund et al., 2015). A lesser known toxic effect of MeHg, is its harmful impact on the immune system by means of a reduction in cytokine levels (Häggqvist et al., 2005). A recent study, however, found that increasing dietary selenium concentrations in mice gradually removed the suppression in immune functions triggered by MeHg exposure (X. Li et al., 2014). The role of selenium in the prevention of mercury’s toxic effects is a widely recognized topic. Therefore, the last objective was to determine the bioaccessibility of selenium in the chosen food types post in-vitro digestion. In contrast to bioaccessible mercury research, studies exploring the bioaccessibility of selenium are few. We hypothesized that a relationship exists between bioaccessible selenium and Hg concentrations in the 4 food items.
Methods

Sample Collection
Traditional foods for this study were sampled from animals found in the village of Kuujjuaq in Nunavik, Northern Quebec between 1996 and 2013. Samples from 10 different organisms, as well as various tissue types were provided by the Nunavik Research Centre. Four food types which previous studies (Laird et al., 2009; M Lemire et al., 2014) have found to exhibit high levels of mercury were selected to undergo bioavailability investigation. These foods included ringed seal liver (n=3), ringed seal muscle (n=3), air-dried beluga muscle (n=3), and lake trout muscle (n=3). Three replicates for each food type indicate samples from 3 different animals. Prior to receiving the samples, the raw foods had undergone total mercury and selenium determination, and the results were used in this research.

In-vitro Digestion
Our in-vitro digestion protocol was based on the method previously developed in our laboratory (Laird & Chan, 2013) to determine the bioaccessibility of metals in fish, shellfish, wild game and seaweed harvested from British Columbia. For each food type studied, three replicates were used from 3 different animals, and each replicate was digested in triplicates for a total of 9 samples per food type. Three blanks were also used as well as a standard reference material (DORM-2), in three replicates. Briefly, 2 grams of each uncooked food type were weighed and transferred to acid-washed serum bottles and samples were left in the fridge overnight. Previously prepared gastric juice (6g pepsin, 8.5g NaCl, adjusted to pH of 2.0 using 12M omnitrace ultra HCL) was added to the samples. Stomach acidity levels were maintained by ensuring that pH levels remained at 2.0 ± 0.2 through drop-wise addition of 0.5M HCL to decrease pH or 0.5M NaOH to increase pH. Samples were shaken at 37° for 2 hours to simulate gastric digestion. After two hours, intestinal digestion was simulated by adding 5mL of 0.5M NaHCO₃ and 15mL of
duodenal solution (12.5 g NaHCO₃, 6.0 g Oxgall Bile, 3.0 g Pancreatin, 8.5 g NaCl) and adjusting the pH to a more basic 6.5 ±0.5. Samples were again shaken at 37º for 3 hours at 180rpm.

Post-digestion, the *in-vitro* extract from each sample was filtered and centrifuged at 19,000 rpm, 4ºC for 15 minutes at which point the supernatant was filtered and samples were stored at -80º until they were ready to be used. The three digested replicates of each food type were then combined to create one composite bioaccessible fraction of each food for a total of four samples.

**[THg] Determination**

To determine the total mercury concentration of the bioaccessible fraction for each food type, the Nippon MA3000 direct combustion mercury analyzer (Nippon North America, College Station, TX) was utilized. Three replicates were used from the composite bioaccessible food sample of each food type. From each bioaccessible fraction, 3 samples of 200ul were measured and added to ceramic boats containing additive B (Aluminum Oxide). To ensure quality control and analytical accuracy, 3 blanks and two duplicates of Standard Reference Materials (SRM) were used. The SRMs used were DORM-4 (fish protein), and DOLT-4 (dogfish liver), which yielded 90% and 95% recovery, respectively. The blanks consisted of the empty ceramic boat containing only the additive, as well as empty boats with no additive. All values for blanks were below the detection limit of the machine (1 ng/g.)
**Hg Species Determination**

To quantify the concentration of MeHg within the bioaccessible fractions, a gas chromatographic separation with cold vapor atomic fluorescence detection protocol was used, which was written by (Mark L. Olson and John F. De Wild, Wisconsin District Mercury Laboratory, U.S. Geological Survey, Madison, Wisconsin).

Two milliliters of each of the four food types were measured and placed into a centrifuge tube. 5mL of Potassium bromide (KBr), 1 mL of copper sulfate (CuSO4), and 10mL of dichloromethane (CH$_2$Cl$_2$/DCM) were then added to the food samples. The samples were then placed onto a shaker (Gyrotary Shaker Model G2) for one hour. Post-shaking, samples were centrifuged at 5000rpm for 10 minutes to break any emulsion that was formed. 2mL of the lower layer of DCM was then pipetted into a 7mL vial ensuring that only clean DCM was obtained. 1mL of Sodium thiosulfate (Na$_2$S$_2$O$_3$) was then added to each sample within the 7mL vials followed by 45 minutes of shaking then centrifugation for 5 minutes at 3500rpm in a RC5 Superspeed Refrigerated Centrifuge (Sorvall, Wilmington, DE). At this point, two distinct phases (aqueous and organic) can be observed in the vials and an exact quantity is removed (0.8 to 0.9 ml) from the top aqueous phase and transferred to a microcentrifuge tube. The bottom (organic) layer containing MeHg was then carefully extracted at a known quantity of 2mL and placed into glass sampling vials for GC-AFS analysis. Two replicates were used from composite bioaccessible fractions for each food type.

**Selenium Determination**

Digested food samples were analyzed in three replicates for total selenium determination by means of Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using the Agilent 7700x ICP-MS (Agilent, California, United States.) Quality assurance was ensured using 3 replicates of the standard reference material, DORM-4 (fish protein) as well as 3 selenium spikes of 10ppb.
Results

Hg Determination in raw Food

The concentrations are presented in (Table 3.1) as total Hg (µg/g wet weight) of the samples taken from three different animals for each food type with standard deviation. Ringed seal liver shows the highest concentrations of total mercury of all the four food types at 24.43µg/g. Air-dried beluga muscle has the second highest concentration at 2.87µg/g, while lake trout muscle and ringed seal muscle are 1.10µg/g and 0.49µg/g total mercury, respectively.
Table 3.1 Total Hg concentrations in four selected raw traditional foods. Three animals per food type were randomly chosen. Total mercury concentrations of each food type (N=3) was calculated and standard deviation is shown.

<table>
<thead>
<tr>
<th>Food Part</th>
<th>Food Prep</th>
<th>Total Hg (µg/g ww)(^1) (std dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ringed Seal (n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Raw</td>
<td>24.43 (0.74)</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>Raw</td>
<td>0.49 (0.53)</td>
</tr>
<tr>
<td>Lake Trout (n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>Raw</td>
<td>1.10 (0.24)</td>
</tr>
<tr>
<td>Beluga Whale (n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>Air-Dried</td>
<td>2.87 (3.51)</td>
</tr>
</tbody>
</table>

\(^1\) Wet weight Hg concentrations
Bioaccessible Total Hg Concentrations

After *in-vitro* digestion, the concentration of total Hg in all four food samples decreased by a minimum of 31%. Ringed seal muscle was found to have the highest concentration of bioaccessible mercury remaining in the food matrix post-digestion at 69.0% of its original mercury content. The percent bioaccessibility of the remaining three foods; lake trout muscle, ringed seal liver and air-dried beluga muscle were relatively similar ranging between 28.8% to 34.0% shown in (Table 3.2) While Ringed seal muscle had the highest percentage of bioaccessible mercury, ringed seal liver still maintained the highest total concentration of mercury at 7.89µg/g. air-dried beluga muscle, ringed seal muscle and lake trout muscle followed in sequence at 0.97µg/g, 0.33µg/g, and 0.32µg/g, respectively.
Table 3.2 Total bioaccessible Hg concentrations (µg/g) in select traditional foods after undergoing *in-vitro* digestion. Three different animal samples were digested for each food type then combined to create one composite sample that was used to determine Hg bioaccessibility. Total bioaccessible Hg concentration for each food type with standard deviation is presented. Percentage of bioaccessible mercury is also shown.

<table>
<thead>
<tr>
<th>Food Part(^1)</th>
<th>Total Hg Pre-Digestion (µg(^3))</th>
<th>Total Hg Pre-Digestion (µg/g)(^2)</th>
<th>Total Hg Post-Digestion (µg)(^3)</th>
<th>Total Hg Post-Digestion (µg/g) (std. Dev)</th>
<th>Percent Total Hg Bioaccessibility</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ringed Seal (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>48.86</td>
<td>24.43</td>
<td>15.78</td>
<td>7.89 (0.07)</td>
<td>32.3</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.98</td>
<td>0.49</td>
<td>0.66</td>
<td>0.33 (0.01)</td>
<td>69.0</td>
</tr>
<tr>
<td><strong>Lake Trout (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>2.20</td>
<td>1.10</td>
<td>0.64</td>
<td>0.32 (0.01)</td>
<td>28.8</td>
</tr>
<tr>
<td><strong>Beluga Whale (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>5.74</td>
<td>2.87</td>
<td>1.94</td>
<td>0.97 (0.01)</td>
<td>34.0</td>
</tr>
</tbody>
</table>

\(^1\) Each bioaccessible experimental unit was a composite sample of 3 digested samples.

\(^2\) Wet weight Hg concentration

\(^3\) Total amount of Hg in a 2 gram sample of the food.
**MeHg Speciation**

The highest percentages of MeHg in the bioaccessible fraction were found in air-dried beluga muscle and lake trout muscle at 88.8% and 86.0% respectively. Slightly more than half of the total mercury in ringed seal muscle was in the form of MeHg at 55.9%. Finally, ringed seal liver showed a very low concentration of MeHg in its bioaccessible fraction at 3.4% (Table 3.3). Standard reference material for this protocol produced 95% recovery. Two extractions were conducted per food type and the averages are presented.
Table 3.3 Concentration of bioaccessible MeHg in four food samples after Hg speciation.

MeHg (µg/g) result using two replicates is shown. Percentage of MeHg within THg of the bioaccessible sample is presented.

<table>
<thead>
<tr>
<th>Food Part</th>
<th>Bioaccessible Total Hg (µg/g)</th>
<th>Bioaccessible MeHg (µg/g)</th>
<th>MeHg in THg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ringed Seal (n=2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>7.89</td>
<td>0.270</td>
<td>3.4</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.34</td>
<td>0.190</td>
<td>55.9</td>
</tr>
<tr>
<td><strong>Lake Trout (n=2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.32</td>
<td>0.275</td>
<td>86.0</td>
</tr>
<tr>
<td><strong>Beluga Whale (n=2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.98</td>
<td>0.870</td>
<td>88.8</td>
</tr>
</tbody>
</table>
**Total Selenium Determination**

The highest fraction of bioaccessible selenium was found in lake trout muscle at 43.52%, while the lowest fraction of bioaccessible selenium was found in ringed seal liver at 1.58%.

Bioaccessible selenium fractions for air-dried beluga muscle and ringed seal muscle were found to be 28.14% and 32.75%, respectively (Table 3.4.) DORM4 standard reference material produced 102% recovery, and the 10ppb selenium spike was 100% recovered.
Table 3.4 Total concentrations of selenium in the raw food and post *in-vitro* digestion.

Selenium (µg/g) results using 3 replicates are shown with standard deviation. Bioaccessibility percentage is presented.

<table>
<thead>
<tr>
<th>Food Part</th>
<th>Total Se Pre-Digestion (µg/g)</th>
<th>Total Se Post-Digestion (µg/g) (Std. Deviation)</th>
<th>Bioaccessible Total Se (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ringed Seal (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>14.488</td>
<td>0.230 (0.004)</td>
<td>1.58</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.513</td>
<td>0.168 (0.012)</td>
<td>32.76</td>
</tr>
<tr>
<td><strong>Lake Trout (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.209</td>
<td>0.091 (0.001)</td>
<td>43.52</td>
</tr>
<tr>
<td><strong>Beluga Whale (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.810</td>
<td>0.228 (0.012)</td>
<td>28.14</td>
</tr>
</tbody>
</table>
Table 3.5 MeHg:Selenium molar ratio based on moles of bioaccessible MeHg and bioaccessible selenium.

<table>
<thead>
<tr>
<th>Food Part</th>
<th>Bioaccessible MeHg (nmol)</th>
<th>Bioaccessible Selenium (nmol)</th>
<th>MeHg:Se Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ringed Seal (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.25</td>
<td>2.91</td>
<td>0.43</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.88</td>
<td>2.12</td>
<td>0.41</td>
</tr>
<tr>
<td><strong>Lake Trout (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>1.28</td>
<td>1.15</td>
<td>1.11</td>
</tr>
<tr>
<td><strong>Beluga Whale (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>4.03</td>
<td>2.91</td>
<td>1.38</td>
</tr>
</tbody>
</table>
Discussion

The Food Directorate of Health Canada in conjunction with the Canadian Food Inspection Agency have enforced a total mercury guideline of 0.5ppm in domestic and imported fish (Health Canada, 2004). For organisms that are higher on the food chain such as marine mammals and caribou, the recommended guideline is 1ppm of total mercury in the food. This guideline however, may potentially overestimate the risks involved in the consumption of mercury-contaminated food.

The four chosen food types for this study all displayed Hg levels higher than 0.5ppm. Ringed seal liver contained the highest concentration of Hg at 24.43µg/g, followed by air-dried beluga muscle at 2.87µg/g, while lake trout muscle and ringed seal muscle contained 1.10µg/g and 0.49µg/g, respectively. Lemire et al. (2014) found total Hg concentrations of ringed seal liver in Nunavik, Northern Quebec were the highest at 10.9 ppm. Air-dried beluga muscle showed Hg levels of approximately 5.4ppm, while ringed seal muscle was found to contain 0.31ppm, and finally Lake Trout muscle at 1.0 ppm (M Lemire et al., 2014). Ringed seal muscle and lake trout muscle THg concentrations in this study are within the range found by (M Lemire et al., 2014).

The relatively lower average Hg concentration of air-dried beluga muscle in our study (2.87 ppm) compared to those previously reported can possibly be attributed to the younger ages of two of the chosen animals (8 and 6 years old) in comparison to the third (27 years old). Younger animals may have lower concentrations of bioaccumulated mercury due to the shorter time span of their life (Piraino & Taylor, 2009).

The sampled ringed seal liver species chosen for this study displayed high THg concentrations between 23-25ppm. Organs such as the liver are target sites for mercury
accumulation. This could be caused by the protein Metallothionein which has been found to contain a large number of amino acids, as well as nitrogen and sulfur which are known to sequester heavy metals (Parsa et al., 2014). It can be postulated that the accumulation of mercury in the liver is a result of the abundance of the protein Metallothionein in the organ (Parsa et al., 2014). Metallothionein-bound inorganic mercury has also been reported in the kidneys and liver of porcine (Chen et al., 2006). A dietary study within 3 Canadian Arctic jurisdictions found that ringed seal liver was the highest contributor of dietary Hg intake (59%) despite its low consumption rate of 32.7 g/week (Laird, Goncharov, et al., 2013). Similar results were found by the 2007-2008 Inuit Health survey conducted in Nunavut. Ringed seal liver was the highest food contributing to mercury intake (25%) despite only being consumed at an average rate of 39 g/week.

Ringed seal liver, air-dried beluga muscle and lake trout muscle were all found to have similar bioaccessible Hg fractions ranging from 28.8-34.0%. The notable exception to this trend was ringed seal muscle which was found to have a higher bioaccessible Hg fraction of 69.0%, despite having the lowest concentration of Hg pre-digestion. Furthermore, the pre-digestion Hg concentration of ringed seal liver was higher than 3 times the average of all four foods, however after digestion, Hg bioaccessibility was found to be the second lowest at 32.3%. Comparison of the bioaccessible mercury results of our experiments to previously reported findings is difficult as to our knowledge, our research is the first to determine the mercury bioaccessibility of ringed seal muscle, air-dried beluga muscle and lake trout muscle. Previous studies researching the bioaccessibility of Hg in other foods have found a wide range of bioaccessibility percentages which may be in part due to factors such as sample storage conditions, quality of enzymes in the digest, or thawing conditions (Calatayud et al., 2012; Torres-Escribano et al., 2010).
However, ringed seal liver was previously found to have a mercury bioaccessibility of 18.9% (Laird et al., 2009). This result is slightly lower than our determined bioaccessible percentage of 32.3%. This could perhaps be due to the difference in age, size or sex of the sampled animal which has been found to be a factor in the bioaccumulative process of mercury (Storelli et al., 2007). Especially likely is the study’s use of the “The Simulator of the Human Intestinal Microbial Ecosystem” or SHIME *in-vitro* digestion model which incorporates the influence of human gastrointestinal microorganisms on digestion, which was not used in our research.

All four of the foods chosen were equivalent to or higher than the HC recommended guideline for mercury in their raw state. After undergoing the *in-vitro* digestion process, the concentration of bioaccessible mercury for two of those foods; ringed seal muscle and lake trout muscle were found to be lower than the guideline value at 0.34ppm and 0.32ppm, respectively. These results reiterate the hypothesis that the total mercury concentration in the raw food is not indicative of the potential risks involved in consumption. The bioaccessible THg concentration for ringed seal liver was still higher than the recommended guideline post-digestion at 7.89ppm. Air-dried beluga muscle was also found to contain a post-digestion THg concentration that was above the recommended guideline at 0.98ppm. Further insight into mercury speciation will provide more information on total Hg bioavailability.

Pre-digestion concentrations of MeHg were not calculated in the raw samples of the chosen food types; however, MeHg determination of the bioaccessible fractions was conducted. Prior to MeHg speciation, ringed seal liver was found to contain the highest concentration of bioaccessible THg at 7.89µg/g, which is approximately 8 times higher than the Health Canada recommended guideline. After MeHg speciation analysis, we found that only 3.4% of the THg in
the bioaccessible ringed seal liver fraction was in the form of MeHg. Therefore, only 0.27µg/g of the initial 7.89µg/g was in the highly absorbed form of MeHg. The results are similar to previously reported findings using 45 collected ringed seals, which found an average of 31.3µg/g of total mercury in the liver and only 0.77µg/g of MeHg in the fraction, which is approximately 2.5% (Rudolf Wagemann et al., 2000).

MeHg concentrations in lake trout muscle were in line with those previously reported in fish species. Our value of 86% of THg being in the form of MeHg is consistent with (Kannan et al., 1998) which found an average of 83% MeHg of the THg in various fish species collected from Florida bay, and more recently (Burger & Gochfeld, 2004) reporting 90% MeHg in tuna. The bioaccessible MeHg concentration of 0.275µg/g in lake trout muscle means that when considering the effects of digestion as well as the fraction of the more toxic form of mercury, lake trout muscle mercury concentrations falls below the recommended guideline of 0.5 ppm.

To the best of our knowledge, MeHg concentrations in the bioaccessible fraction of ringed seal muscle have not been previously determined in research. A MeHg percentage of 55.9% indicates that the food sample only contains 0.19 µg/g of bioaccessible MeHg, the lowest of all four food type.

The MeHg/THg value in air-dried beluga muscle was 88.8% which is consistent with a previous study using 10 different beluga whale samples and finding an average of 84% MeHg of the THg concentration (Lemes et al., 2011). This value means that the bioaccessible MeHg concentration is 0.870µg/g which is well above the Health Canada recommended guideline of 0.5ppm for fish species and slightly below the 1 ppm guideline for marine mammals. For MeHg to exert its neurotoxic effects and impact the optimal activity of the central nervous system, it must first enter the bloodstream after its uptake by the gut. Therefore, to determine what
percentage of the bioaccessible Hg concentration remaining in the food digest will be absorbed in the gastrointestinal tract and enter systemic circulation, the bioavailability of the chosen food types must be investigated.

Lastly, bioaccessible selenium fractions in our food were determined to range between 1.58% and 43.52% of pre-digestion selenium concentrations. Previous selenium bioaccessibility results in seafood have varied. In 2007, Cabañero et al. digested samples of swordfish, sardines, and tuna to determine the bioaccessibility of selenium and found a range of 50% to 83%. More recently, Calatayud et al. (2012), used 16 species of seafood consumed in Spain and found a bioaccessible selenium range of 17% to 125%. However, the aforementioned studies have only digested fish and shellfish species and therefore bioaccessible selenium data for ringed seal and air-dried beluga are novel results. Similar to its bioaccessible MeHg percentage, the bioaccessible selenium percentage in ringed seal liver was the lowest of all food types at 1.58% despite having the highest selenium concentration in the raw food. Furthermore, both ringed seal muscle and liver were found to have a MeHg:Se molar ratios below 1:1 at 0.41 and 0.43, respectively. Lake trout muscle and air-dried beluga muscle MeHg:Se ratios were found to exceed a 1:1 ratio at 1.11 and 1.38, respectively (Table 3.5). The fact that the ratio of Se and Hg vary and deviate from 1:1 suggest that the Se and Hg in the bioaccessible digest are not bound to each other as reported in the organs of marine mammals (R. Wagemann et al., 1998)

**Conclusion**

Prior to the digestion of the four chosen food samples, the THg concentrations were highest in ringed seal liver (24.43µg/g) and lowest in ringed seal muscle (0.49µg/g). After digestion, the bioaccessible Hg fraction was highest in ringed seal muscle (69.0%), while the bioaccessible Hg fraction in ringed seal liver was the second lowest (32.3%). These results were in opposite to our
hypothesis that bioaccessible mercury fractions will be dependent on total original Hg concentrations in the raw food. Based on previous studies (Bridges & Zalups, 2010; Korbas et al., 2012), we hypothesized that mercury in the muscle food samples was primarily in the form of MeHg while ringed seal liver would contain the lowest levels of MeHg due to the accumulation of inorganic mercury in organs such as the liver and kidney. The results showed that ringed seal liver contained the least amount of MeHg at 3.4% of THg, while the three remaining food types showed MeHg concentrations higher than 50% of THg. Lastly, the bioaccessible selenium fractions were determined in the four food items and were found to range between 1.58% and 43.52%. Ringed seal liver bioaccessible selenium fraction was found to be similar to the fraction of bioaccessible MeHg at 1.58% and 3.4%, respectively. These results are useful for risk assessors to more accurately estimate MeHg exposure from the consumption of these traditional foods.
Chapter 4: Determination of Mercury Bioavailability in Food

Introduction
For the past few decades, toxicology laboratories have attempted to advance the intestinal in-vitro model to gain a better understanding of absorption in the human body. Several methods have emerged to be widely accepted by the research committee. The Ussing chamber created by Ussing and Zehran in the 1950’s was used to determine the rate of active transport across the epithelium via elimination of electrical and chemical gradients (Acra & Ghishan, 1991). The drawback was a lack of replication potential due to the variability caused by the excision procedure involved in preparing intestinal segments for the model. The everted gut sac model, a technique founded by Wilson & Wiseman, (1953), presented the possibility of studying regional absorption within the gastrointestinal tract. However, preparation of the intestinal segment often requires euthanization of the source animal. Additionally, the model was found to degrade when placed in simple salt medium (Barthe et al., 1999).

Arguably, the most popular in-vitro intestinal models for studying uptake in the gastrointestinal tract are cell models. However, several efforts at the isolation and culture of human enterocytic cells were unsuccessful due to the low viability of the cells after isolation and the difficult requirements for differentiation and the formation of a viable monolayer (Hillgren et al., 1995).

A study using a collection of cell lines established from gastrointestinal tumors was the first to isolate the Caco-2 cell line (Fogh et al., 1977). Caco-2 cells are derived from human colon adenocarcinoma and were found to show differentiation under standard culture conditions. Post-differentiation, the cells formed a monolayer with a brush border membrane, and tight junctions
reminiscent of the enterocytic lining of the gastrointestinal tract (Meunier et al., 1995). Most importantly, they expressed factors such as microvillus hydrolases and protein transporters which are a hallmark of the small intestines (Meunier et al., 1995).

An advantage of using the Caco-2 cell line is due to being a human cell line, there is no drawback in potential interspecies differences in the morphological aspects of intestinal cells (Barthe et al., 1999). Previous studies have shown that human in vivo transport rates show a strong association with uptake rates found in Caco-2 cell models. Lennernäs et al. (1996) compared the rate of drugs transported via the transcellular passive pathway (naproxen, antipyrine, naproxen and metaprolol) and found similar rates of uptake in the Caco-2 cell model as in-vivo results of uptake rates in the human jejunum. Similarly, a study using Caco-2 cells to determine the mechanisms of uptake of methylmercury (MeHg) have found that it occurs primarily through transcellular passive transport (Marta Vázquez et al., 2014). MeHg was transported across the Caco-2 cell monolayer at a comparable percentage (64%) to a previously researched molecule, verapamil (82%), known to primarily undergo transcellular passive transport (Marta Vázquez et al., 2014).

Past studies using fish and marine mammals have concentrated on determining the bioaccessibility of Hg in food while research on the bioavailability of Hg is scarce. Recently, a study by Calatayud et al. (2012) evaluated the transport of Hg across the Caco-2 cell model using species of swordfish. Frozen swordfish samples had an initial Hg concentration of 1004ng/g. Post-digestion, the Hg concentration in the samples was decreased to an average of 549ng/g. Finally, Caco-2 cell transport studies found a bioavailable Hg concentration of only 55ng/g. This study also compared the Caco-2 cell Hg transport results of the bioaccessible swordfish fraction to a MeHg standard and found similar rates of transport and cellular retention.
(Calatayud et al., 2012). The similarity of these two results indicated that the dominant species of Hg in the bioaccessible fraction was MeHg and that the presence of nutrients in the food digest did not affect the uptake of MeHg (Calatayud et al., 2012). In 2016, a study used Caco-2 cells to investigate the bioavailability of MeHg found in various samples of seafood after in-vitro digestion. Results showed that in all food types except salmon, bioavailable MeHg concentrations were significantly lower than initial MeHg in raw food (Siedlikowski et al., 2016). Bioavailability of MeHg in the food digest ranged from 29% to 67%. However, the true figures may be slightly lower as this study incorporated the concentrations of transported MeHg as well as MeHg retained within the cells into the bioavailability calculation (Siedlikowski et al., 2016).

The objective of the first phase of this chapter is to use Caco-2 cells to determine the bioavailability of Hg in the digested samples of the chosen food types. This chapter will provide insight into quantifying the fraction of Hg that is transported across the intestinal epithelium and into the bloodstream after consumption of mercury-contaminated foods. Of all species of mercury, MeHg is known to have one of the highest rates of absorption in the gastrointestinal tract at approximately 90-95% (Nielsen, 1992). Therefore, when determining bioavailability, we hypothesize that foods with higher concentrations of MeHg in their bioaccessible digest will have a higher percentage of mercury uptake after a 24 hour period. Species such as lake trout muscle and beluga whale muscle which were found to contain 86.0% and 88.8% MeHg content, respectively, will show higher mercury uptake than ringed seal muscle and more so ringed seal liver which contain 55.9% and 3.4% MeHg.
One of the many benefits of a marine diet is its high nutritional content of selenium (Lemire et al., 2014). Many studies have researched at great lengths, the antagonistic relationship between mercury and selenium in the body (Bjørklund, 2015; Y. F. Li et al., 2012; Penglase et al., 2014). However, a recent study used rice grown in different conditions with ranging concentrations of selenium and mercury to determine if selenium supplementation will decrease Hg uptake in rice grown within mercury-contaminated fields (Wang et al., 2014). This study showed that high selenium treatments significantly decreased total and MeHg uptake in the rice by a total of 47-55% in comparison with the control treatments (Wang et al., 2014). In addition to rice, mercury-selenium antagonism research has also been conducted in animals such as the Brown Shrimp (Bjerregaard & Christensen, 2012). The study found that selenium-enriched diets significantly reduced the retention of MeHg in the animals. Furthermore, when fecal pellets of the organism were studied, the researchers found a dose-dependent increase in mercury excretion through increases in selenium-exposure concentration (Bjerregaard & Christensen, 2012). This effect has also been seen in plants such as radish (Shanker et al., 1996).

Though these studies in plants and animals have provided preliminary insight into the effects of selenium on mercury uptake, the detailed interactions between the two elements at environmentally representative doses through the human gastrointestinal tract is not known. The objective of the second phase of this chapter will be to determine the effects of co-incubation with selenium on the uptake of mercury in Caco-2 cells. We hypothesize that co-incubation with selenium will significantly decrease mercury uptake after 24 hours.
**Methods**

**Colorectal adenocarcinoma cells (Caco-2 cells) Culture**

The human colorectal adenocarcinoma cell line (Caco-2 cells) was purchased from the American Type Culture Collection (ATCC, HTB-37, Manassas, VA). Once received, the cells were stored in -80°C liquid nitrogen. Upon use, the cells were thawed and added to a 75cm² flask which contained 10mL of Eagle's Minimum Essential Medium (EMEM) at pH 7.4. The EMEM was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated at 37°C, within a 95% relative humidity atmosphere and a CO₂ flow of 5%. Every 2-3 days, the Caco-2 cell medium would be discarded and 10mL of fresh media was added. After approximately 1-2 weeks, the cells reached 70% confluency as they formed a monolayer. At that point, the cells required sub culturing. They were pre-washed with phosphate-buffered saline solution with added MgCl₂ and CaCl₂. The added calcium and magnesium are promoters of cell adhesion which helped in the formation of the Caco-2 cell monolayer. 3 mL of 0.25% Trypsin/0.53 mM EDTA 1X solution was added and the cells were incubated for 9 minutes to allow detachment from the flask. Once the cells had detached, they were harvested and reseeded in a new flask containing EMEM at a density of approximately 6 × 10⁴ cells/cm². All experiments were performed at passages 16-28.

**Caco-2 Cells Experimental Methods**

Once the cells reached the pre-determined passage number and had shown consistent growth and continued formation of a monolayer, they were considered ready for plating. Cells were collected from the 75cm² flask using trypsin and centrifuged. The supernatant containing trypsin was discarded and the cells were resuspended using EMEM.
For all experiments, we used 12-well plates containing polyester membrane inserts (10.5 mm diameter, pore size 0.4 μm; BD Biosciences, Franklin Lakes, NJ, USA). The plates and inserts created a dual chamber system with apical and basolateral compartments. After collecting the cells, we used a hematocytometer for cell counting which allowed us to seed the cells on the porous membrane within the apical compartment of the transwell plate at a density of \((5.0 \times 10^4 \text{ cells/cm}^2)\). The apical compartment contained 1mL of EMEM, while the basolateral compartment contained 2mL. In this system, the apical compartment represented the gastrointestinal tract which would normally hold the investigational substance. The Caco-2 cells were seeded on the porous membrane of the apical compartment representing the enterocytes lining the GI tract. The cells in this system act as a semi-permeable membrane between the apical and basolateral compartments. Finally, the basolateral compartment represents the bloodstream. Therefore, uptake of a particular substance from the apical compartment, through the Caco-2 cells and into the basolateral compartment is representative of its movement from the GI tract through the enterocytes and into the bloodstream.

Two days after seeding the cells in the transwell plate, we performed a synchronization protocol to ensure that growth of the monolayer occurred collectively at the same time. This protocol used incomplete media (EMEM including 1% non-essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin but missing Fetal Bovine Serum.) The original media was pipetted out of the wells, and incomplete media was added at the same ratios and incubated for a period of 4 hours to allow for synchronization. After 4 hours, the incomplete media was discarded and complete media was added again. From that point, the transwell plate was incubated at 37°C, within a 95% relative humidity atmosphere and a CO₂ flow of 5%, and the medium was changed every 2-3 days.
To verify monolayer formation, TEER (Trans-epithelial electrical resistance) values were taken approximately twice a week. TEER values measure the electrical resistance between the apical and the basolateral chamber which indicates the permeability of the Caco-2 cell monolayer. To take these values, we used a Millicell®-ERS voltohmmeter (Millipore Corporation, Billerica, MA, USA), which when used to probe the two chambers, provides a value within the range of 0 – 2000 ohms (Ω cm²). Previous research has shown that TEER values that are ≥ 250 Ω cm² indicated that differentiation has been attained and the formation of an intact Caco-2 cell monolayer (Leblondel et al., 2001; M Vázquez et al., 2013). However, TEER values that were too high (≥ 1500) were considered to be too tight and could be a sign of a multilayer formation. If values were too low, ≤ 250 Ω cm², it was a sign that the monolayer had either not fully formed and would require more time or contained inherent issues in monolayer formation and adherence to the insert. For our research, experiments were only carried out on cells in wells showing TEER values within the range of 600 to 900 Ω cm² which were found to produce the most consistent results (Leblondel et al., 2001; M. Vázquez et al., 2014). Cell differentiation producing TEER values within that range usually occurred within 2-3 weeks. Additionally, TEER values were taken at each time point with the 24 hour duration of the experiments (2, 6 and 24 hours.) In some wells, TEER values were found to decrease slightly throughout experiments due to the inclusion of foreign substances other than cell media (MeHg, selenium, or food digest), and thus a TEER value drop within the range of 1-10% was considered acceptable. However, a TEER value decrease in excess of 35% of the original value or to a value below 250 Ω cm² necessitated the removal of those specific wells from the final results as it indicated the Caco-2 cells have either died or the integrity of the monolayer had been compromised. This was evident in experiments using higher concentrations of MeHg. Therefore,
experiments required a balance between the use of high enough concentrations of MeHg to be measurable by the mercury analyzer but low enough to ensure that cell death did not occur over time throughout the experiment.

**Determination of Bioavailability in Traditional Food**

Once cells reached differentiation, as indicated by TEER values within the optimal range of 600 to 900 Ω cm², they were ready to be used for experiments. However prior to using the cells to test the bioavailability of Hg within the digested food samples, we determined the highest concentration of Hg that would be tolerable for experiments without resulting in cell death. Various dilutions of MeHg spikes were administered in each well and observed for cell death over a 24h period, the experimental duration. Using this method, it was determined that a MeHg concentration within the range of 0.005µM to 0.02µM was optimal for viability. TEER values of each well were taken to ensure that the Caco-2 cell monolayer was within the optimal range of experimentation. Our food digest experiments tested the mercury concentrations in each chamber at 3 different time points (2, 6 and 24 hours.) Therefore, at each time point, aliquots were taken from 2 different wells. Averages of THg concentrations were calculated using the two wells. Two wells were used as blanks containing only cell culture media and one well contained the cell medium with no Caco-2 cells to correct for basal TEER values. Each experimental plate counted as 1 replicate, and each food experiment was carried out in triplicates.

The treatment solutions consisted of 18 mL of 0.005µM to 0.02µM Hg food digest (based on food type) diluted in Caco-2 cell media. Each well required 1.5mL of treatment solution (1.5mL x 12 wells = 18mL.)

All cell culture was conducted in the fume hood to ensure sterile conditions. To start the experiment, we took the experimental plate out of incubation conditions and measured the TEER
values of each well prior to starting the experiment. We then aspirated all Caco-2 cell medium out of the plate and placed 2mL of fresh medium into the basolateral chamber. Noting down the starting time point, we dosed 1.5mL of the food treatment into the apical compartment. In the two designated blank wells, we added 1.5mL of cell culture medium. The plates were then placed back into incubation conditions (37ºC, within a 95% relative humidity atmosphere and a CO₂ flow of 5%).

At each time point, 1mL aliquots were taken from the apical and basolateral compartments of each well designated for that time point. Then, the Caco-2 cell monolayers were detached from the insert of each well using 100µl of trypsin, followed by 9 minutes of incubation and then collected using 100ul of PBS solution. The plate was then placed back into incubation and this process was repeated at each time point. All mercury aliquots were frozen at -20ºC until ready to be analyzed.

**MeHg Uptake.**

To determine a baseline for the rate of absorption of Hg across the gastrointestinal tract, a MeHg spike experiment was required. 1000ppm CH₃HgCl was diluted to a concentration of 0.02µM MeHg in Caco-2 cell media, similar to the mercury concentration used in the food digest experiment. The spike treatment was used to determine the rate of absorption of MeHg in the absence of any food digest as well as other nutrients such as selenium.

The treatment solution of 0.02µM CH₃HgCl spike was added to the Caco-2 cells and incubated for two time points (6, and 24 hours.) This experiment was performed in replicates of 2 and the same parameters for blanks and quality assurance as in the food bioavailability experiment were used.
Selenium-MeHg Co-incubation

The last experiment was designed to determine if the presence of equimolar selenium had an effect on the rate of absorption of MeHg. A 50g stock of Seleno-L-methionine was diluted to 1µM Selenomethionine in milli-Q water. The 1µM stock was then used to achieve a further dilution of 0.02µM Selenomethionine in Caco-2 cell media, which is the dominant species of selenium in our chosen foods.

This experiment used a modified design from the previous two protocols. One day prior to conducting the experiment, all the Caco-2 cell media was pipetted from the apical and basolateral chambers of the wells. Then, 2mL of fresh EMEM were added into the basolateral chamber. However, in the apical chamber, 0.75mL of 0.02µM Seleno-L-methionine diluted in Caco-2 cell media was added. This was followed by 24 hours of incubation in the appropriate growth conditions. On the day of the experiment, TEER values were taken to ensure that cells are still viable 24h post incubation with selenium. At experimental time point 0, 0.75mL of 0.02µM CH₃HgCl spike treatment was added in addition to the already present selenium treatment for a total volume of 1.5mL. This experiment was conducted in replicates of 2 and similar to the previous MeHg spike experiment, aliquots were taken of both chambers at the 6h and 24h time points.

Mercury Determination

Total Hg for all experiments was determined using the MA-3000 Total Mercury Analyzer (Nippon Instrument North America, College Station, USA.) The machine had a mercury detection limit of 0.002 ng. Quality control was ensured using sample standards, replicates of three for each time point, and blanks. The standard reference materials (SRMs) used were DORM-4 (fish protein; National Research Council, ON, Canada), and DOLT-4 (dogfish liver; National Research Council, ON, Canada), which yielded 90% and 95% recovery, respectively.
The SRMs were analyzed in unison with our samples and were found to be in range of the certified standard (4.10 ± 0.55 µg Hg /g dw.) The blanks consisted of empty ceramic boats as well as boats containing only the additive. All values for blanks were below the detection limit of the machine (1 ng/g.)

Each experimental plate was analyzed separately using the MA-3000 mercury analyzer. Aliquots taken from both compartments (apical and basolateral) as well as the cells at all three time points, vortexed, and 200µl samples were added to ceramic wells containing additive B (Aluminum Oxide). Results were obtained as nanograms of mercury found within the 200µl sample as well as total concentration of Hg in the sample (µg/kg). To calculate the total amounts of mercury transferred between compartments, the amount of mercury (ng) found in the 200µl sample was multiplied by the total volume within the original compartment. Mass balance was done to ensure that total Hg in all chambers reflected the amount of Hg within the treatment solution.

Once all values were adjusted for their respective compartments, the sum of the amounts of Hg in the apical and basolateral chambers as well as within the cells were calculated to find the total amount of Hg in each well. The amount of Hg in each individual chamber was then divided by the total Hg in the well and converted to percentages.

**Statistical Analysis**

Results from all experiments were analyzed using one-tailed student’s t-test on “R” software to determine the statistical significance of the increase in THg in the basolateral compartment at each time point. In all cases, the results were considered significant at $p \leq 0.05$, however, cases where $p \leq 0.01$ are outlined in the results. The experimental means were calculated
using triplicates in all food bioavailability experiments and duplicates in MeHg and selenium spike experiments. The averages for all time points are expressed as ±standard deviation.

Results

Determination of Bioavailability in Traditional Food

Of all our food samples, ringed seal liver showed the least uptake of mercury across the Caco-2 cells and into the basolateral compartment over time (Table 4.1). There were no significant differences between the amount of mercury found in the basolateral compartment at the 2h time point and the 24 hour time point. The THg (%) in the apical compartment ranged from 96.78 to 98.32 over the span of 24 hours with a standard deviation ranging from 0.63 to 0.94%. The basolateral compartment also remained consistent with 0.42 to 1.07 (%THg). Standard deviation ranged between 0.32 to 0.44%. The percent recovery of Hg ranged from 102% to 105% for all time points.

The ringed seal muscle sample showed the second least uptake of mercury in the basolateral chamber after a 24 hour period (Table 4.2) Between the 2 hour and 6 hour time point, the amount of mercury in the basal chamber remained similar and no significant increase was found. At the 2 hour time point, only 1.94% of total mercury was found in the basal chamber, and at the 6 hour time point, 0.99%. However, between the 2 hour and 24 hour time points, the amount of total Hg found in the basolateral compartment significantly increased ($p < 0.05$) to 5.24%. Standard deviation for all results was very low ranging from 0.48 to 2.22%. The percent recovery for Hg ranged from 90% to 98% for all time points.

Using air-dried beluga muscle (Table 4.3), we found that similar to the ringed Seal muscle sample, there was no significant increase in the amount of mercury in the basal compartment between the 2 hour and 6 hour time points. At 2 hours, only 0.18% of the THg was
found in the basal compartment, while 98.9% was in the apical compartment and 0.89% was in the cells. At the 6 hour time point, that mercury fraction in the basal compartment was 1.24%, while the apical compartment contained 98.4% and the remaining 0.5% was in the cells. However, after 24 hours of incubation, the amount of THg in the basolateral compartment significantly increased in comparison to both the 2 hour and 6 hour time points to 7.3% (p < 0.01). The amount of mercury found within the Caco-2 cells remained consistent throughout the 24 hour incubation at levels ranging from 0.5% to 0.89%. The standard deviation for all results was at the lowest 0.1% to the highest value of 1.05%. The percent recovery for Hg ranged from 86% to 94% for all time points.

The food sample that was found to have the highest rate of mercury uptake by the Caco-2 cells was lake trout muscle (Table 4.4.) In contrast to the remaining food samples, we saw a significant increase (p < 0.05) in the amount of THg in the basolateral compartment at the 6 hour time point in relation to the 2 hour time point. At the 2 hour time point, 1.49% of THg was found in the basolateral compartment, while 95.91% was in the apical compartment and 2.97% in the cells. At the 6 hour time point, the amount of THg in the basal compartment significantly increased to 4.07%. At the 24 hour time point, we saw the highest rate of mercury uptake of all the food samples. There was a significant increase (p < 0.01) in mercury uptake with 12.7% of the THg found in the basolateral compartment, accompanied by a significant decrease (p < 0.01) to 84.9% in the apical compartment. The amount of mercury in the cells remained consistent ranging between 2.38% and 2.97. The standard deviation for all results ranged from 0.11% to 2.23%. The percent recovery for Hg ranged from 88 to 95% for all time points.
Table 4.1 Mercury values in the three compartment system of Caco-2 cells for ringed seal liver food digest diluted to 0.015μM at three time points following a 24 hour incubation.

Mean Hg amount and percent of total Hg ± standard deviation is shown (N=3.)

<table>
<thead>
<tr>
<th></th>
<th>2 Hours</th>
<th>6 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of Hg (ng)</td>
<td>Amount of Hg (ng)</td>
<td>Amount of Hg (ng)</td>
</tr>
<tr>
<td></td>
<td>[% of recovered Hg±StdDev]</td>
<td>[% of recovered Hg±StdDev]</td>
<td>[% of recovered Hg±StdDev]</td>
</tr>
<tr>
<td>Apical</td>
<td>4.56 [97.23 ± 0.81]</td>
<td>4.51 [96.78 ± 0.63]</td>
<td>4.67 [98.32 ± 0.94]</td>
</tr>
<tr>
<td>Basal</td>
<td>0.03 [0.64 ± 0.32]</td>
<td>0.05 [1.07 ± 0.44]</td>
<td>0.02 [0.42 ± 0.42]</td>
</tr>
<tr>
<td>Cells</td>
<td>0.1 [2.13 ± 0.65]</td>
<td>0.094 [1.93 ± 0.46]</td>
<td>0.06 [1.26 ± 1.25]</td>
</tr>
<tr>
<td>Mercury Recovered</td>
<td>[ % of Added Mercury]</td>
<td>[ % of Added Mercury]</td>
<td>[ % of Added Mercury]</td>
</tr>
<tr>
<td></td>
<td>4.69 [103.30 ± 8.09]</td>
<td>4.66 [102.64 ± 7.05]</td>
<td>4.75 [104.63 ± 5.68]</td>
</tr>
<tr>
<td>Mercury Added</td>
<td>4.54±0.30</td>
<td>4.54±0.30</td>
<td>4.54±0.30</td>
</tr>
</tbody>
</table>
Table 4.2. Mercury values in the three compartment system of Caco-2 cells for ringed seal muscle food digest diluted to 0.007µM at three time points following a 24 hour incubation.

Mean Hg amount and percent of total Hg ± standard deviation is shown (N=3.) Statistical significance was calculated using one-tailed student’s t-test on “R” software, * represents p≤0.05, and ** represents p≤0.01.

<table>
<thead>
<tr>
<th></th>
<th>2 Hours</th>
<th>6 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of Hg (ng) [±STD]</td>
<td>Amount of Hg (ng) [±STD]</td>
<td>Amount of Hg (ng) [±STD]</td>
</tr>
<tr>
<td></td>
<td>[% of recovered Hg±StdDev]</td>
<td>[% of recovered Hg±StdDev]</td>
<td>[% of recovered Hg±StdDev]</td>
</tr>
<tr>
<td><strong>Apical</strong></td>
<td>2.01 [97.57 ± 2.22]</td>
<td>1.99 [98.51 ± 1.20]</td>
<td>1.8 [94.24 ± 1.24]</td>
</tr>
<tr>
<td><strong>Basal</strong></td>
<td>0.04 [1.94 ± 1.27]</td>
<td>0.02 [0.99 ± 0.68]</td>
<td>0.1 [5.24 ± 1.15] *</td>
</tr>
<tr>
<td><strong>Cells</strong></td>
<td>0.02 [0.97 ± 0.95]</td>
<td>0.01 [0.50 ± 0.49]</td>
<td>0.01 [0.52 ± 0.48]</td>
</tr>
<tr>
<td><strong>Mercury Added</strong></td>
<td>2.11±0.93</td>
<td>2.11±0.93</td>
<td>2.11±0.93</td>
</tr>
<tr>
<td><strong>Mercury Added</strong></td>
<td>2.11±0.93</td>
<td>2.11±0.93</td>
<td>2.11±0.93</td>
</tr>
</tbody>
</table>
Table 4.3 Mercury values in the three compartment system of Caco-2 cells for air-dried beluga muscle food digest diluted to 0.02µM at three time points following a 24 hour incubation. Mean Hg amount and percent of total Hg ± standard deviation is shown (N=3.) Statistical significance was calculated using one-tailed student’s t-test on “R” software, * represents p≤0.05, and ** represents p≤0.01.

<table>
<thead>
<tr>
<th></th>
<th>2 Hours</th>
<th>6 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of Hg (ng) [ % of recovered Hg±StdDev]</td>
<td>Amount of Hg (ng) [ % of recovered Hg±StdDev]</td>
<td>Amount of Hg (ng) [ % of recovered Hg±StdDev]</td>
</tr>
<tr>
<td>Apical</td>
<td>5.53 [98.9 ± 0.87]</td>
<td>5.55 [98.4 ± 0.98]</td>
<td>4.78 [91.9 ± 0.68]</td>
</tr>
<tr>
<td>Basal</td>
<td>0.01 [0.18 ± 0.10]</td>
<td>0.07 [1.24 ± 1.05]</td>
<td>0.38 [7.30 ± 0.90]**</td>
</tr>
<tr>
<td>Cells</td>
<td>0.05 [0.89 ± 0.79]</td>
<td>0.03 [0.5 ± 0.35]</td>
<td>0.03 [0.58 ± 0.34]</td>
</tr>
<tr>
<td>Mercury Recovered [ % of Added Mercury]</td>
<td>5.59 [92.7 ± 2.24]</td>
<td>5.64 [93.4 ± 2.59]</td>
<td>5.2 [86.1 ± 3.36]</td>
</tr>
<tr>
<td>Mercury Added</td>
<td>6.04±0.99</td>
<td>6.04±0.99</td>
<td>6.04±0.99</td>
</tr>
</tbody>
</table>
Table 4.4 Mercury values in the three compartment system of Caco-2 cells for lake trout muscle food digest diluted to 0.01µM at three time points following a 24 hour incubation. Mean Hg amount and percent of total Hg ± standard deviation is shown (N=3.) Statistical significance was calculated using one-tailed student’s t-test on “R” software, * represents p≤0.05, and ** represents p≤0.01.

<table>
<thead>
<tr>
<th></th>
<th>2 Hours</th>
<th>6 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of Hg (ng) [% of recovered Hg±StdDev]</td>
<td>Amount of Hg (ng) [% of recovered Hg±StdDev]</td>
<td>Amount of Hg (ng) [% of recovered Hg±StdDev]</td>
</tr>
<tr>
<td>Apical</td>
<td>2.58 [95.91 ± 1.58]</td>
<td>2.52 [93.33 ± 1.04]</td>
<td>2.14 [84.92 ± 1.38]</td>
</tr>
<tr>
<td>Basal</td>
<td>0.04 [1.49 ± 1.02]</td>
<td>0.11 [4.07 ± 1.14]*</td>
<td>0.32 [12.70 ± 0.85]**</td>
</tr>
<tr>
<td>Cells</td>
<td>0.08 [2.97 ± 2.23]</td>
<td>0.08 [2.96 ± 0.11]</td>
<td>0.06 [2.38 ± 0.58]</td>
</tr>
<tr>
<td>Mercury Recovered [% of Added Mercury]</td>
<td>2.69 [94.06 ± 3.60]</td>
<td>2.7 [94.40 ± 1.53]</td>
<td>2.52 [88.25 ± 6.06]</td>
</tr>
<tr>
<td>Mercury Added</td>
<td>2.86±0.27</td>
<td>2.86±0.27</td>
<td>2.86±0.27</td>
</tr>
</tbody>
</table>
Figure 2. Percentage of Hg found in the basolateral compartment of Caco-2 cells at the 24 hour time point for three experiments as a function of increasing MeHg fraction in the bioaccessible sample of the four chosen food types. R squared value is presented with regression line (n=3.)
Figure 3. Percentage of Hg found in the basolateral compartment of Caco-2 cells at the 24 hour time point for three experiments as a function of increasing selenium concentrations in the bioaccessible sample of the four chosen food types. R squared value is presented with regression line (n=3.)
Table 4.5 Mean concentrations of mercury in raw food samples, bioaccessible fractions after *in-vitro* digestion and transported across Caco-2 cells into the basolateral chamber as well as bioaccessible MeHg concentrations.

<table>
<thead>
<tr>
<th>Food Part</th>
<th>Total raw Hg (µg/g ww)</th>
<th>IVBA Total Hg (µg/g) [% of total raw Hg]</th>
<th>IVBA MeHg (µg/g) [% of total BA³ Hg]</th>
<th>Hg IVTbasolateral (µg/g) [% of total IVBA Hg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>24.3</td>
<td>7.89 [32.3]</td>
<td>0.270 [3.4]</td>
<td>0.033 [0.42]</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.49</td>
<td>0.33 [69.0]</td>
<td>0.190 [55.9]</td>
<td>0.017 [5.24]</td>
</tr>
<tr>
<td>Lake Trout (n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>1.1</td>
<td>0.32 [28.8]</td>
<td>0.275 [86.0]</td>
<td>0.040 [12.70]</td>
</tr>
<tr>
<td>Beluga Whale (n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>2.87</td>
<td>0.97 [34.0]</td>
<td>0.870 [88.0]</td>
<td>0.070 [7.30]</td>
</tr>
</tbody>
</table>

¹ Bioaccessible Hg concentration after undergoing *in-vitro* digestion (*In-vitro* bioaccessible)

² Bioavailable Hg concentration found in the basolateral compartment in Caco-2 cells

³ Bioaccessible
**Mercury and Selenium Uptake**

After a 6 hour incubation of 0.02µM MeHg, an uptake of 21.32% of the added mercury was observed with a recovery rate of 73.66% of the total added Hg. After 24 hours, the amount of mercury in the basolateral compartment significantly increased (p≤0.01) to 61.61% of the total added Hg and recovered at 118.27% of total added Hg. Also observed was a corresponding significant decrease in the concentration of mercury in the apical compartment from 72.44% to 35.46% at the 6 hour and 24 hour time point.

The second experiment involved pre-incubation with 0.02µM selenium for 24 hours, followed by addition of an equimolar concentration of MeHg. At the 6 hour time point, the concentration of MeHg in the basolateral compartment was 27.03% of the total added Hg. This result is significantly higher (p≥0.05) than the sole MeHg treatment at the 6 hour time point. At the 24 hour time point, the concentration of MeHg in the basolateral compartment remained consistent at 28.32% of the total added Hg, which is significantly less (p≤0.01) than the sole MeHg treatment at the 24 hour time point. A mean recovery rate of 95.69% and 94.0% was observed for the 6 hour and 24 hour time points, respectively.
Table 4.6 Mercury values in the three compartment system of Caco-2 cells after a 0.02 µM MeHg spike treatment at two time points following 24 hour incubation. Mean Hg amount and percent of total Hg ± standard deviation is shown (N=2.) Statistical significance was calculated using one-tailed student’s t-test on “R” software, * represents p≤0.05, and ** represents p≤0.01.

<table>
<thead>
<tr>
<th></th>
<th>6 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of Hg (ng) [ % of recovered Hg±StdDev]</td>
<td>Amount of Hg (ng) [ % of recovered Hg±StdDev]</td>
</tr>
<tr>
<td>Apical</td>
<td>5.23 [72.44 ± 0.58]</td>
<td>3.75 [35.46 ± 9.16]</td>
</tr>
<tr>
<td>Basal</td>
<td>1.53 [21.32 ± 1.15]</td>
<td>7.50 [61.61 ± 9.34]**</td>
</tr>
<tr>
<td>Cells</td>
<td>0.46 [6.31 ± 1.73]</td>
<td>0.21 [2.93 ± 1.23]</td>
</tr>
<tr>
<td>Mercury Recovered [% of Added Mercury]</td>
<td>7.22 [73.66 ± 8.76]</td>
<td>11.59 [118.27 ± 54.28]</td>
</tr>
<tr>
<td>Mercury Added</td>
<td>9.80±0.30</td>
<td>9.80±0.30</td>
</tr>
</tbody>
</table>
Table 4.7 Mercury values in the three compartment system of Caco-2 cells after a 0.02µM MeHg and Selenium co-incubation treatment. Mean Hg amount and percent of total Hg ± standard deviation is shown (N=2.) Statistical significance was calculated using one-tailed student’s t-test on “R” software, * represents p≤0.05, and ** represents p≤0.01.

<table>
<thead>
<tr>
<th></th>
<th>6 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of Hg (ng) [% of recovered Hg±StdDev]</td>
<td>Amount of Hg (ng) [% of recovered Hg±StdDev]</td>
</tr>
<tr>
<td><strong>Apical</strong></td>
<td>3.98 [62.91 ± 8.36]</td>
<td>4.28 [68.92 ± 12.07]</td>
</tr>
<tr>
<td><strong>Basal</strong></td>
<td>1.71 [27.03 ± 8.82]</td>
<td>1.76 [28.32 ± 14.21]</td>
</tr>
<tr>
<td><strong>Cells</strong></td>
<td>0.64 [10.05± 6.70]</td>
<td>0.17 [2.74 ± 2.19]</td>
</tr>
<tr>
<td><strong>Mercury Recovered [% of Added Mercury]</strong></td>
<td>6.33 [95.69 ± 14.52]</td>
<td>6.21 [94.0% ± 29.67]</td>
</tr>
<tr>
<td><strong>Mercury Added</strong></td>
<td>6.61±0.42</td>
<td>6.61±0.42</td>
</tr>
</tbody>
</table>
**Discussion**

In our previous chapter, the oral bioaccessibility of Hg in ringed seal liver, ringed seal muscle, lake trout muscle and air-dried beluga muscle was determined. Oral bioaccessibility is defined as the fraction of the consumed substance that is released from the food matrix during the process of digestion. The results showed that when incorporating the effects of the digestive process, the concentration of ingested mercury in our body is significantly reduced for all food types. Despite initial Hg concentrations in the raw food being higher than the Health Canada recommended guideline of 0.5 ppm, the bioaccessible Hg concentrations fell below the guideline for ringed seal muscle and lake trout muscle. However, ringed seal liver and air-dried beluga muscle maintained Hg concentrations higher than the recommended guideline at 7.89µg/g and 0.97µg/g. Therefore, the objective of this chapter was to determine the fraction of remaining Hg that would be absorbed across the gastrointestinal tract and into the bloodstream where it may cross the blood-brain barrier and exert its neurodegenerative effects (Van de Wiele *et al.*, 2007).

Our food bioavailability experiment results found that bioavailable Hg concentrations in all four of our chosen foods were significantly less than bioaccessible Hg concentrations. In all experimental replicates for all food types, the percentage of total mercury absorbed through the Caco-2 cells and into the basolateral compartment did not exceed 13% of bioaccessible Hg concentrations and ranged from 0.42% to 12.70%. These results are very similar to those previously reported by (Calatayud *et al.*, 2012) of 3% to 14% Hg uptake in samples of frozen swordfish. This finding suggests that while all our chosen foods contained Hg concentrations that met or exceeded the recommended guideline of 0.5µg/g, only 0.14% of the initial 24.3µg/g of ringed seal liver is absorbed into the bloodstream after ingestion. Similarly, 3.7% of the initial 0.49µg/g of Hg in ringed seal muscle is absorbed and 3.63% of the initial 1.1µg/g for lake trout muscle. Lastly 2.44% of the initial 2.87µg/g of Hg in air-dried beluga muscle was found to be
absorbed (Table 4.5), demonstrating that when incorporating the effects of digestion as well as permeability of the gastrointestinal tract, the concentration of ingested Hg from food that is absorbed into the bloodstream is significantly reduced. This finding advocates for the importance of integrating the aforementioned parameters in the process of mercury risk assessment.

Furthermore, the current study relies on using digested food from its raw state that had not gone through any process of cooking, steaming or grilling, which all have been found to also significantly reduce the bioaccessibility of Hg (He & Wang, 2011).

The second objective of this study was to determine the effects of bioaccessible MeHg concentration in the food on uptake by Caco-2 cells. We hypothesized that foods containing higher percentages of MeHg in their bioaccessible total mercury fraction (THg) would be absorbed through the Caco-2 cells and into the basal chamber at higher concentrations. Specifically, the bioaccessible fractions of air-dried beluga muscle and lake trout muscle containing a MeHg percentage of 88.0% and 86.0% should show higher Hg uptake than ringed seal muscle and even more so ringed seal liver fractions containing 55.9% and 3.4% MeHg, respectively. Our experiments showed that 12.7% and 7.30% of the total added Hg using samples of lake trout muscle and air-dried beluga muscle was transported across the Caco-2 cells. These percentages were significantly higher than those found using ringed seal muscle and especially ringed seal liver, which were 5.24% and 0.42%, respectively. Our results indicate that the percentage of MeHg in the bioaccessible THg fraction of a food may play a role in determining its bioavailability. Furthermore, (Figure 4.1) shows the correlation between increasing fractions of MeHg in the bioaccessible digest and the percentage of Hg found in the basolateral compartment ($R^2 = 0.51$.) These findings are similar to those previously reported in zebra fish exposed to two diets of MeHg (CH$_3$HgCl) or inorganic mercury (HgCl$_2$) (Korbas et
Analysis of Hg accumulation found concentrations as high as (0.4 – 0.96 µg/cm$^2$) in zebra fish exposed to a MeHg diet, while in zebra fish fed a diet of HgCl$_2$, Hg concentrations were significantly lower at (0.05 to 0.16 µg/cm$^2$), indicating preferential accumulation for MeHg (Korbas et al., 2012.)

As mercury speciation analysis was not conducted on the mercury found in the basolateral compartment, the percentage of MeHg absorbed cannot be concluded. However, even if it is assumed that all Hg found in the basolateral compartment is in the form of MeHg, then only 8.0% to 14.5% of bioaccessible MeHg in the food was transported through the Caco-2 cells. These results are contradictory to the previously reported finding that 95% of ingested MeHg is bioavailable. This percentage was reported in human studies (Aberg et al., 1969) as well as in monkeys (Berlin et al., 1975). However, a common aspect of these studies was that the MeHg used was bound to a nitrate rather than the commonly found form of sulfur-bound MeHg and more importantly, it was used in the absence of a food matrix as it would normally be ingested. Traditional food has been found to be a remarkable source of nutrients such as selenium, omega-3 fatty acids, vitamins A, D, and E as well as proteins (Laird, Goncharov, et al., 2013). These nutrients have been found to affect the bioavailability, metabolism, and toxicity of Hg (Beyrouty & Chan, 2006; Kaur et al., 2007; Zeng et al., 2011).

The final objective for this research was to determine the effects of selenium on the uptake of mercury using Caco-2 cells. A baseline for MeHg uptake was established using a spike treatment of 0.02µM MeHg chloride. After an incubation period of 24 hours, 61.61% of total added MeHg was transported through the Caco-2 cells and into the basolateral compartment. This was a significant increase ($p<0.01$) from 21.32% uptake of added Hg at the 6H time point. The significantly higher percentage of mercury transported through the Caco-2 cells when using
only MeHg-chloride in the absence of food digest suggests that components of the food may play a role in the reduction of mercury uptake. To test this possibility, the cells were pre-incubated in an equimolar (0.02µM) concentration of selenium 24 hours prior to the start of the experiment as well as throughout the duration of the experiment. We hypothesized that the inclusion of selenium would result in a significant reduction in the uptake of Hg after a 24 hour period. At the end of the experiment, only 28.32% of the total added Hg was transported through the Caco-2 cells and into the basal chamber which is a significant decrease (p≤0.01) compared to 61.61% of transported Hg in the absence of selenium. No increase in Hg uptake was found between the 6H and the 24H time point. Additionally, the concentration of selenium was determined in the bioaccessible fractions of all four foods. Ringed seal liver, which was found to contain the highest concentration of selenium (0.230 µg/g) in its bioaccessible fraction showed a negligible percentage of Hg transport into the basolateral compartment after 24 hours at 0.42%. In comparison, lake trout muscle which was found to contain the least amount of bioaccessible selenium (0.091µg/g), showed the highest rates of Hg transport across the Caco-2 cells and into the basolateral compartment. When comparing the concentration of bioaccessible selenium in the food digest with the decreasing percentage of Hg transport in Caco-2 cells after 24 hours of incubation, a coefficient of determination (R^2) value of 0.78 is found (Figure 4.2.) The molecular mechanisms by which selenium impacts the uptake rate of Hg are still relatively unknown. However, the phenomenon of selenium’s impact on the uptake of mercury has been reported in several studies. A recent study by (Huang et al., 2013) used sturgeon fed a diet of MeHg chloride, Selenomethionine, as well as a combination diet of Se/Hg. An increase in blood Hg concentrations was found in both the MeHg group as well as the Se/Hg group. However, Hg concentrations in the MeHg group peaked at 12 hours and maintained its elevation for 48 hours,
while the Se/Hg group peaked at 1.5 hours then promptly decreased to approximately half the value of the peak and remained low for the remaining time period (Huang et al., 2013). Similarly, in rats fed a diet of Hg or a Hg/Se diet with increasing concentrations of selenium, it was found that the concentration of Hg in the brain and kidneys decreased with increased selenium dosage. Brain Hg concentrations were 3 times higher and kidney concentrations were 12 times higher in the Hg-fed group than in the group fed the highest concentration of selenium (Orct et al., 2009). These studies show that selenium not only impacts the uptake of mercury, but the retention and distribution as well.

**Conclusion**

The purpose of this chapter was to determine the *in-vitro* uptake of mercury in four food types and to determine the potential impact of factors such as MeHg and selenium concentration on the absorption of Hg. We determined that lake trout muscle was found to have the highest concentration of transported Hg in the basolateral chamber at 12.70%, while ringed seal liver was found to have the lowest amount of Hg in the basal chamber at 0.42%. These results agree with our initial hypothesis that the fraction of bioaccessible MeHg in the food digest will show a positive correlation with Hg uptake. The percentage of MeHg in the bioaccessible fraction of ringed seal liver was the lowest at only 3.40%, while MeHg percentage in lake trout muscle was 86.0%, second only to air-dried beluga muscle at 88.8%.

The impact of selenium on the uptake of mercury was studied in two ways. Initially, the concentrations of bioaccessible selenium in the food digest were determined in the previous chapter. In this chapter, results showed that Hg transport was highest in foods such as lake trout muscle which contained the lowest concentration of bioaccessible selenium (0.091µg/g) and lowest in ringed seal liver which contained the highest amount of bioaccessible selenium.
(0.230µg/g). The coefficient of determination ($R^2$) value found by this relationship was 0.78. Secondly, after a 24 hour incubation, the percentage of Hg transported to the basolateral compartment decreased significantly from 61.61% of added Hg to only 28.32% when co-incubating with selenium. These results are in line with our hypothesis that selenium co-incubation will significantly reduce the uptake of Hg after 24 hours.
Chapter 5: Conclusions and Future Direction

Overall Conclusions

The purpose of this study was to determine the bioaccessibility and bioavailability of four key foods of the traditional Inuit diet to provide better estimation of Hg exposure and improve the characterization of risk of Hg exposure to human health. The impact, if any, that selenium may have on the uptake of Hg was also investigated using an \textit{in-vitro} Caco-2 cell model. It was hypothesized that bioaccessible Hg fractions would be dependent on original Hg concentrations in the food, which was determined through an \textit{in-vitro} digestion process. Our results showed that while ringed seal liver was found to contain the highest concentration of Hg in the raw food (24.43µg/g), after digestion, the bioaccessible Hg fraction was second lowest (32.3%). Similarly, ringed seal muscle contained the lowest concentration of Hg in the raw food (0.49µg/g), but post \textit{in-vitro} digestion, was found to contain the highest bioaccessible Hg fraction (69.0%). These findings are contrary to our hypothesis and highlight the need for risk assessment to include factors such as bioaccessibility when estimating MeHg exposure from traditional food consumption.

Secondly, the objective of chapter 4 of this study was to use Caco-2 cell model was used to determine the uptake of mercury into the bloodstream from digested samples of traditional food. A key finding of this experiment was that ringed seal liver having an initial Hg concentration (24.43µg/g) approximately 30x higher than the mean of the three remaining foods, was absorbed at the lowest percentage in Caco-2 cells (0.42%). This result is in line with our hypothesis that the fraction of bioaccessible MeHg in the food digest will show a positive correlation with the percentage of Hg uptake in Caco-2 cells. Ringed seal liver was found to have the lowest fraction of bioaccessible MeHg in its food digest at 3.4%. Hg from lake trout muscle was transported at
the highest percentage of (12.70%) which corresponded to its high bioaccessible MeHg fraction of 86.0%.

Summarizing our analysis of Hg uptake, the Hg bioaccessibility percentage of ringed seal liver was 32.3%, ringed seal muscle was 69.0%, lake trout muscle was 28.8%, and lastly air-dried beluga muscle was 34.0% after in-vitro digestion. Furthermore, Hg bioavailability percentages were determined to be 0.42% for ringed seal liver, 5.24% for ringed seal muscle, 7.30% for air-dried beluga muscle, and finally, 12.70% for lake trout muscle.

Lastly, the impact of the nutrient, selenium, was determined as Hg transport percentages in Caco-2 cells were found to decrease with increasing selenium concentrations in the bioaccessible fraction of the food. The concentration of bioaccessible selenium in lake trout muscle was the lowest at (0.091µg/g) while the percentage of transported Hg was the highest. Additionally, ringed seal liver bioaccessible selenium concentration was the highest at (0.230µg/g), and was found exhibit the lowest fractions of transported Hg in Caco-2 cells. This relationship was highlighted in (Figure 4.2) resulting in an $R^2$ value of 0.78.

A baseline of MeHg uptake after a 24 hour incubation was found to be 61.61% of total added MeHg. The effects of selenium co-incubation on Hg uptake were determined using an equimolar concentration of selenium and MeHg treatment. A significant decrease in mercury uptake from 61.61% to 28.32% was observed after 24 hours. These results agree with our initial hypothesis that selenium co-incubation will result in a significant reduction in Hg uptake after an incubation period of 24 hours. The significance of this experiment is to emphasize the importance of looking at a traditional diet as a whole. Selenium is one of many nutrients in traditional food which includes omega-3 fatty acids, a multitude of vitamins, as well as other beneficial nutrients. Aside from the ability of these nutrients to mitigate the neurotoxicity, uptake and overall
metabolism of mercury, a traditional diet also encourages a healthy active lifestyle and reduces the risk of diseases (ex. diabetes and obesity) (Laird et al, 2013). Therefore, when considering the risks of traditional food consumption due to potential Hg exposure, it is important to also incorporate factors such as bioaccessibility, bioavailability as well as the host of inherent benefits of a traditional Inuit diet.

**Future Directions**

The results presented in this study add to the recently growing research against the long-held assumption that the fraction of ingested dietary Hg reaching systemic circulation is 95-100%. Using an *in-vitro* digestion model as well as the caco-2 cell *in-vitro* gastrointestinal uptake model, the bioaccessibility and bioavailability of Hg from the four chosen food types were determined. When incorporating these factors, it was found that only 0.001% to 0.04% of Hg from food was absorbed into the bloodstream. These results can be used to inform future Hg risk assessments through the use of adjustment factors. The following adjustments factors can be applied to future risk assessments; for RSL, Original THg (32.3 x 0.42) %, for RSM, Orig. THg (69.0 x 5.24) %, for ADB, Orig. THg (34.0 x 7.30) %, and lastly, for LT, Orig. THg (28.8 x 12.70) %. Further research into the bioaccessibility and bioavailability of more traditional food can increase the accuracy of risk assessment studies and reduce the overestimation of Hg risks.

For future studies, a more in-depth analysis of bioavailability experiments could be carried out. A limitation of this study was that original MeHg concentrations in the food were not determined and therefore the change in concentration of MeHg prior to and post-digestion is unknown. This would provide further insight into the influence of the digestion process on specifically MeHg rather than THg, which would, in turn, depict a more accurate representation of the process of MeHg retention and elimination after digestion.
Inherent limitations still exist when using the *in-vitro* gastrointestinal model of Caco-2 cells. Cell monolayer growth on the insert will differ based on many factors and therefore maximizing the use of one 12-well plate is difficult. Due to cost and availability of material, this could potentially lead to less experimental replicates. Further research into Caco-2 cell culture may in the future result in more consistent growth and viability.

Lastly, in this research, reduced Hg uptake was observed when using the same concentration of Hg from food digest instead of MeHg chloride spike treatment. A negative correlation between increased selenium bioaccessible concentrations and Hg uptake was also seen. However, traditional food is host to a variety of beneficial nutrients. Future studies can expand on our current research by determining the impact of omega-3 fatty acids, and Vitamins A, D and E on Hg uptake.
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