Université du Québec
Institut National de la Recherche Scientifique
Centre Eau Terre Environnement

Spéciation hépatique et distribution tissulaire et sub-cellulaire du mercure : effets sur la toxicité chez les poissons

Par

Benjamin D. Barst

Thèse présentée pour l'obtention du grade de
Philosophiae doctor (Ph.D.)
en sciences de l'eau

Jury d'évaluation

Président du jury et examinateur interne	Prof. Patrice Couture INRS-ETE
Examinateur externe	Dr. John Chételat Environment Canada
Examinateur externe	Prof. Feiyue Wang University of Manitoba
Directeur de recherche	Prof. Paul Drevnick University of Michigan
Codirecteur de recherche	Prof. Peter G.C. Campbell INRS-ETE

RÉSUMÉ

Le mercure est un contaminant d'intérêt mondial, car il affecte même des environnements éloignés comme l'Alaska et l'extrême-arctique canadien. La consommation de poissons contaminés est la principale source de mercure chez les humains et la faune et cela entraîne des effets négatifs sur la santé. Le mercure est également toxique pour les poissons eux-mêmes. Les poissons sont principalement exposés au mercure, comme le méthylmercure, via leurs régimes alimentaires. Le méthylmercure ingéré traverse la paroi intestinale de manière plus efficace que le mercure inorganique. Suite à son ingestion, il est distribué par la veine porte vers le foie. C'est pour cette raison, que la majorité du mercure dans le foie des poissons devrait être sous la forme de méthylmercure, mais des recherches récentes suggèrent que ce n'est pas toujours le cas. En raison du positionnement stratégique du foie dans le système circulatoire, il joue un rôle important dans la détoxication du mercure avant d'atteindre d'autres tissus du poisson.

La première partie de cette thèse décrit une nouvelle méthode afin de déterminer la spéciation du mercure dans les tissus des poissons par un analyseur de mercure direct. Le méthylmercure dans le foie et les muscles des poissons est estimé par la différence des mesures directes du mercure inorganique dans un extrait acide et du mercure total dans les tissus entiers. La méthode a été validée par l'analyse d'une substance de référence certifiée (DOLT-4, le foie d'aiguillat), et la comparaison des résultats obtenus sur les tissus de poissons contaminés naturellement, avec une méthode de spéciation du mercure traditionnelle (chromatographie en phase gazeuse couplée à la spectrométrie de fluorescence atomique à vapeur froide). Le rendement du mercure organique de DOLT-4, estimé par différence, est en moyenne 99 ± 5 % de la valeur moyenne certifiée pour le méthylmercure. Dans la majorité des échantillons de foie et dans tous les échantillons de muscles, les estimations de mercure organique à partir de la méthode proposée étaient indiscernables des mesures directes de spéciation de méthylmercure (99 ± 6 %). L'estimation de mercure organique par différence entre le mercure et le mercure inorganique totale était moins précise dans les échantillons de foie comportant des proportions de mercure inorganique élevées (≥90 %).

Par la suite, la nouvelle méthode a été utilisée pour déterminer les proportions relatives de méthylmercure dans les foies de plusieurs poissons osseux, représentant des espèces primitives et des plus évoluées. Dans les trois espèces de salmonidés, la majorité du mercure total hépatique était présente sous forme de méthylmercure. Inversement, le barbue de rivière (*Ictalurus punctatus*) et deux espèces primitives, le lépisostée tacheté (*Lepisosteus oculatus*) et le poisson-castor (*Amia calva*), comportaient des proportions élevées de mercure inorganique dans leurs foies. Pour les autres espèces de poissons, le méthylmercure composait environ la moitié du mercure hépatique totale. Chez une espèce de poisson donnée, la spéciation hépatique du mercure observée était similaire sur toute la gamme de concentrations de mercure total mesurées. Les différences entre espèces peuvent être issues de différences alimentaires ou encore de métabolismes différents du mercure pour chaque espèce de poisson.

Les agrégats de mélano-macrophages (MA) sont des collections de cellules spécialisées du système immunitaire de poissons. Dans les foies de certaines espèces, les MA augmentent en taille et en nombre en fonction de l'exposition aux contaminants, et peuvent être impliqués dans le métabolisme et le stockage du mercure.

Afin d'élucider la relation entre les MA et l'exposition aux métaux, nous avons échantillonné des sébastes aux yeux jaunes (*Sebastes ruberrimus*), une espèce à vie longue qui vit en milieu côtier; l’échantillonnage a eu lieu à l‘est et à l’ouest de l'île Prince of Wales, en Alaska. Les concentrations en métaux (c.-à-d., le méthylmercure, le mercure inorganique, le sélénium, le nickel, le cadmium, le cuivre, le zinc) dans le foie, ainsi que leurs corrélations avec la superficie globale des mélano-macrophages, ont été déterminées. Des sections de tissu hépatique ont été analysées par spectrométrie de masse à plasma induit couplée à l’ablation laser afin de déterminer la répartition des métaux entre les hépatocytes et les MA. La concentration de mercure inorganique dans tout le tissu était le meilleur facteur prédictif de la superficie globale des mélano-macrophages dans le foie et la rate des sébastes aux yeux jaunes. Comparativement aux hépatocytes, les mélano-macrophages comportaient des concentrations relativement plus élevées pour la plupart des métaux mesurés. Cependant, tous les métaux n’ont pas été accumulés de la même manière dans les macrophages, comme

indiqué par les différences de proportions de métaux dans les macrophages par rapport aux hépatocytes. Les données d'ablation laser ont été corroborées avec les résultats d'imagerie obtenus par la spectrométrie de fluorescence X sur d'une section du foie d'un sébaste aux yeux jaunes, ces mesures ayant été obtenues sur le synchrotron canadien à Saskatoon.

De plus, des ombles chevaliers (*Salvelinus alpinus*) ont été échantillonnés dans quatre lacs (c.-à-d., Small, 9-Mile, North, et Amituk) couvrant un gradient de contamination au mercure. De la même manière que pour les sébastes aux yeux jaunes, leurs foies ont été examinés afin de déterminer la relation entre les MA et le mercure hépatique. Les MA des quatres populations d'ombles chevaliers étaient plus petits et moins bien définis que ceux des sébastes aux yeux jaunes et n'étaient pas corrélés avec les concentrations de mercure. En revanche, les MA dans le foie de l'omble chevalier peuvent augmenter en nombre en présence de niveaux élevés de fer. Les ombles chevaliers du lac Amituk, qui comportaient les niveaux de fer les plus bas et les concentrations de mercure les plus élevées, possédaient moins de MA et significativement plus d'occurrence de fibrose du foie.

Une procédure a été utilisée pour déterminer la répartition sub-cellulaire des métaux entre les fractions potentiellement sensibles et les fractions détoxiquées, chez les foies des ombles chevaliers des lacs Small et Amituk. Au niveau sub-cellulaire, le mercure a été trouvé principalement dans les fractions potentiellement sensibles, ce qui indique que les ombles chevaliers ne détoxiquent pas efficacement ce métal.

Enfin, les indices généraux de santé (facteur de condition, l'indice hépatosomatique l'indice gonadosomatique) ont été calculés. Pour les sébastes aux yeux jaunes, les augmentations du facteur de condition et de l'indice hépatosomatique étaient probablement le résultat d'une augmentation de la disponibilité alimentaire. Pour l'omble chevalier, les concentrations de mercure étaient positivement corrélées avec l'indice hépatosomatique, qui était probablement un reflet de l'écologie alimentaire de l'omble. L'alimentation au niveau trophique supérieur conduit à de plus grandes réserves de lipides, mais aussi à une plus grande exposition au mercure. Le facteur de condition et l'indice gonadosomatique n'étaient pas liés aux concentrations de mercure chez l'omble

chevalier. En conclusion, selon les indices de santé communs utilisés dans cette étude, le mercure ne semble pas affecter négativement la santé générale des sébastes aux yeux jaunes et de l'omble chevalier.

ABSTRACT

Mercury is a contaminant of global concern as it affects even remote environments such as Alaska and the Canadian High Arctic. Consumption of contaminated fish is the principal source of mercury in humans and wildlife and results in negative health effects. Mercury is also toxic to the fish themselves. Fish are primarily exposed to mercury, as methylmercury, through their diets. Ingested methylmercury crosses the intestinal wall much more efficiently than inorganic mercury, and is circulated via the portal vein to the liver. Therefore, the majority of mercury in fish liver should be methylmercury, however recent research suggests that this is not always the case. Due to the liver's strategic positioning within the circulatory system it plays an important role in the detoxification of mercury before it reaches other fish tissues.

The first part of this research describes a novel method to determine mercury speciation in fish tissue using a direct mercury analyzer. Methylmercury in fish liver and muscle is estimated by difference from direct measurements of inorganic mercury in an acid extract and total mercury in whole tissue. The method was validated by analysis of a certified reference material (DOLT-4 dogfish liver) and naturally contaminated fish tissues with comparison to an established mercury speciation method (gas chromatography cold vapor atomic fluorescence spectrometry). Recovery of organic mercury from DOLT-4, estimated by difference, averaged 99 ± 5% of the mean certified value for methylmercury. In the majority of liver samples and all muscle samples, estimates of organic mercury from the proposed method were indiscernible from direct speciation measurements of methylmercury (99 ± 6%). Estimation of organic mercury by difference between total mercury and inorganic mercury was less accurate in liver samples with high percent inorganic mercury (90%).

The outlined method was then used to determine the relative proportions of methylmercury in the livers of several bony fish, representing both primitive and more evolved species. In the three salmonid species, the majority of hepatic total mercury was present as methylmercury. Conversely, channel catfish (*Ictalurus punctatus)* and the more primitive spotted gar (*Lepisosteus oculatus*) and bowfin (*Amia calva*) had elevated proportions of inorganic mercury in their livers. In the remaining species of fish,

methylmercury made up approximately half of the total hepatic mercury. These trends in hepatic mercury speciation are similar over a range of total mercury concentrations and may be due to differences in diet or how mercury is metabolized. Melano-macrophage aggregates (MA), collections of specialized cells of the innate immune system of fish. In the livers of some species, MA increase in size and number as a result of contaminant exposure, and may be involved in mercury metabolism and storage.

In order to elucidate further the relationship between macrophage aggregates and metals exposure, we sampled yelloweye rockfish (*Sebastes ruberrimus*), a long-lived species, from the east and west coasts of Prince of Wales Island, Alaska. Metal concentrations in livers (methylmercury, inorganic mercury, selenium, nickel, cadmium, copper, zinc) were determined as well as their correlations with melano-macrophage aggregate area. Sections of liver tissue were analyzed by laser ablation inductively coupled plasma mass spectrometry to determine how metals were spatially distributed between hepatocytes and macrophage aggregates. The concentration of inorganic Hg in whole tissue was the best predictor of macrophage area in yelloweye livers and spleens. Macrophage aggregates had higher relative concentrations of most metals compared to the surrounding hepatocytes. However, not all metals were accumulated to the same degree as evidenced by differences in the ratios of metals in macrophages compared to hepatocytes. Laser ablation data were corroborated with the results of X-ray synchrotron fluorescence imaging of a yelloweye liver section.

Landlocked Arctic char (*Salvelinus alpinus*) were sampled from four lakes (Small, 9-Mile, North, and Amituk) spanning a gradient of mercury contamination and, similar to yelloweye rockfish, their livers were examined to determine how melano-macrophage aggregates were related to hepatic mercury. The melano-macrophage aggregates of Arctic char were smaller and less well defined than those of yelloweye rockfish and were not correlated with mercury concentrations when including fish from all four char populations. Rather, melano-macrophage aggregates in the livers of Arctic char may increase in number as a result of elevated levels of iron. Amituk Lake char, which had the lowest iron and highest mercury concentrations, had relatively few melano-macrophage aggregates and significantly greater numbers of char with liver fibrosis.

A sub-cellular partitioning procedure was employed to determine how metals were distributed between potentially sensitive and detoxified compartments of Arctic char livers from Small Lake and Amituk Lake. At the sub-cellular level mercury was found mainly in potentially sensitive fractions of both Small and Amituk Lake char, indicating that Arctic char are not effectively detoxifying this metal.

Finally, common health indices (condition factor, hepatosomatic index, gonadosomatic index) were calculated. For yelloweye, increased condition factor and hepatosomatic index were likely a result of food availability. For Arctic char, concentrations of mercury were positively correlated with the hepatosomatic index, which was most likely a reflection of the feeding ecology of char; feeding at a higher trophic position results in greater lipid reserves, but also a greater exposure to mercury. The condition factor and gonadosomatic index were not related to mercury concentrations in Arctic char. In conclusion, mercury does not appear to be negatively affecting the general health of yelloweye rockfish and Arctic char, based on the common health indices employed in this study.

AVANT-PROPOS

Cette thèse comprend un résumé récapitulatif (en français) ainsi qu'une première partie (en anglais) qui comporte une synthèse de l'ensemble de la recherche doctorale. Celle-ci comprend une introduction générale, les objectifs de la recherche ainsi que des hypothèses connexes, les méthodes, résultats, discussion et conclusions en plus d'orientations pour de futures recherches. La deuxième partie est composée de trois articles, lesquels sont énumérés ci-dessous incluant les contributions de chaque auteur. Deux articles ont déjà été publiés et le troisième est en préparation.

Article 1 :

Barst BD, Hammerschmidt CR, Chumchal MM, Muir DC, Smith JD, Roberts AP, Rainwater TR & Drevnick PE (2013) Determination of mercury speciation in fish tissue with a direct mercury analyzer. *Environ. Toxicol. Chem.* 32(6) :1237-1241.

Barst BD : Conception de l'étude, réalisation des expériences, traitement et interprétation des données, rédaction initiale et finale du manuscrit.

Drevnick PE : Conception de l'étude et contribution à la rédaction du manuscrit.

Hammerschmidt CR : Participation à la réalisation des analyses et contribution à la rédaction du manuscrit.

Chumchal MM : Participation à l'échantillonnage, participation à la réalisation des analyses et contribution à la rédaction du manuscrit.

Muir DC : Conception de l'étude, de l'échantillonnage et contribution à la rédaction du manuscrit.

Smith JD : Conception de l'étude et contribution à la rédaction du manuscrit.

Roberts AP : Participation à l'échantillonnage et contribution à la rédaction du manuscrit.

Rainwater TR : Participation à l'échantillonnage et contribution à la rédaction du manuscrit.

Article 2 :

Barst BD, Bridges K, Korbas M, Roberts AP, Van Kirk K, McNeel K & Drevnick PE (2015) The role of melano-macrophage aggregates in the storage of mercury and other metals: an example from yelloweye rockfish (*Sebastes ruberrimus*). *Environ. Toxicol. Chem.* DOI: 10.1002/etc.3009

Barst BD : Conception de l'étude, réalisation des expériences, traitement et interprétation des données, rédaction initiale et finale du manuscrit.

Drevnick PE : Conception de l'étude et contribution à la rédaction du manuscrit.

Bridges K : Participation à la réalisation des analyses et contribution à la rédaction du manuscrit.

Korbas M : Participation à la réalisation des analyses et contribution à la rédaction du manuscrit.

Roberts AP : Participation à la réalisation des analyses et contribution à la rédaction du manuscrit.

Van Kirk K : Participation à la réalisation des analyses et contribution à la rédaction du manuscrit.

McNeel K : Participation à la réalisation des analyses et contribution à la rédaction du manuscrit.

Article 3 :

Barst BD, Rosabal M, Campbell PGC, Muir DC, Wang X, Köck G & Drevnick PE Sub-cellular distribution of trace elements and liver histology of landlocked Arctic char (*Salvelinus alpinus*) sampled along a mercury contamination gradient.

Barst BD : Conception de l'étude, réalisation des expériences, traitement et interprétation des données, rédaction initiale et finale du manuscrit.

Drevnick PE : Conception, sampling, and contribution to article writing.

Rosabal M : Conception de l'étude et contribution à la rédaction du manuscrit.

Campbell PGC : Conception de l'étude et contribution à la rédaction du manuscrit.

Muir DC : Conception de l'étude et contribution à la rédaction du manuscrit.

Wang X : Participation à la réalisation des analyses et contribution à la rédaction du manuscrit.

Köck G : Conception de l’étude et contribution à la rédaction du manuscrit.

REMERCIEMENTS

Au cours de mon travail de doctorat, j'ai reçu de l'aide du soutien de plusieurs personnes à qui je suis reconnaissant. Je remercie en premier lieu mes parents, Geoffrey et Elizabeth; merci d'avoir toujours cru en moi, pour votre patience, votre amour et votre soutien. Je remercie Pascale Marquis pour sa générosité et son amour inconditionnel. Je remercie mes trois sœurs Annabel, Jessica, et Tina.

Je remercie Dr Paul Drevnick d'avoir assumé le rôle d'être mon directeur de recherche à l'INRS. Paul m'a aidé à me développer en tant que scientifique et m'a encouragé vers les sujets qui m'intéressaient. Ses encouragements n'ont jamais faibli, ni passé inaperçus. Je remercie également Dr Peter Campbell pour ses précieux conseils, sa disponibilité, et d'avoir accepté d'être mon codirecteur. Je remercie Dr Patrice Couture, Dr John Chételat et Dr Feiyue Wang qui ont tous les trois consenti à servir en tant que membres du comité.

Je remercie Dr Jason Ahad pour sa vision unique sur tous les sujets et son partage d'opinions. J'apprécie aussi grandement qu'il m'ait permis de travailler dans son laboratoire, ce qui a augmenté mes connaissances sur les contaminants organiques.

Mes remerciements vont également à l'ensemble de l'équipe de techniciens de l'INRS-ETE; je remercie Julie Perreault, Lise Rancourt, René Rodrigue, Philippe Gérard et Stéfane Prémont.

Je remercie Dr Charles Gobeil pour ses nombreux conseils, Dre Isabelle Lavoie pour son temps et son aide avec la langue française ainsi que Dr Aaron Roberts pour m'avoir rendu accessible son laboratoire à UNT.

Je remercie les chercheurs du ministère de la Faune de l'Alaska pour leur aide (Kevin McNeel, Kara Hilwig, Kray Van Kirk, Kristin Munk). Je remercie Dr Derek Muir, Dr Günter Köck, et Debbie Iqaluk pour toute l'aide qu'ils m'ont apportée en Arctique. Je remercie également Tracy, Christopher, Melton Sr., et Melton "Bubba" Griggers de m'avoir gentiment accueilli dans leur maison pendant mes quatre merveilleux séjours en Alaska. Votre famille connaît le sens de l'hospitalité.

TABLE DE MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES ÉQUATIONS

LISTE DES ABBREVIATIONS

Cd : Cadmium

Cu : Cuivre

DMA-80 : Analyseur de mercure direct 80

DOLT : Matériau de référence certifié de foie de chien de mer

EXAFS : Spectroscopie d'absorption des rayons X, structure fine

Fe : Fer

Fe(III) : Fer ferrique

GC-CVAFS : Spectrométrie de fluorescence atomique à vapeur froide couplée à chromatographie en phase gazeuse

GSH : Glutathion

GSI : Indice gonadosomatique

GPx : Glutathion peroxydase

HDP : Protéines thermosensibles

H&E : Hématoxyline et l'éosine

Hg : Mercure

Hg(0) : Mercure élémentaire

Hg(II) : Mercure inorganique

HgSe : Séléniure de mercure

HPLC-ICP-MS: Chromatographie liquide à haute performance couplée à la spectrométrie de masse avec plasma à couplage inductif

HSP : Protéines thermostables

ICP-AES : Spectroscopie d’émission atomique avec atomisation par plasma à couplage inductif

ICP-MS : Spectrométrie de masse à plasma inductif

IHS : Indice hépatosomatique

LA-ICP-MS : Spectrométrie de masse à plasma induit couplée à l'ablation laser

LC-CVAFS : Spectrométrie de fluorescence atomique à vapeur froide couplée à chromatographie liquide

MA : Agrégats mélanomacrophages

M_{MA}:M_{Hep} : Métal dans les MA / métal dans les hépatocytes

MeHg : Méthylmercure

Ni : Nickel

POPs : Polluants organiques persistants

POW : Île du Prince de Galles

Se : Sélénium

SOD : Superoxydes dismutases

TBARS : Substances réactives à l'acide thiobarbiturique

XANES : Spectroscopie de structure près du front d'absorption de rayons X

Zn : Zinc

LIST OF ABBREVIATIONS

Cd : cadmium

Cu : copper

DMA-80 : direct mercury analyzer-80

DOLT : dogfish liver

EXAFS : extended X-Ray absorption fine structure

Fe : iron

Fe(III) : ferric iron

GC-CVAFS : gas chromatography cold vapor atomic fluorescence spectrophotometry

GSH : glutathione

GSI : gonadosomatic index

GPx : glutathione peroxidase

HDP : heat-denatured proteins

H&E : hematoxylin and eosin

Hg : mercury

Hg(0) : elemental mercury

Hg(II) : inorganic mercury

HgSe : mercuric selenide

HPLC-ICP-MS: high performance liquid chromatography inductively coupled plasma mass spectrometry

HSI : hepatosomatic index

HSP : heat-stable proteins

ICP-AES : inductively coupled plasma atomic emission spectroscopy

ICP-MS : inductively coupled plasma mass spectrometry

LA-ICP-MS : laser ablation inductively coupled plasma mass spectrometry

LC-CVAFS : liquid chromatography cold vapor atomic fluorescence spectrophotometry

MA : melano-macrophage aggregates

M_{MA}:M_{Hep} : ratio of metal in melano-macrophage aggregate to metal in hepatocytes

MeHg : methylmercury

Ni : nickel

POPs : persistant organic pollutants

POW : Prince of Wales Island

Se : selenium

SOD : superoxide dismutase

TBARS : thiobarbituric acid reactive substances

XANES : X-ray absorption near edge structure

Zn : zinc

RÉSUMÉ RÉCAPITULATIF

1 INTRODUCTION

1.1 Le cycle du mercure dans l'environnement aquatique

Le mercure (Hg) est un métal qui appartient au groupe IIB du tableau périodique des éléments. Trouvé naturellement dans la croûte terrestre, le Hg a des propriétés chimiques intéressantes et un cycle biogéochimique unique qui implique la libération des réserves géologiques, le transport atmosphérique, le dépôt atmosphérique, et la re-volatilisation. Les activités anthropiques, notamment l'exploitation minière, l'incinération des déchets et la combustion de combustibles fossiles, ont entraîné la mobilisation accrue de mercure dans l'environnement. Des données récentes suggèrent que l'extraction artisanale de l'or et de la combustion de charbon représentent environ les deux tiers des émissions anthropiques de mercure, dont la majorité est libérée par l'Asie (~50 % de toutes les émissions anthropiques). Le mercure, émis par la combustion du charbon, est introduit dans l'atmosphère sous les formes élémentaire (Hg(0)) et inorganique (Hg(II)); la forme Hg(0) peut séjourner et voyager dans l'atmosphère pendant des périodes de 0,5 à 2 ans avant d'être oxydé en Hg(II) et déposé dans les écosystèmes aquatiques et terrestres. Pour cette raison, la mobilisation anthropique de mercure est un problème mondial et par conséquent même les régions éloignées sont soumises à la contamination. Dans les milieux aquatiques, le Hg(II) est méthylé pour former du méthylmercure (MeHg), qui s’accumule facilement et se bioamplifie dans les réseaux trophiques, résultant en des concentrations élevées de mercure chez les poissons.

1.2 Les effets toxiques du mercure chez les poissons

La toxicité du MeHg peut être causée par sa capacité de provoquer un stress oxydatif (Scheuhammer *et al.*, 2012). Par exemple, Berntssen *et al.* (2003) ont rapporté une diminution des activités des superoxydes dismutases (SOD) et de la glutathion (GSH)

peroxydase (GPx) dans le cerveau des jeunes saumons de l'atlantique (*Salmo salar*) se nourrissant de régimes enrichis en MeHg. Les auteurs ont également noté une augmentation des produits de peroxydation lipidique (mesurés en tant que substances réactives à l'acide thiobarbiturique ou TBARS), des dommages pathologiques au cerveau ainsi qu'une réduction du comportement suivant l'alimentation chez les poissons nourris avec une diète riche en MeHg. La diminution des activités des SOD et de la GPx, ainsi que l'augmentation de la peroxydation lipidique, suggèrent une atteinte au système de défense contre le stress oxydatif (Berntssen *et al.*, 2003).

Des changements histologiques associés à l'exposition au mercure ont également été décrits comme étant présents dans le foie des poissons. Par exemple, la variation de la couleur du foie était positivement corrélée avec la concentration de mercure totale dans le tissu musculaire du grand brochet (*Esox lucius*). Le pigment responsable de la variation de couleur a été identifié comme étant la lipofuscine. Ce pigment est composé de lipides et de protéines oxydées et se forme suite à un stress oxydatif (Drevnick *et al.*, 2008; Brunk &Terman, 2004). La lipofuscine et d'autres pigments sont souvent trouvés dans les agrégats mélano-macrophage (MA), qui sont des cellules phagocytaires spécialisées du système immunitaire inné chez les poissons. Ces agrégats servent de sites de détoxication, de destruction et de recyclage du matériel cellulaire endogène et exogène, y compris les cellules endommagées et affaiblies**.** Bien que les MA peuvent augmenter en taille et en nombre en réponse à une foule de processus (y compris le jeûne, le vieillissement, les infections pathogènes et l'exposition à d'autres contaminants) (Agius & Roberts, 2003), plusieurs études ont montré un plus grand nombre de MA avec une exposition accrue au Hg. Dans une étude en laboratoire, un nombre important de MA et de nécroses ont été signalés dans les foies de *Hoplias malabaricus* qui ont été nourris avec une diète contenant 0,075 μg $MeHg \cdot g^{-1}$ poids humide pendant 70 jours (Mela *et al.*, 2007). Raldúa *et al.* (2007) ont trouvé des niveaux élevés de lipofuscine, de noyaux pycnotiques, et de MA dans le foie des poissons sauvages en aval d'une usine de chlore-alcali (Raldúa *et al.*, 2007). Des corrélations entre les concentrations de mercure et les MA ont également été documentées dans le foie et les reins de plusieurs espèces de salmonidés (Schwindt *et al.*, 2008), ainsi que dans les reins, le foie et la rate de brochet (Meinelt *et al.*, 1997).

Une étude visant une compréhension plus approfondie de la relation entre les MA et le Hg a été réalisée par Barst et ses collègues (2011), qui ont mesuré le Hg dans les MA hépatiques et les hépatocytes adjacents par spectrométrie de masse à plasma induit couplée à l'ablation laser (LA-ICP-MS), et a révélé que les MA avaient des concentrations relatives plus élevées de Hg que les tissus environnants (Barst *et al.*, 2011). Ces résultats, ainsi que ceux de deux études récentes sur la perchaude (*Perca flavescens*), démontrent que le Hg s'accumule dans ces cellules immunitaires, probablement en raison de la phagocytose des cellules endommagées par le Hg (Barst *et al.*, 2011; Batchelar et al., 2013; Müller et al., 2015). Comme il a été démontré que les MA augmentent en taille et en nombre avec l'âge et l'exposition à d'autres métaux, ces variables doivent être également considérées lors de la détermination des liens entre les MA et le Hg chez les poissons sauvages.

En plus des effets sur le foie, les récentes revues de la littérature indiquent que la reproduction est un des processus biologiques des plus sensibles à la toxicité du Hg chez les poissons sauvages (Crump et Trudeau, 2009; Scheuhammer *et al.*, 2007). Des études en laboratoire menées sur des têtes-de-boules nourris avec une diète riche en MeHg (à des concentrations environnementales réalistes) ont induit l'apoptose dans les cellules des gonades qui synthétisent les hormones nécessaires pour la reproduction (Drevnick *et al.*, 2006). Pour l'instant, cependant, aucune étude concluante n'a lié l'exposition au Hg et les effets négatifs sur la reproduction chez les poissons sauvages.

Les changements dans la condition physique, l'indice gonadosomatique (GSI), ou l'indice hépatosomatique (IHS) des poissons exposés au mercure ont également été documentés. Ces mesures sont simples à réaliser et sont fréquemment rapportées dans la littérature. Le facteur de condition est le rapport entre la longueur d'un poisson à son poids; les indices hépatosomatique et gonadosomatique donnent le poids relatif du foie et des gonades, respectivement, par rapport au poids total du poisson (Di Giulio et Hinton, 2008). Drevnick *et al.* (2008) ont noté une réduction des réserves de lipides dans le foie des grands brochets et une diminution de la condition physique des poissons ayant des concentrations élevées de mercure. Des résultats similaires ont été rapportés pour le brochet du Lac Clay, au Canada (Lockhart *et al.*, 1972). L'indice hépatosomatique était corrélé négativement avec les concentrations de mercure dans

le doré et la perchaude sauvage (Larose *et al.*, 2008). De plus, dans une étude en laboratoire, l'exposition au MeHg alimentaire a conduit à une diminution de l'indice gonadosomatique du doré jaune (Friedmann *et al.*, 1996).

1.3 La détoxication du mercure dans le foie de poisson

Grâce à son positionnement stratégique dans le système circulatoire, le foie est exposé au MeHg et Hg(II) alimentaire avant les autres tissus. La capacité du foie à détoxiquer le Hg dicte donc le niveau d'exposition et les effets toxiques résultant dans les autres tissus des poissons. L'étude de la détoxication hépatique et de la toxicité du mercure nécessite une compréhension de la façon dont les espèces de Hg réagissent dans les systèmes biologiques. Le MeHg et le Hg(II) ont de fortes affinités pour le sélénium (Se) et le soufre réduit, qui sont généralement retrouvés dans les organismes sous forme de sélénols et de thiols (Wang *et al.*, 2012). Bien que la liaison du MeHg et du Hg(II) avec les sélénols et les thiols de certaines biomolécules hépatiques puisse entraîner des effets délétères, elle peut également aider à prévenir la toxicité en facilitant l'excrétion, la transformation ou la séquestration du MeHg et du Hg(II), empêchant ainsi leur interaction avec les sites potentiellement sensibles physiologiquement.

Comme un mécanisme de détoxication primaire, les hépatocytes produisent de la GSH qui est conjuguée au MeHg et excrétée dans la bile (Dutczak & Ballatori, 1994). L'excrétion est cependant inefficace et le MeHg est en grande partie réabsorbé dans l'intestin (Dutczak et al., 1991; Roberts et al., 2002). Le Hg inorganique se lie également avec la GSH et est excrété dans la bile, mais la réabsorption de l'intestin est moins efficace et une grande partie du Hg(II) est éliminée dans les fèces (Zalups *et al.*, 1999). En outre, la détoxication de Hg est obtenue par la séquestration de Hg(II) avec les métallothionéines, qui sont des protéines de faible poids moléculaire à haute teneur en cystéine (Sigel *et al.*, 2009). La synthèse de métallothionéine peut augmenter en raison de l'exposition au Hg(II) (Monteiro *et al.*, 2010) et à d'autres métaux (Sigel *et al.*, 2009). En revanche, l'interaction entre la métallothionéine et le MeHg n’est pas bien comprise. Leiva-Presa et ses collègues (2004) ont suggéré la formation d’un complexe de MeHg-métallothionéine, basée sur des expériences *in vitro* (Leiva-Presa *et al.*, 2004).

Cependant, il est présumé que la métallothionéine ne se lie pas au MeHg ou n'est pas induite par le MeHg chez les poissons (Kidd & Batchelar, 2011; Wiener & Spry, 1996).

Le sélénium peut également être impliqué dans la détoxication de Hg dans les foies de poissons. L'une des premières études impliquant un effet antagoniste entre le Hg et le Se a montré qu'une dose de sélénite de sodium, administrée après une dose de Hg(II), pourrait protéger contre la néphrotoxicité chez le rat (Pařízek & Ošťádalová, 1967). Depuis cette découverte, de nombreuses études ont démontré que le Se donné en supplément peut prévenir les effets toxiques de Hg chez plusieurs espèces (Cuvin-Aralar et Furness, 1991; El-Begearmi *et al.*, 1977). L'effet de protection du Se contre le Hg était présumé être le résultat de la séquestration du Hg par le Se, diminuant ainsi les effets toxiques. Cependant, des analyses récentes suggèrent que l'interaction Hg-Se pourrait aussi représenter des effets négatifs. Comme le Se est nécessaire pour la production de sélénocystéines, qui sont incorporées dans les sites actifs de sélénoenzymes, une réduction de la disponibilité du Se en raison de sa complexation avec le Hg pourrait diminuer la fonction et la synthèse des sélénoenzymes (Ralston & Raymond, 2010). Comme le MeHg est facilement réabsorbé dans l'intestin et recyclé par le foie, une exposition continue peut porter atteinte aux défenses cellulaires, conduisant à une interaction entre le MeHg et des molécules intracellulaires sensibles. Une transformation du MeHg en Hg(II), la forme la plus facilement excrétée, serait donc bénéfique. Cette conversion, connue sous le nom déméthylation, a été rapportée chez des bactéries, des mammifères marins et des oiseaux (Barkay *et al.*, 2003; Eagles-Smith et al., 2009; Palmisano et al., 1995), mais un mécanisme clair n'a pas encore été décrit chez les poissons.

1.4 **La répartition sub-cellulaire de mercure**

Récemment, dans le domaine de la toxicologie des métaux, des procédures de partitionnement ont été employées pour comprendre comment les métaux sont répartis entre les fractions sub-cellulaires potentiellement sensibles et les fractions détoxiquées (Giguère *et al.*, 2006; Rosabal et al., 2012; Rosabal et al., 2015; Wallace *et al.*, 2003). Les procédures de partitionnement sub-cellulaires permettent de mieux comprendre

comment les organismes gèrent les métaux et d'évaluer si des effets toxicologiques sont susceptibles de se produire. Les procédures de partitionnement ont souvent recours à la centrifugation différentielle, qui sépare le contenu sub-cellulaire après homogénéisation d'un tissu. Les centrifugations différentielles n'aboutissent pas en des fractions parfaitement séparées puisqu'un certain chevauchement des fractions est probable. Par conséquent, les fractions sont définies sur le plan opérationnel et l'interprétation des résultats doit être effectuée avec soin. Idéalement, des calculs de bilan de masse devraient être effectués pour déterminer si les métaux ont été perdus ou si des contaminations se sont produites au cours de la procédure (Campbell & Hare, 2009).

Peu d'études ont été menées pour déterminer la distribution sub-cellulaire de Hg chez le poisson (Araújo *et al*., 2015; Onsanit & Wang, 2011). Onsanit *et al.* (2011) ont déterminé la répartition de Hg total et de MeHg dans les fractions sub-cellulaires isolées à partir de tissu musculaire de la dorade rose (*Pagrus major*), de la dorade noire (*Acanthopagrus schlegelii*), et du tambour rouge (*Sciaenops ocellatus*). Les auteurs ont constaté que la majorité du Hg total et du MeHg accumulés étaient contenus dans la fraction des débris cellulaires[1], suivis par la fraction de protéines thermostables (HSP), les granules riches en métaux, la fraction de protéines dénaturées à la chaleur (HDP), et des organites. Comme la majorité du Hg n'a pas été trouvée dans les fractions sensibles, les auteurs ont donc suggéré que le Hg avait peu de toxicité pour le muscle des poissons. Plus récemment, Araújo *et al.* (2015) ont mesuré le Hg total dans les fractions sub-cellulaires des foies de rougets sauvages (*Liza aurata*) et ont observé de faibles concentrations de Hg dans la fraction HSP et la fraction contenant des granules, ce que les auteurs ont attribué à des concentrations de Hg en dessous du seuil physiologique nécessaire pour activer des mécanismes de détoxication (Araújo *et al.*, 2015). Bien que ces auteurs aient été parmi les premiers à présenter des données de partitionnement sub-cellulaire de Hg chez les poissons sauvages, ils n'ont pas fourni de calcul du bilan de masse et donc des pertes ou des ajouts de Hg pendant la procédure

[1] A noter – la présence d'une forte proportion d'un métal dans la fraction "débris cellulaires" est un « mauvaise signe », indiquant que l'homogénéisation de l'organe a été inadéquate (Giguère et al., 2006).

de séparation n'ont pas pu être déterminés. Étant donné que la recherche dans ce domaine est limitée, il serait justifié que de futures études se concentrent sur la distribution du Hg entre les fractions sub-cellulaires qui sont potentiellement sensibles ou détoxiquées chez les poissons sauvages.

1.5 La spéciation du mercure dans les tissus de poissons

Le méthylmercure est la forme prédominante de Hg dans le tissu musculaire de poisson (Bloom, 1992), tandis qu'une proportion importante de Hg peut être présente comme Hg(II) dans le foie des poissons (Barst *et al*., 2011; Drevnick *et al*., 2008). Des proportions élevées de Hg(II) hépatique peuvent être le résultat d'une source alimentaire importante de Hg(II), de la séquestration et la liaison préférentielle de Hg(II) dans le foie, et/ou d'un mécanisme de déméthylation hépatique. Drevnick *et al.* (2008) ont rapporté qu'une fraction importante de Hg dans les foies de brochet était présente comme Hg(II). Ces auteurs ont émis l'hypothèse que la forte proportion de Hg(II) hépatique n'était pas dû à une déméthylation, mais plutôt le résultat de la concentration élevée de Hg(II) dans les tissus des odonates larvaires qui étaient une proie commune du grand brochet dans cet écosystème (Drevnick *et al.*, 2008). Toutefois, des différences dans le régime alimentaire ne pouvaient pas expliquer les divergences observées dans la spéciation du Hg hépatique chez l'achigan à grande bouche (*Micropterus salmoides*) et le lépisosté tacheté (*Lepisosteus oculatus*) de Caddo Lake aux États-Unis. Dans ce lac, le lépisosté tacheté et l'achigan à grande bouche se nourrissent en positions trophiques similaires (positions établies par $\delta^{15}N$), mais la spéciation du Hg hépatique est très différente dans les deux espèces. Chumchal et ses collègues ont indiqué que seulement 2 % du Hg total dans les foies de lépisosté tacheté était présent sous forme de MeHg, alors que dans les foies d'achigan à grande bouche le MeHg représentait 74 % du Hg total, suggérant que ces poissons métabolisent le Hg différemment (Barst *et al.*, 2011; Chumchal & Hambright, 2009; Chumchal et al., 2011). La déméthylation a été rapportée comme étant spécifique à l'espèce pour les oiseaux aquatiques (Eagles-Smith *et al.*, 2009) et pourrait également l'être chez des poissons. Des recherches récentes suggèrent que les différences dans la spéciation hépatique de

Hg chez les poissons peuvent être liées aux différences phylogénétiques des MA (Smith, 2012).

Selon Agius & Roberts (2003), les MA sont des versions primitives des centres germinaux des ganglions lymphatiques chez les oiseaux et les mammifères et sont répandus dans les tissus hématopoïétiques. L'hématopoïèse ou la synthèse du sang ont lieu dans la moelle osseuse des mammifères. Cependant, les poissons ne possèdent pas de moelle osseuse (Moyle & Cech, 2004) et, par conséquent, la création de cellules sanguines se produit principalement dans la rate, les reins et le foie (Agius 1980). Le tissu primaire où se produit le sang varie selon les groupes phylogénétiques de poissons, et donc la distribution tissulaire des MA varie aussi dans ce sens. Ces résultats sont cohérents avec la distribution tissulaire des MA hépatiques chez le lépisosté tacheté et l'achigan à grande bouche de Caddo Lake; les foies de lépisosté tacheté sont riches en MA, alors que les MA sont plus répandues dans les rates et les foies d'achigan à grande bouche. On ne sait pas si les MA hépatiques sont directement responsables de la déméthylation du MeHg ou si elles ne servent qu'à stocker le Hg(II) après que la déméthylation ait eu lieu ailleurs dans le foie. L'évidence pour leur rôle dans la déméthylation peut être tirée d'études sur les mammifères, qui montrent que le MeHg est converti en Hg(II) dans les ganglions lymphatiques riches en macrophages (Havarinasab *et al.*, 2007). De plus, la conversion du MeHg en Hg(II) a été documentée dans d'autres types de cellules phagocytaires de rats, d'humains, et de lapins, probablement dues à la présence à l'intérieur des phagocytes de radicaux libres produits lors de la phagocytose (Suda *et al.*, 1992). Si la déméthylation du MeHg se produit de la même manière chez les poissons, on pourrait conclure que les MA peuvent servir à la fois à déméthyler et à stocker le Hg.

1.6 Techniques analytiques pour déterminer la spéciation du mercure dans les tissus de poissons

Les techniques analytiques actuelles pour la détermination de la spéciation du Hg dans les tissus de poissons comprennent le HPLC-ICP-MS (Bushee, 1988), le LC-CVAFS

(Shade, 2008) et le GC-CVAFS[2] (Bloom, 1989), cette dernière étant probablement l'approche la plus populaire. Bien que ces techniques aient des limites de détection basses et permettent la quantification du Hg(II) et du MeHg, le coût de l'instrumentation ou les frais d'analyses faites à l'externe peuvent être considérables. Pour cette raison, plusieurs études se sont limitées à des analyses de Hg total, tout en analysant seulement quelques échantillons pour la spéciation du Hg (Chumchal *et al.*, 2011). Récemment, pour la détermination du Hg total, les analyseurs directs de Hg ont gagné en popularité (Haynes *et al.*, 2006). Des études antérieures ont également utilisé l'analyseur direct de Hg pour quantifier le MeHg dans différentes matrices (Carbonell *et al.*, 2009; Maggi et al., 2009; Nam & Basu, 2011; Scerbo & Barghigiani, 1998). Maggi et ses collègues (2009) ont utilisé une méthode de digestion avec le HBr, suivie d'une extraction au toluène et d'une rétro-extraction avec une solution aqueuse de L-cystéine, puis finalement une analyse par DMA-80 pour mesurer le Hg dans les différentes substances de référence certifiées ainsi que dans des échantillons environnementaux. En utilisant cette approche, ils ont récupéré plus de 80 % du MeHg à partir de plusieurs substances de référence, mais la récupération était en moyenne de seulement 74 % à partir de l'étalon de référence du foie de l'aiguillat (DOLT-3). De même, Scerbo et ses collègues (1998) ont constaté un accroissement de la variabilité dans la récupération du mercure organique à partir de DOLT-1, ce qu'ils ont attribué à la teneur en lipides relativement élevée dans le foie de ce poisson. Compte tenu des résultats de ces études, des méthodes peu coûteuses fournissant des données précises de la spéciation du Hg pour les tissus des poissons riches en lipides sont nécessaires.

[2] L'explication des sigles HPLC-ICP-MS, LC-CVAFS et GC-CVAFS se trouve dans le glossaire.

2 LACUNES DE CONNAISSANCES

2.1 Les techniques analytiques pour déterminer la spéciation du mercure dans les tissus des poissons

Les analyses pour la détermination de la spéciation du mercure dans les tissus de poissons peuvent être coûteuses en raison du prix de l'instrumentation ou des frais de sous-traitance. Des études antérieures ont eu des niveaux de succès variables en utilisant des analyseurs de Hg directs pour déterminer la spéciation du Hg dans les tissus des poissons. La quantification précise de la spéciation du Hg dans les tissus riches en lipides, comme le foie, a été un problème dans le passé (Maggi et al., 2009; Scerbo & Barghigiani, 1998). Par conséquent, des méthodes peu coûteuses qui fournissent les données de spéciation du Hg précises pour les tissus riches en lipides sont nécessaires.

2.2 La spéciation de mercure hépatique et la phylogénie de poissons

Il y a très peu d'informations concernant les proportions relatives du MeHg et du Hg(II) dans le foie des poissons sauvages et, par conséquent, on ne comprend pas bien comment la spéciation de Hg hépatique peut différer entre les espèces. Dans cet esprit, la spéciation du Hg hépatique doit être évaluée pour différentes espèces de poissons. Cette information peut donner un aperçu des différences de sensibilité des espèces au Hg et des mécanismes de détoxication.

2.3 Répartition de mercure dans les tissus de foie de poisson

Des études antérieures ont rapporté des corrélations positives entre le nombre de MA hépatiques chez les poissons et les concentrations de Hg (Barst *et al*., 2011; Batchelar *et al*., 2013; Mela *et al*., 2007; Raldúa *et al*., 2007). De plus, d'autres métaux ont également été étudiés et semblent se retrouver dans ces cellules immunitaires (Barst *et al*., 2011; Pulsford *et al*., 1992). Toutefois, on ne comprend pas bien dans quelle

mesure le Hg s'accumule dans les cellules par rapport à d'autres métaux essentiels et non essentiels.

2.4 **Répartition sub-cellulaire du mercure dans le foie de poissons**

Très peu d'études ont déterminé la répartition sub-cellulaire du Hg chez les poissons. Le nombre d'études portant la répartition sub-cellulaire du Hg dans le foie des poissons (Araújo *et al.*, 2015) est encore plus faible, ce qui est surprenant étant donné le rôle que cet organe joue dans la détoxication du Hg. Par conséquent, d'autres études sur la répartition sub-cellulaire du Hg dans les foies de poissons sauvages sont nécessaires. Les études qui visent à déterminer la répartition sub-cellulaire du Hg dans les poissons en utilisant une procédure de centrifugation différentielle devraient également calculer les bilans de masse pour déterminer la récupération du Hg.

2.5 **Les effets toxiques de mercure chez le poisson**

Des études en laboratoire et sur le terrain ont démontré que l'exposition alimentaire au MeHg affecte négativement la santé des poissons (Kidd & Batchelar, 2011; Sandheinrich et Wiener, 2011; Scheuhammer et al., 2012). Toutefois, on ne comprend pas bien comment l'exposition au mercure affecte les poissons sauvages, en particulier dans les régions éloignées comme l'Arctique. Cette constatation a été démontrée clairement par une étude récente sur les effets biologiques du mercure dans le poisson et la faune de l'Arctique canadien : « *There is an explicit need for Hg effects information for Arctic species themselves. Therefore, bioeffects studies should comprise a major focus of future Hg research in the Canadian Arctic.*» (Scheuhammer *et al.*, 2014). Il y a donc un besoin critique et justifié pour plus de recherche sur les concentrations de mercure dans les poissons afin d'améliorer les connaissances concernant leur santé et ainsi optimiser leur conservation (Wiener *et al.*, 2003).

3 ORGANISMES D'ÉTUDE

3.1 Les sébastes aux yeux jaunes

Les sébastes aux yeux jaunes (*Sebastes ruberrimus*) sont originaires de la côte Pacifique des États-Unis et du Canada, et font partie des pêches récréatives et commerciales importantes dans le Pacifique du Nord (MacLellan & Station, 1997; Yamanaka *et al*., 2006). Les sébastes aux yeux jaunes ont été sélectionnés pour l'étude, car ils ont une très longue durée de vie (plus vieux spécimen > 120 ans) (MacLellan & Station, 1997) et étaient susceptibles d'avoir des concentrations élevées de mercure dans leurs tissus.

3.2 L'omble chevalier

Les ombles chevaliers (*Salvelinus alpinus*) ont été choisis pour l'étude parce qu'ils sont souvent le seul poisson présent dans les lacs de l'Arctique canadien et sont considérés comme une espèce clé (Gantner *et al*., 2010; Power *et al.*, 2008). Des résultats antérieurs ont montré que plusieurs populations d'omble chevalier du nord du Canada et du Groenland ont, dans leurs tissus, des concentrations de mercure s'élevant à des niveaux qui dépassent les seuils de toxicité pour le mercure (Drevnick, 2013), mais on en sait très peu sur la façon dont le Hg pourrait affecter la santé de cette espèce.

3.3 Autres espèces

D'autres espèces ont également été étudiées dont le lépisosté tacheté (*Lepisosteus oculatus*), le poisson-castor (*Amia calva*), la barbue de rivière (*Ictalurus punctatus*), le grand brochet (*Esox lucius*), l'achigan à grande bouche (*Micropterus salmoides*), l'omble de fontaine (*Salvelinus fontinalis*), et le touladi (*Salvelinus namaycush*). Ces espèces ont été choisies parce qu'elles représentent plusieurs familles taxonomiques, qui vont de la plus primitive (Lepisosteidae et Amiidae) à la plus récemment évoluée (Centrarchidae et salmonidés) des poissons osseux. Les foies de sébastes aux yeux jaunes, d'omble chevalier, et des espèces de poissons mentionnées ci-haut ont été

analysés pour 1) aider à valider la méthode de spéciation du Hg et 2) déterminer si la spéciation du Hg hépatique diffère entre les groupes de poissons. Les ombles de fontaine ont été capturés dans huit lacs situés dans le parc national de la Mauricie au Québec. Les touladis et les grands brochets ont été prélevés dans les lacs situés dans le Parc national d'Isle Royale, au Michigan dans les États-Unis. Les achigans à grande bouche, les barbues de rivière, les poissons-castors, et les lépisostés tachetés ont été échantillonnés au Lac Caddo, situé au Texas et en Louisiane dans les États-Unis.

4 OBJECTIFS ET HYPOTHÈSES

Les principaux objectifs et leurs hypothèses respectives sont présentés ci-dessous. Les deux premiers objectifs ont été atteints par l'étude de plusieurs espèces de poissons. Les autres objectifs sont axés uniquement sur le sébaste aux yeux jaunes et l'omble chevalier.

Objectif 1 : Développer une méthode pour déterminer la spéciation du mercure dans les tissus des poissons en utilisant un analyseur direct de Hg.

Hypothèse : Les concentrations de MeHg, calculées par la méthode proposée, ne seront pas significativement différentes de la valeur certifiée de MeHg dans le DOLT-4.

Hypothèse : Les concentrations de MeHg, calculées à partir du Hg total et des concentrations de Hg(II), ne différeront pas de manière significative des mesures de MeHg quantifiées par GC-CVAFS.

Objectif 2 : Déterminer la spéciation du mercure hépatique chez les différentes espèces de poissons osseux.

Hypothèse : Le MeHg constituera la majorité du Hg total dans les foies des poissons osseux.

Objectif 3 : Déterminer comment le mercure est lié aux pathologies hépatiques chez les sébastes aux yeux jaunes et les ombles chevaliers.

Hypothèse : Le nombre ou la superficie des MA seront corrélés positivement avec le Hg et d'autres métaux dans le foie des sébastes aux yeux jaunes et le foie des ombles chevaliers.

Hypothèse : D’autres pathologies hépatiques seront corrélées positivement avec le Hg et d'autres métaux dans les foies de sébastes aux yeux jaunes et les foies d’ombles chevaliers.

Objectif 4 : Déterminer comment le mercure et d'autres métaux sont distribués au niveau des tissus dans le foie des sébastes aux yeux jaunes.

Hypothèse : Les concentrations relatives de Hg et d'autres métaux seront plus élevées dans les MA que dans le tissu adjacent.

Objectif 5 : Déterminer la répartition sub-cellulaire du mercure total dans les foies des ombles chevaliers.

Hypothèse : Le Hg total sera principalement associé à la fraction de protéines thermostables (HSP).

Objectif 6 : Déterminer comment le mercure est lié à la santé reproductive et à la santé générale des sébastes aux yeux jaunes et des ombles chevaliers.

Hypothèse : L'indice hépatosomatique sera corrélé négativement avec le Hg total dans le foie des sébastes aux yeux jaunes et des ombles chevaliers.

Hypothèse : L'indice gonadosomatique et la condition physique seront corrélés négativement avec le Hg total dans le muscle des ombles chevaliers.

5 MÉTHODES EXPÉRIMENTALES

Les méthodes spécifiques utilisées pour cette étude sont décrites de façon détaillée dans les articles annexés à cette thèse. Par conséquent, seuls les points principaux de ces méthodes sont présentés dans les paragraphes suivants.

5.1 Les zones d'étude

5.1.1 Sud-est de l'Alaska

L'île de Prince-de-Galles (POW) est une île relativement grande située dans l'Archipel d'Alexandre dans le sud-est de l'Alaska, aux États-Unis. L'échantillonnage des sébastes aux yeux jaunes a eu lieu sur deux sites; un situé à l'est de l'île (55°52'N, 132°13'O) et, un à l'ouest, entre l'île de Warren et l'île de Coronation (55°52'N, 134°03'O). Cette zone de l'Alaska a été choisie pour l'étude parce qu'elle est à distance de toutes sources industrielles locales du Hg, conjointement au fait que les eaux côtières sont facilement accessibles à partir du village de pêcheurs de Thorne Bay. L'augmentation rapide des émissions de Hg en Asie (Fitzgerald & Lamborg 2007; Selin, 2009), qui sont transportés à travers le Pacifique, ont pu provoquer une augmentation des dépôts de Hg en Alaska et au Canada (Dan Engstrom, données non publiées). Une récente étude de modélisation a indiqué que 20 % du Hg déposé dans le nord de la Colombie-Britannique, du Yukon et de l'Alaska provient de sources anthropiques de l'Asie (Jaffe & Strode, 2008). Les concentrations de Hg dans les poissons du Pacifique nord pourraient augmenter avec l'augmentation de l'émission et le dépôt de Hg de l'Asie.

5.1.2 L'extrême-arctique canadien

Les Ombles chevaliers ont été échantillonnés à partir de quatre lacs (Small, 9-Mile, Nord, et Amituk) tous situés sur l'île Cornwallis, au Nunavut dans l'extrême-arctique canadien (75°08'N, 95°00'O). Ces lacs ont été inclu s dans le programme de surveillance en lien avec un projet d'Environnement Canada, qui est conçu pour fournir des informations sur les tendances temporelles de Hg et sur les polluants organiques persistants (POPs), sur une période de plusieurs années (Lac Amituk depuis 1989).

Basés sur des informations provenant du programme de surveillance, les lacs à l'étude ont été sélectionnés pour enjamber un gradient de contamination par le mercure et parce que les concentrations de POPs y sont faibles.

5.2 Analyses de métaux

5.2.1 Analyses de mercure

Tous les échantillons de tissus et de fractions sous-cellulaires ont été lyophilisés et ensuite analysés pour le Hg total avec un DMA-80 (Milestone Inc.). Celui-ci utilise une décomposition thermique suivie de l'amalgamation de l'or et de détection de la spectrométrie d'absorption atomique selon la méthode 7473 du USEPA (United States Environmental Protection Agency). Des informations spécifiques sur le total des analyses de Hg sont disponibles dans les trois articles suivant cette thèse.

La nouvelle méthode pour la détermination de Hg(II) dans des tissus de poissons est présentée dans l'article 1, à la fin de cette thèse. En bref, un sous-échantillon homogénéisé de tissus de poissons lyophilisés est digéré avec du HCl à l'aide de chauffage aux micro-ondes. Ensuite du toluène est ajouté afin de séparer le Hg organique du Hg(II). Suivant l'enlèvement de la couche de toluène, la fraction aqueuse acide restante est diluée et analysée pour le Hg total avec un DMA-80. Un autre sous-échantillon du même tissu de poissons est analysé pour le Hg total comme décrit dans la section précédente. La concentration de Hg(II) est ensuite soustraite du total de Hg pour obtenir la concentration de Hg dans l'échantillon biologique.

La précision de cette méthode a été validée en comparant les concentrations de MeHg calculées à des concentrations de MeHg certifiées dans un matériau de référence. Les concentrations de MeHg calculées ont été comparées aux concentrations de MeHg mesurées dans les mêmes tissus de poissons contaminés naturellement. Le MeHg mesuré a été déterminé par GC-CVAFS, selon des méthodes décrites précédemment (Bloom, 1989; Gill & Fitzgerald, 1987; Hammerschmidt & Fitzgerald, 2006; Tseng et al., 2004). D'autres détails sont également fournis dans l'article 1.

5.2.1 D’autres métaux

D'autres métaux ont été mesurés par la digestion des sous-échantillons lyophilisés de foie avec le HNO_3 concentré (Aristar) suivis par 30 % (w/w) de peroxyde d'hydrogène (Trace Select). L'eau ultra-pure a été ajoutée aux digestats afin d'atteindre une concentration de HNO_3 de 10 % avant l'analyse par ICP-MS (Thermo Elemental X Series) ou la spectroscopie d'émission atomique de plasma à couplage inductif (ICP-AES; AX Vista). D'autres détails sont présentés dans les articles 2 et 3.

5.3 Les indices de santé

L’indice hépatosomatique (IHS), l’indice gonadosomatique, et les indices de condition ont été calculés selon les équations suivantes.

Équation 1. L’indice hépatosomatique

$$IHS = \left(\frac{W_L}{W_T}\right) \times 100$$

W_L est le poids du foie et W_T est le poids total du poisson.

Équation 2. L’indice gonadosomatique

$$GSI = \left(\frac{W_g}{W_T}\right) \times 100$$

W_g est le poids de la gonade et W_T est le poids total du poisson.

Les indices de condition ont été calculés selon une des équations suivantes.

Équation 3. Facteur de condition de Fulton

$$facteur\ de\ condition\ de\ Fulton = \frac{1{,}000 \times W_T}{L^3}$$

W_T est le poids total du poisson et L^3 est la longueur totale du poisson au cube.

Équation 4. Facteur de condition relatif

$$facteur\ de\ condition\ relatif = \frac{W_T}{W'}$$

W_T est le poids total du poisson et W' est le poids calculé pour une longueur observée basée sur le rapport longueur-poids.

Le facteur de condition de Fulton suppose que la pente de la droite de régression entre la longueur et le poids est égale à trois et que la croissance est isométrique. Cela a été utilisé pour évaluer la condition de sébastes aux yeux jaunes parce que ces hypothèses ont été respectées. Le facteur de condition relatif était plus approprié pour les ombles chevaliers, car une variation significative était présente entre les pentes des quatre populations et que toutes les régressions n'ont pas eu des pentes égales à trois. Afin de déterminer les facteurs de condition, la pente utilisée pour les quatre populations était égale à 3.23.

5.4 **Histologie**

Les tissus du foie fixés des sébastes aux yeux jaunes et des ombles chevaliers ont été inclus dans la paraffine, sectionnés, et montés sur des lames de verre. Les sections de foie ont été colorées avec de l'hématoxyline et de l'éosine (H & E) et évaluées par la microscopie optique, ou archivées pour l'imagerie des métaux par LA-ICP-MS ou de la fluorescence à rayons X. Pour les sections de tissus de sébaste aux yeux jaunes colorés, chacun des trois champs de vision a subi un grossissement de 100X et a été photographié. La moyenne de pourcentage de couverture de MA, par rapport à la surface totale du champ de vision, a été calculée pour chaque photographie en utilisant le logiciel ImageJ (NIH http://rsbweb.nih.gov/ij/). Un pourcentage de couverture moyen de MA a ensuite été calculé sur la base des trois photographies.

Comme noté par Agius (1980), les MA chez les salmonidés ont tendance à être petits et mal définis. De ce fait, il est difficile de déterminer leur superficie de la même manière que démontré ci-dessus. Par conséquent, le nombre de MA dans chacun des trois champs de vision à grossissement de 100X a été utilisé pour déterminer une moyenne de MA hépatique pour chaque omble chevalier.

5.5 L’imagerie des métaux dans les tissus de foie

5.5.1 LA-ICP-MS

Les sections de tissu ont été utilisées pour étudier la distribution des métaux dans les sections du foie des sébastes par LA-ICP-MS, selon les méthodes de Barst et al. (2011) avec une légère modification. En bref, une lame de microscope comprenant un tissu non coloré inclus dans la paraffine, a été placée dans la chambre d'un 213 nm Nd:YAG source d'ablation laser. Des régions d'hépatocytes normaux et de MA ont été choisies au hasard et analysées avec un laser de 55 µm diamètre. Une ICP-MS coupée au laser a été utilisée pour surveiller le Hg, le Se, le Cd, le Ni, le Cu, et le Zn dans les sections de tissus. Les moyennes de comptes isotopiques de chaque élément précédant l’utilisation du laser ont été utilisées comme une estimation du bruit de fond et soustraites de la moyenne des comptes pour le reste du parcours du laser, afin de calculer un signal. Nous avons calculé le rapport des comptes du métal dans le MA par rapport aux comptes dans le tissu normal environnant (M_{MA}: M_{Hep}), pour Cu, Zn, Cd, Hg et Se. Après l'ablation, les lames ont été regardées à nouveau en microscopie optique afin de s'assurer que le MA a été ciblé avec précision.

5.5.2 L’analyse par fluorescence à rayons X

Des images de la fluorescence à rayons X d’un foie de sébaste aux yeux jaunes ont été prélevées à l’Advanced Photon Source, Argonne National Laboratory, selon les méthodes décrites précédemment (Prince *et al.*, 2014). Les intensités des lignes de la fluorescence de rayons X : Hg Lα, Se Kα, Cu Kα, et Zn Kα ont été collectées. Malheureusement, nous ne sommes pas en mesure de déterminer la concentration de Cd dans la section du foie avec la fluorescence des rayons X, comme l'énergie du Cd Lα chevauche avec celle des photons Ar Kα présents dans l'air.

5.6 La procédure de la répartition sub-cellulaire

Le protocole expérimental pour déterminer la répartition sub-cellulaire des métaux a été décrit précédemment (Campbell & Hare, 2009; Giguère *et al.*, 2006; Rosabal *et al.*,

2012; Rosabal *et al.*, 2015). Cette procédure a séparé les cellules du foie des ombles chevaliers en six fractions définies sur le plan opérationnel : (1) « Débris » (débris cellulaires plus noyaux), (2) « Granules », (3) Mitochondries (4) « Organelles » (lysosomes plus microsomes), (5) protéines thermosensibles (HDP) incluant les enzymes, (6) protéines thermostables (HSP) incluant les métallothionéines et GSH. Ces fractions ont été combinées en compartiments sensibles (mitochondries + HDP + organelles) et compartiments détoxiqués (HSP + granule) afin de déterminer le potentiel d'effets. Le protocole comprend des étapes de centrifugation différentielle, une digestion avec l'hydroxyde de sodium et un traitement thermique. Une description plus approfondie des méthodes est présentée dans l'article 3 à la fin de cette thèse.

6 RÉSULTATS ET DISCUSSION

6.1 La détermination de la spéciation du mercure dans les tissus de poissons par le DMA-80

Les concentrations de Hg(II) mesurées dans les fractions acides de DOLT-4 n'étaient pas significativement différentes de celles qui ont été estimées par la différence entre les valeurs moyennes certifiées de Hg total et de MeHg. La récupération du Hg(II) à partir de DOLT-4 représente en moyenne 99 ± 5 % de la différence entre les valeurs moyennes certifiées. Le Hg organique peut être estimé comme étant la différence entre les concentrations mesurées de Hg total et de Hg(II). Avec la méthode proposée, la moyenne de récupération estimée de Hg organique de DOLT-4, par différence, était de 100 ± 5 % de la valeur moyenne certifiée pour le MeHg.

Les estimations de MeHg, calculées par différence entre les concentrations mesurées de Hg total et de Hg(II) avec la méthode proposée, étaient en accord avec les mesures de spéciation directes de MeHg par la technique bien établie de GC-CVAFS (récupération moyenne = 99 % ± 6 %) pour tous les échantillons de foie et de muscles, à l'exception des foies de lépisostés tachetés.

Contrairement aux autres foies de poissons analysés, les foies des lépisostés tachetés avaient des concentrations beaucoup plus élevées de Hg total, dont seulement une petite fraction était présente comme Hg organique (moyenne = 4,1 %). Les concentrations de Hg organique dans le foie des lépisostés tachetés, estimées par la différence entre le Hg(II) et le Hg total mesuré, étaient en moyenne 183 % plus élevées que celles déterminées par GC-CVAFS. Ces différences dans l'estimation du Hg organique par rapport au MeHg mesuré sont probablement dues à l'incertitude accrue qui résulte de l'estimation d'une troisième valeur (Hg organique) par différence entre deux valeurs élevées (le Hg(II) et le Hg total).

En utilisant les différences de pourcentage entre les duplicatas comme une estimation d'erreur, il a été possible de modéliser le pourcentage de récupération du MeHg par rapport au pourcentage de Hg(II) pour un groupe d'échantillons hypothétiques et d'ainsi déterminer le moment où la méthode proposée ne fournit plus d'estimation précise de

MeHg. Ces échantillons hypothétiques ont été attribués à des concentrations de Hg totales différentes (0,5, 5 ou 50 pg g^{-1}) et à des pourcentages différents de Hg(II) (concentrations comprises entre 0 et 100 %) avant que leurs valeurs n'aient été ajustées en utilisant les différences de pourcentage calculées. Le MeHg, comme les valeurs de MeHg corrigées, a été calculé (par différence entre le total de Hg et du Hg(II)) et son pourcentage de récupération a été déterminé. Basée sur les données hypothétiques et les données de cette étude, une estimation imprécise du MeHg se manifeste lorsque la proportion de Hg(II) dépasse 90 %, mais cette estimation semble indépendante de la concentration totale de mercure.

Ainsi, la méthode proposée est une excellente approche pour quantifier directement le Hg(II) dans le foie et les muscles de poissons et pour estimer le Hg organique dans des échantillons où il y a moins de 90 % de Hg(II). Les estimations de Hg organique ou de MeHg dans les échantillons avec des fractions relativement élevées de Hg(II) et des concentrations élevées de mercure total doivent être interprétées avec prudence et une mesure directe plus traditionnelle de MeHg semble être plus appropriée.

6.2 La spéciation du mercure hépatique dans les poissons osseux

La méthode décrite précédemment a été utilisée pour calculer les concentrations de MeHg dans des échantillons de foie de neuf espèces de poissons osseux. En raison du manque de confiance dans les concentrations de MeHg calculées pour les poissons avec des fractions relativement élevées (≥90 %) de Hg(II) dans le foie, le MeHg a également été mesuré dans les foies de lépisosté tacheté et de poissons castors par GC-CVAFS.

Dans les trois espèces de salmonidés, la majorité du Hg total hépatique était présent sous forme de MeHg. Par ailleurs, la barbue de rivière ainsi que des espèces plus primitives, comme le lépisosté tacheté et le poisson castor, avaient des proportions de Hg(II) élevées dans le foie. Dans les autres espèces de poissons, le MeHg représentait environ la moitié du Hg total hépatique. Ces tendances en matière de spéciation sont similaires sur une gamme de concentrations de Hg total hépatique.

Les différences dans la spéciation hépatique du Hg pourraient être dues à des différences de régimes alimentaires entre les espèces. Par exemple, le grand brochet de l'Isle Royale (Michigan,États-Unis) se nourrit d'odonates larvaires qui sont riches en Hg(II) (Drevnick *et al.*, 2008). Les proportions élevées de Hg(II) pourraient également être dues à des différences dans la façon dont le Hg est accumulé dans le foie des différentes espèces de poissons. Des études antérieures ont montré que les lépisostés tachetés ont un grand nombre de MA dans leurs foies qui accumulent le Hg (Barst et al., 2011; Smith, 2012). Cependant, on ignore si le Hg est présent sous forme de MeHg ou de Hg(II). Les proportions élevées de Hg(II) dans les foies de certaines espèces de poissons pourraient aussi être dues à une déméthylation hépatique de MeHg.

6.3 **Pathologies et métaux dans le foie des sébastes aux yeux jaunes et des ombles chevaliers**

Les concentrations de MeHg, Hg(II), Se, Cd et Cu étaient corrélées positivement avec la superficie des MA dans les foies des sébastes aux yeux jaunes. La superficie des MA augmentait également avec l'âge du poisson. Par conséquent, les concentrations de MeHg, Hg(II), Se, Cd et Cu ont été intégrés, avec l'âge des poissons, dans un modèle de régression multiple par étapes afin d'identifier le meilleur facteur déterminant la superficie des MA. Les résultats montrent que le Hg(II) était le meilleur facteur déterminant la superficie des MA dans les foies des sébastes aux yeux jaunes, ce qui suggère que le Hg(II) a été le principal contributeur à l'augmentation de la superficie de ces pathologies.

Les résultats statistiques ont été soutenus par les deux techniques d'imagerie de métaux, qui ont démontré que non seulement le Hg s'accumule dans les MA des sébastes aux yeux jaunes, mais que cette accumulation se fait à un degré plus élevé que pour la plupart des autres métaux. La seule exception est pour le Se, qui s'est accumulé dans les MA à environ le même ordre de grandeur que le Hg par rapport aux hépatocytes adjacents. Ni la technique LA-ICP-MS, ni la fluorescence de rayons X ne fournissent des informations sur la forme chimique exacte de Hg ou de Se dans les MA. Cependant, la majorité du Hg total dans les foies des sébastes aux yeux jaunes était

présente sous forme de Hg(II), le meilleur facteur déterminant de la superficie des MA. En outre, des corrélations significatives ont été obtenues entre les concentrations de Se et le Hg dans les foies ainsi qu'entre le rapport <Se dans les MA / Se dans les hépatocytes> et les comptes de Hg dans les MA. Il est donc possible que le Hg(II) soit présent dans les MA sous la forme HgSe. Dans les sébastes aux yeux jaunes, les MA hépatiques semblent être d'excellents biomarqueurs de l'exposition au Hg.

Contrairement aux MA dans les foies des sébastes aux yeux jaunes, les MA hépatiques de l'omble chevalier étaient plus petites et leurs contours étaient moins bien définis. Cette observation est cohérente avec les rapports précédents sur les MA des salmonidés (Agius, 1980). Le nombre de MA hépatiques était positivement corrélé avec l'âge de l'omble chevalier. Lors de cette étude, des corrélations positives ont également été observées entre le nombre de MA et les concentrations de MeHg et de Hg(II) dans les foies d'omble chevalier provenant des lacs Small, 9-Mile et North. Cependant, dans les foies d'omble chevalier du Lac Amituk, il y avait très peu de MA. En combinant les données pour l'omble chevalier de tous les lacs ensemble, il n'y avait pas de relation entre les concentrations de Hg et les MA. Malheureusement, les MA d'omble chevalier étaient trop petits et les concentrations de Hg étaient trop faibles (dans les ombles chevaliers des lacs Small et 9-Mile) pour déterminer par LA-ICP-MS si le Hg et d'autres métaux ont été accumulés dans les MA. Néanmoins, les résultats statistiques ne suggèrent pas d'augmentation de MA en nombre dans les foies d'omble chevalier à la suite de l'exposition au Hg.

En plus des MA, les foies des deux espèces de poissons ont été évalués pour la pathologie générale. Les foies des sébastes aux yeux jaunes semblaient avoir une morphologie normale sur toute la gamme de concentrations de Hg totales étudiée. Ce ne fut pas le cas pour les ombles chevaliers, dont la pathologie générale des foies variait d'une apparence saine à une morphologie anormale. La pathologie la plus apparente dans les foies anormaux était la fibrose dans la région périsinusoïdale. Un nombre significativement plus élevé de poissons provenant du lac Amituk présentait cette anomalie par rapport à ceux des trois autres lacs étudiés. La présence de lésions du foie chez les ombles chevaliers du lac Amituk et non chez les sébastes aux yeux jaunes peut être due à des différences dans la spéciation de Hg hépatique; la plupart du

Hg dans le foie des sébastes aux yeux jaunes était présent comme Hg(II), alors que dans les foies des ombles chevaliers le MeHg prédominait. Les concentrations totales de mercure ont également tendance à être plus élevées dans les foies des ombles chevaliers du Lac Amituk en comparaison avec celles des sébastes aux yeux jaunes. Il est également possible que chez les sébastes aux yeux jaunes, le foie soit mieux protégé du Hg dû à la présence de MA, qui pourraient séquestrer le Hg et ainsi empêcher la poursuite de l'interaction du Hg avec d'autres cellules du foie.

6.4 La répartition sub-cellulaire de mercure dans les foies d'ombles chevaliers

Malgré les concentrations de mercure significativement plus élevées dans les foies des poissons du Lac Amituk par rapport à celles du Lac Small, le Hg a été distribué de façon similaire dans les cellules hépatiques des ombles chevalier des deux lacs; les compartiments sensibles contenaient 73 % du Hg total pour le Lac Small et 61 % du Hg total pour le Lac Amituk. Ceci suggère que le mercure n'ait pas été bien détoxiqué dans le foie des ombles chevaliers dans ces deux lacs. Le Hg restant a été trouvé dans le compartiment détoxiqué, respectivement de 10 % et de 19 % pour le Lac Small et le Lac Amituk. Presque tout le Hg dans le compartiment détoxiqué a été associé à la fraction HSP, contenant vraisemblablement de la métallothionéine, et très peu a été trouvé dans la fraction de granules. Dans le compartiment potentiellement sensible, le Hg s'est accumulé principalement dans les mitochondries et la fraction HDP.

Une faible proportion de Hg a été associée à la fraction contenant des HSP, et probablement plus précisément à la métallothionéine. Araújo et collègues (2015) ont également trouvé un faible pourcentage de Hg dans la fraction HSP des foies de rouget sauvages, qu'ils attribuaient à des concentrations de mercure qui ne dépassaient pas le seuil physiologique pour l'induction de métallothionéines. Étant donné les concentrations de Hg extrêmes dans les foies des ombles chevaliers du Lac Amituk, il semble peu probable qu'une faible proportion de Hg dans la fraction HSP soit le résultat de la métallothionéine non induite. Une explication possible pour un faible pourcentage de Hg dans cette fraction peut être liée à la spéciation du Hg. La spéciation du Hg n'a

pas été déterminée dans des fractions sub-cellulaires des foies individuels et pour l'instant les processus de distribution du MeHg et du Hg(II) à l'échelle sub-cellulaire ne sont pas bien compris. Toutefois, le MeHg était la forme prédominante de Hg total dans les homogénats de foies des ombles chevaliers. Selon Kidd et Batchelar (2011), le Hg(II) peut induire la production de métallothionéine et s'y lier. Inversement, le MeHg n'est pas connu pour induire la synthèse de la métallothionéine (Kidd & Batchelar, 2011) et probablement se lie moins fortement aux résidus de cystéine (Wang *et al.*, 2012). Toutefois, l'hypothèse selon laquelle une plus grande proportion de Hg(II) dans le foie conduirait à une plus grande proportion de Hg dans la fraction HSP n'a pas été testée chez les poissons. Fait intéressant, les invertébrés aquatiques exposés au Hg(II) ont également accumulé très peu de Hg dans la fraction HSP (Xie *et al.*, 2008). Par conséquent, il est possible qu'aucune forme de Hg ne tende à s'associer avec la fraction HSP des organismes aquatiques.

L'accumulation de Hg dans les mitochondries et les fractions HDP peut avoir des conséquences négatives pour la respiration cellulaire et les enzymes du système de défense contre le stress oxydatif. Cette affirmation est appuyée par des études en laboratoire, qui ont établi que l'exposition au MeHg alimentaire conduit à des anomalies structurelles des mitochondries et à l'inhibition de la respiration (Cambier *et al.*, 2009), ainsi qu'à une diminution de l'activité de la GPx (Berntssen *et al.*, 2003).

6.5 La comparaison entre les indices de santé et les concentrations de mercure dans les sébastes aux yeux jaunes et les ombles chevaliers

Le facteur de condition et l'IHS étaient significativement plus élevés chez les sébastes aux yeux jaunes de l'ouest que chez les poissons de la région de l'est. Les sébastes aux yeux jaunes de l'ouest ont également eu des taux de croissance plus rapides que les poissons échantillonnés dans l'est. Pris ensemble, le taux élevé de croissance et d'IHS et le bon état physique des sébastes aux yeux jaunes originaires de l'ouest sont probablement le résultat de températures plus élevées ou d'une meilleure disponibilité de la nourriture, les deux pouvant influencer le taux de croissance. Les sébastes aux

yeux jaunes de l'est ont probablement eu des concentrations plus élevées de Hg parce qu'ils ont des taux de croissance plus lents. Pour les sébastes aux yeux jaunes, ni le facteur de condition ni l'IHS n'étaient significativement corrélés avec les concentrations de Hg.

L'IHS était positivement corrélé avec le Hg dans les foies d'ombles chevaliers mâles et femelles. L'IHS peut augmenter après l'exposition aux métaux due à une hyperplasie (Adams *et al.*, 1990), qui est une augmentation du nombre de cellules du foie. L'IHS a également été corrélé positivement avec la position trophique, qui varie avec le Hg. Par conséquent, l'augmentation de l'IHS dans les ombles chevaliers mâles et femelles peut être interprétée comme un effet positif de l'alimentation à une position trophique supérieure, les ombles chevaliers ayant de plus grandes réserves de graisse à la suite de la consommation d'autres ombles chevaliers. Cela a été pris en charge par la corrélation positive entre l'IHS et les valeurs de C:N. Le facteur de condition de l'omble chevalier n'était pas corrélé à la position trophique, aux valeurs de C:N, ou aux concentrations de Hg.

Le GSI des ombles chevaliers, mâles ou femelles, variait en fonction du développement des gonades et était proportionnel à la taille du corps, mais ne différait pas significativement entre les lacs. Pour les femelles matures, le GSI a augmenté avec les concentrations totales de Hg. En ce qui concerne la taille du corps, tant que les ombles chevaliers grossissent, ils ont proportionnellement de plus grandes gonades, mais aussi plus de Hg. Si l'«effet» de la taille du corps sur le GSI est isolé (par analyse de covariance), les résultats indiquent que le Hg n'est pas lié au GSI. Les quatre lacs étudiés sont peu productifs, ce qui signifie que les ombles chevaliers doivent restreindre leurs budgets énergétiques. Puisque l'énergie est affectée d'abord au maintien des fonctions biologiques de base, puis pour la croissance et la reproduction, les ombles chevalier ne frayent pas chaque année. Même après l'échantillonnage pour les deux étés consécutifs, seulement quelques ombles chevaliers étaient sexuellement matures. Il est donc difficile de tirer des conclusions solides sur les effets possibles du Hg sur la reproduction chez les ombles chevaliers. Après correction pour la taille du corps, en calculant la fécondité relative (nombre d'œufs / 100 g de poids corporel), une régression quantile a été utilisée afin de déterminer si le Hg peut limiter le rendement de

reproduction des ombles chevaliers. Les résultats de cette analyse ne sont cependant pas significatifs, ce qui peut être expliqué par de la taille limitée de l'échantillon.

7 CONCLUSION

La méthode pour déterminer la spéciation du Hg dans les tissus des poissons avec un analyseur de Hg direct fournit des concentrations de MeHg précises, pour les échantillons de moins de 90 % de Hg(II), couvrant une gamme de concentration de Hg totale. Le Hg(II) peut être mesuré avec précision dans tous les échantillons, indépendamment du pourcentage de Hg(II). En plus de fournir des données précises, cette méthode est également rapide et peu coûteuse. Cette approche devrait faciliter l'incorporation de la spéciation du Hg dans les futures études sur les poissons sauvages. La spéciation est importante à considérer dans le contexte de l'étude de la toxicité du Hg chez les poissons sauvages parce qu'il semble que toutes les espèces de poissons n'ont pas les mêmes proportions de MeHg dans leurs foies. Cette observation pourrait entraîner des différences dans la sensibilité des espèces contaminées par le Hg. La majorité du Hg total était sous forme de Hg(II) dans les foies des sébastes aux yeux jaunes. Au niveau des tissus, le Hg s'accumule dans les MA des sébastes aux yeux jaunes, possiblement comme HgSe. Ce complexe peut servir à éliminer le Hg de la circulation et réduire l'exposition au Hg des autres cellules, tissus et organes. Inversement, les ombles chevaliers avaient des proportions élevées de MeHg dans leurs foies, et leurs MA ne semblaient pas répondre au Hg de la même manière. Dans les ombles chevaliers du Lac Small et du Lac Amituk, le Hg a été trouvé principalement dans les fractions potentiellement sensibles, ce qui suggère que le Hg n'était pas bien détoxiqué dans leurs foies. Ceci peut expliquer les dommages observés dans les foies des ombles chevaliers du Lac Amituk. Cependant, l'alimentation au niveau trophique supérieur conduit à une plus grande exposition au Hg et peut aussi conduire à des charges de parasites élevées; il s'ensuit que l'on ne peut conclure que le mercure est la seule cause de la fibrose hépatique chez l'omble chevalier. Enfin, selon les indices de santé communs utilisés dans cette étude, le mercure ne semble pas affecter négativement la santé générale des sébastes aux yeux jaunes ni de l'omble chevalier. Des travaux supplémentaires sont nécessaires pour comprendre comment la reproduction des ombles chevaliers peut être affectée par la contamination de Hg.

PREMIÈRE PARTIE: SYNTHÈSE

8 INTRODUCTION

8.1 Mercury cycling in the aquatic environment

Mercury (Hg) is a non-transition metal which belongs to group II B of the periodic table of elements. Found naturally in the Earth's crust, Hg has interesting chemical properties and a unique biogeochemical cycle which involves release from geological stores, atmospheric transport, deposition, and revolatilization. Anthropogenic activities including mining, waste incineration, and the combustion of fossil-fuels have resulted in the increased mobilization of Hg in the environment. Recent data suggest that the artisanal gold mining and the combustion of coal account for approximately two-thirds of anthropogenic Hg emissions, the majority of which are released from Asia (~50% of all anthropogenic emissions (UNEP, 2013). Mercury, emitted from coal combustion, enters the atmosphere as both elemental (Hg(0)) and inorganic (Hg(II)) forms; Hg(0) can remain and travel in the atmosphere for 0.5 - 2 years before it is oxidized to Hg(II) and deposited to aquatic and terrestrial ecosystems (Pacyna *et al.*, 2006; Selin, 2009). For this reason, anthropogenic mobilization of Hg is a global problem as even remote regions are subject to contamination (Fitzgerald *et al.*, 1998; Fitzgerald & Lamborg, 2007; Selin, 2009). Evidence for this has been found in lake sediment cores, which show a 3-fold increase in Hg deposition since the preindustrial period, in remote regions of the Canadian High Arctic, Alaska, and Greenland (Muir *et al.*, 2009; Yang *et al.*, 2010). In aquatic environments, deposited Hg(II) is methylated to form methylmercury (MeHg), which readily bioaccumulates and biomagnifies in food webs, leading to elevated Hg concentrations in fish (Fitzgerald *et al.*, 2007; Selin, 2009) (Figure 1). Larger and older fish, feeding at higher trophic positions, often have the highest Hg concentrations (Sandheinrich & Wiener, 2011). The consumption of contaminated fish is the primary route of exposure for humans and this has been shown to negatively impact health (Harada, 1995; Mergler *et al.*, 2007). Mercury may also be toxic to the fish themselves (Scheuhammer *et al.*, 2007).

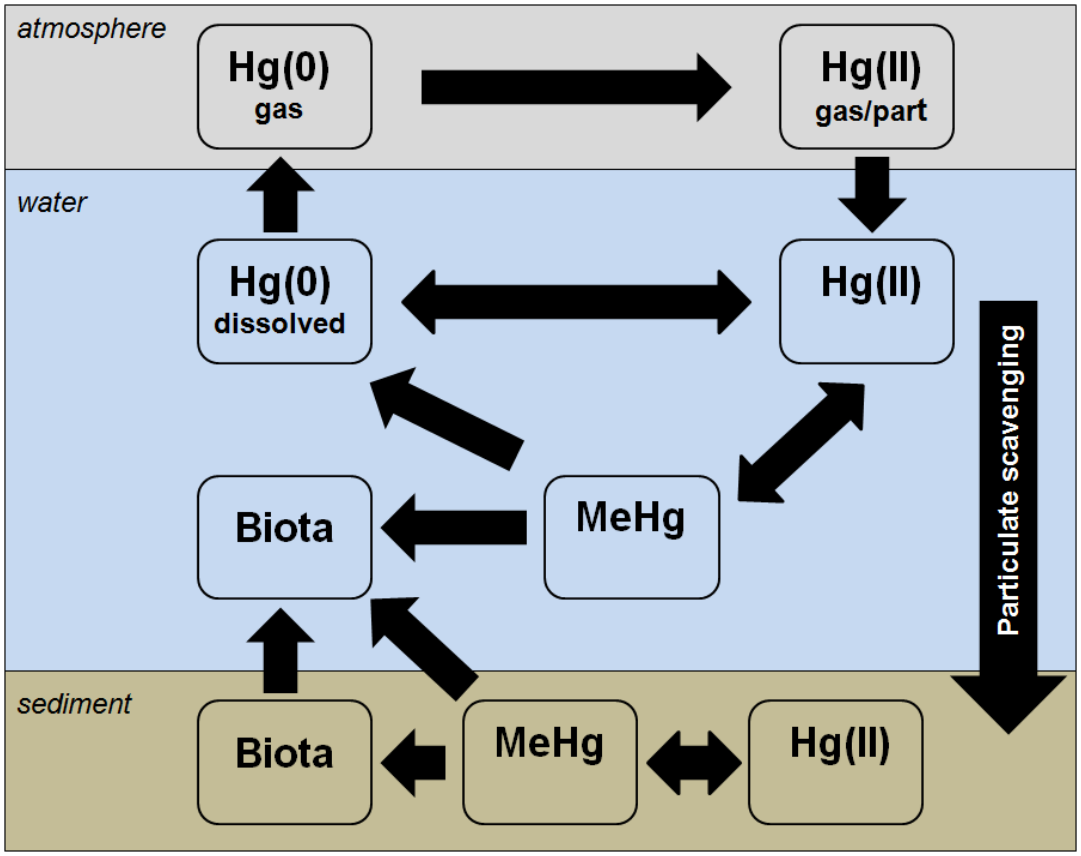

Figure 1. A simplified biogeochemical cycle of Hg. Adapted from (Fitzgerald *et al.*, 2007).

8.2 Toxic effects of Hg in fish

Laboratory and field studies have provided compelling evidence that environmentally-relevant concentrations of MeHg in the tissues of fish, as well as in their diets, negatively impact their health. Recent analyses of the available data for Hg toxicity in fish indicate that effects are likely to occur at whole-body concentrations (wet wt) exceeding 0.2 µg g^{-1} (Beckvar *et al.*, 2005), or 0.3 µg g^{-1} (Dillon *et al.*, 2010; Sandheinrich & Wiener, 2011) (equivalent concentrations in edible muscle are 0.33 and 0.5 µg g^{-1}, respectively), or for dietary concentrations above 0.2 µg g^{-1} (Depew *et al.*, 2012). The toxicity of MeHg may be due to its ability to cause oxidative stress (Scheuhammer *et al.*, 2012). For example, Berntssen *et al.* (2003) reported decreased activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the brains of juvenile Atlantic salmon (*Salmo salar*) fed diets enriched with MeHg. The authors also

noted increased lipid peroxidative products (measured as thiobarbituric acid reactive substances or TBARS), pathological brain damage (necrosis and vacoulation), and reduced post-feeding behavior in fish fed MeHg diets. The decreased activities of SOD and GPx, as well as the increase in lipid peroxidation suggest a collapse of the redox defense system (Berntssen *et al.*, 2003).

Changes in histology associated with Hg exposure have also been described in the livers of fish. For example, variation in liver color was positively correlated with total Hg concentration in the muscle tissue of northern pike (*Esox lucius*). The pigment responsible for the color variation was identified as lipofuscin, which is composed of oxidized lipids and proteins and forms during oxidative stress (Drevnick *et al.*, 2008; Terman & Brunk, 2004). Lipofuscin and other pigments are often found in melano-macrophage aggregates (MA), which are specialized phagocytic cells of the innate immune system of fish. These aggregations serve as sites of detoxification, destruction, and recycling of both exogenous and endogenous cellular materials, including damaged and weakened cells. Though MA may increase in size and number in response to a host of processes (including starvation, aging, pathogenic infection, and exposure to other contaminants) (Agius & Roberts, 2003), several studies have reported greater numbers of MA with increased Hg exposure. In a laboratory study, increased numbers of MA and necrosis were reported in the livers of *Hoplias malabaricus* that were fed a diet containing 0.075 µg $MeHg \cdot g^{-1}$ wet wt. for 70 days (Mela *et al.*, 2007). Raldúa *et al.* (2007) found elevated levels of lipofuscin, pycnotic nuclei, and MA in the livers of wild fish downstream from a chlor-alkali plant (Raldúa *et al.*, 2007). Similar, correlations between Hg concentrations and MA have been documented in the kidneys and spleens of several salmonid species (Schwindt *et al.*, 2008), and in the kidneys, livers, and spleens of northern pike (Meinelt *et al.*, 1997). The relationship between MA and Hg was further explored by Barst and colleagues (2011), who measured Hg in hepatic MA and adjacent hepatocytes using laser ablation inductively coupled mass spectrometry (LA-ICP-MS) and determined that MA had higher relative concentrations of Hg than the surrounding tissues (Barst *et al.*, 2011). These results, along with those from two recent studies with yellow perch (*Perca flavescens*), demonstrate that Hg accumulates in these immune cells presumably due to the phagocytosis of cells damaged by Hg (Barst *et al.*,

2011; Batchelar *et al.*, 2013; Müller *et al.*, 2015). Since MA have been shown to increase in size and number with age and exposure to other metals, these variables should be included when determining how MA and Hg are related in wild fish.

In addition to effects in the liver, recent literature reviews point to reproduction as the biological process most susceptible to Hg toxicity in wild fish (Crump & Trudeau, 2009; Scheuhammer *et al.*, 2007). Feeding walleye (*Sander vitreus*) diets enriched in MeHg (up to 0.987 µg g^{-1}) resulted in reduced fish length and weight, as well as testicular atrophy in male fish (Friedmann *et al.*, 1996). In laboratory studies with fathead minnows (*Pimephales promelas*), environmentally-relevant concentrations of dietary MeHg induced apoptosis in gonad cells that synthesize hormones necessary for reproduction (Drevnick *et al.*, 2006). In fatheads, apoptosis of ovarian follicular cells resulted in suppressed estrogen levels, diminished production of egg yolk protein, and less fecundity (Drevnick & Sandheinrich, 2003; Klaper *et al.*, 2006). In male fatheads, suppressed testosterone levels resulted in altered reproductive behavior (Sandheinrich & Miller, 2006). The combined effects of Hg exposure on females and males resulted in reproductive failure (Hammerschmidt et al. 2002). Suppressed levels of hormones have also been documented in wild fish with elevated concentrations of Hg, though results may have been confounded by other environmental contaminants (Webb *et al.*, 2006). As of yet, no study has conclusively linked Hg exposure and negative effects on reproduction in wild fish.

Changes in the body condition, gonadosomatic index, or hepatosomatic index of fish exposed to Hg have also been documented. These metrics are simple to perform and are widely reported in the literature. The condition factor relates the length of a fish to its weight; the hepatosomatic and gonadosomatic indices give the relative weights of the liver and gonads, respectively, as compared to the total fish weight (Di Giulio & Hinton, 2008). Drevnick et al. (2008) noted reduced lipid reserves in the livers of pike and decreased body condition of fish with elevated concentrations of Hg. Similar results were reported for emaciated pike from Clay Lake, Canada (Lockhart *et al.*, 1972). The hepatosomatic index was negatively correlated with concentrations of Hg in wild walleye and perch (Larose *et al.*, 2008). Additionally, dietary exposure to MeHg decreased the gonadosomatic index of walleye in a laboratory study (Friedmann *et al.*, 1996).

8.3 **Hg uptake and distribution in fish**

Fish are exposed to Hg(II) and MeHg from water and diet, though dietary uptake is considered to be more significant (Hall *et al.*, 1997). The majority of Hg(II) that enters fish via the diet is excreted as it does not efficiently cross the intestinal tract (Berntssen *et al.*, 2004). Unlike Hg(II), the majority of dietary MeHg (up to ~ 90%) crosses the intestinal epithelium and is transferred to blood within vessels associated with the gut (Leaner & Mason, 2002; Wang & Wong, 2003). This process may occur by both passive and facilitated uptake mechanisms (Leaner & Mason, 2002). Once in the blood, Hg(II) binds with cysteine, albumin, and glutathione (GSH) in plasma, whereas MeHg tends to associate with hemoglobin in erythrocytes. Blood, containing both forms of Hg, moves from the intestinal tract to the liver via the portal vein. Mercury species are then distributed throughout sinusoids, the microvasculature of the liver, thus coming into close contact with hepatocytes. Uptake of MeHg (complexed with cysteine), into hepatocytes and other cells, depends on L-type large neutral amino acid transporters (Simmons-Willis *et al.*, 2002). Inorganic Hg may diffuse through the cell membrane as $HgCl_2$ (Gutknecht, 1981), or is carried across in Na^+-independent transporters (Bridges *et al.*, 2004). The MeHg and Hg(II) remaining in blood continue to travel through the circulatory system to other parts of the body. Long-term storage of MeHg occurs in muscle tissue, bound to cysteine (Harris *et al.*, 2003; Lemes & Wang, 2009). Muscle represents the largest pool of MeHg in the body where it makes up approximately 90% of the total Hg (Bloom, 1992). MeHg and Hg(II) also accumulate in spleen, kidney, and brain to varying degrees (Berntssen *et al.*, 2003; Kidd & Batchelar, 2011). Due to its strategic positioning within the circulatory system, the liver is exposed to dietary MeHg and Hg(II) before other tissues. The liver's ability to detoxify Hg therefore dictates the level of exposure and toxic effects in other fish tissues.

8.4 **Hg detoxification in fish liver**

The study of the hepatic detoxification and toxicity of Hg requires an understanding of how Hg species react in biological systems. Both MeHg and Hg(II) have strong binding affinities for selenium (Se) and sulfur, which within organisms are mostly present as

selenols and thiols. Selenocysteine, the twenty-first amino acid, is the predominate selenol-containing biomolecule found in selenoproteins. Thiols are found in the amino acid cysteine, cysteine residues of proteins, and the tri-peptide GSH (Wang *et al.*, 2012). Although the binding of MeHg and Hg(II) with the selenols and thiols of certain hepatic biomolecules may result in deleterious effects, binding with others may help to avoid toxicity. The latter occurs either by facilitating the excretion, transformation, or sequestration of MeHg and Hg(II), thus preventing their interaction with potentially physiologically sensitive sites.

As a primary detoxification mechanism, hepatocytes produce GSH which is conjugated to MeHg and excreted in bile (Dutczak & Ballatori, 1994). Excretion is inefficient and MeHg is largely reabsorbed in the intestine (Dutczak *et al.*, 1991; Roberts *et al.*, 2002). Inorganic Hg also binds with GSH and is excreted in bile, however reabsorption in the intestine is less efficient and much of the Hg(II) is removed in feces (Zalups *et al.*, 1999). Further detoxification of Hg, is achieved by sequestration of Hg(II) with metallothioneins, which are low molecular weight proteins with high cysteine contents (Sigel *et al.*, 2009). Metallothionein synthesis may increase as a result of exposure to Hg(II) (Monteiro *et al.*, 2010) and other metals (Sigel *et al.*, 2009). In contrast, the interaction between MeHg and metallothionein is not well understood. Leiva-Presa and colleagues (2004) found evidence for an MeHg-metallothionein complex based on *in vitro* experiments (Leiva-Presa *et al.*, 2004). However, metallothionein is not thought to bind with or be induced by MeHg in fish (Kidd & Batchelar, 2011; Wiener & Spry, 1996).

Selenium may also be involved in the detoxification of Hg in fish livers. One of the earliest studies involving Hg and Se antagonism showed that a dose of sodium selenite, administered after a dose of Hg(II), could protect against nephrotoxicity in rats (Pařízek & Ošťádalová, 1967). Since this discovery, there have been numerous studies which demonstrate that supplementation of Se can prevent the toxic effects of Hg in a variety of species (Cuvin-Aralar & Furness, 1991; El-Begearmi *et al.*, 1977). The protective effect of supplemental Se was presumed to be a result of the sequestration of Hg by Se, which then averts toxic effects. However, recent analyses suggest an alternative way of interpreting the Hg/Se interaction. As Se is required for production of selenocysteines which are incorporated into the active sites of selenoenzymes, a reduction of the

available Se due to complexation with Hg, may diminish selenoenzyme function and even synthesis (Ralston & Raymond, 2010). As MeHg is readily reabsorbed in the intestine and recycled to the liver, continued exposure may overwhelm cellular defenses leading to an interaction between MeHg and sensitive target molecules. A transformation of MeHg to Hg(II), the more easily excreted form, would therefore be beneficial. This conversion, known as demethylation, has been reported in bacteria, marine mammals, and birds (Barkay *et al.*, 2003; Eagles-Smith *et al.*, 2009; Palmisano *et al.*, 1995), but a clear mechanism has not yet been described in fish. In bacteria, the demethylation of MeHg occurs enzymatically, but this process has not been found in vertebrates. Several studies have implicated Se in the demethylation of MeHg in biological systems. A proposed mechanism for the demethylation of MeHg involves a bis(methylmercuric selenide) intermediate and a mercuric selenide (HgSe) end product (Khan & Wang, 2010). This end product has high stability and low solubility, and is therefore not thought to be biologically active. Evidence of the presence of HgSe has been found in the organs of several mammals and birds (Arai *et al.*, 2004; Palmisano *et al.*, 1995), and most recently in the brains of humans (Korbas *et al.*, 2010). Although Hg(II) will also form HgSe complexes directly (Gailer *et al.*, 2000; Wang *et al.*, 2012), the previously mentioned demethylation reaction is more likely the cause of elevated proportions of Hg(II) given that marine mammals and humans are primarily exposed to MeHg through their diet. As demethylation of MeHg has been reported in several taxa, it is also likely to occur in fish and may result in a similar HgSe end product. Selenium may therefore be involved in the conversion of MeHg to Hg(II), and also sequester it in a non-bioavailable form.

8.5 Sub-cellular partitioning of Hg in fish

For metals in general, resistance or tolerance may correspond with an organism's ability to prevent the binding of "inappropriate" metals to potentially metal-sensitive sub-cellular sites (e.g. heat-denaturable proteins, mitochondria, organelles) by sequestering metals in "detoxified" compartments (e.g. metal-binding proteins, lysosomes, and metal-rich granules) (Mason & Jenkins, 1995). Recently in the field of metal toxicology, partitioning

procedures have been employed to understand how metals are distributed between these potentially sensitive and detoxified compartments (Giguère *et al.*, 2006; Rosabal *et al.*, 2012; Rosabal *et al.*, 2015; Wallace *et al.*, 2003). Sub-cellular partitioning procedures provide insight into how organisms cope with metals and whether toxicological effects are likely to occur. Partitioning procedures often rely upon differential centrifugation, to separate sub-cellular contents after a tissue has been homogenized. Differential centrifugations do not result in perfectly separated fractions, as some overlap is likely. Therefore, the fractions are operationally defined and the interpretation of results should be carried out with care. Ideally, mass balance calculations should be used to determine if metals were lost or contamination occurred during the procedure (Campbell & Hare, 2009).

Few studies have been carried out to determine the sub-cellular distribution of Hg in fish (Araújo *et al.*, 2015; Onsanit & Wang, 2011). Onsanit et al. (2011) determined the sub-cellular distribution of both total Hg and MeHg in sub-cellular fractions isolated from muscle tissue of red seabream (*Pagrus major*), black seabream (*Acanthopagrus schlegelii)*, and red drum (*Sciaenops ocellatus*). The authors found that the majority of total Hg and MeHg accumulated in fractions containing cellular debris, followed by metallothionein-like proteins, metal-rich granules, heat-denatured proteins, and organelles. As the majority of Hg was not found in metal-sensitive fractions, the authors suggested that Hg had little toxicity to fish muscle. Most recently, Araújo *et al.* (2015) measured total Hg in sub-cellular fractions of the livers of wild mullets (*Liza aurata*), and found low contributions of Hg in the heat-stable protein and granule fractions, which the authors attributed to Hg concentrations below the physiological threshold to activate detoxification mechanisms (Araújo *et al.*, 2015). Though the latter was one of the first studies to report sub-cellular partitioning of Hg in wild fish, a mass balance calculation was not reported, and therefore any losses or additions of Hg during the separation procedure could not be determined. Given that research in this area is limited, future studies which investigate how Hg is distributed between potentially sensitive and detoxified sub-cellular compartments in wild fish are justified.

8.6 **Hg speciation in fish tissues**

Methylmercury is the predominate form of Hg in fish muscle tissue (Bloom, 1992), whereas a substantial proportion of Hg may be present as Hg(II) in the livers of fish (Barst *et al.*, 2011; Drevnick *et al.*, 2008). Elevated proportions of hepatic Hg(II) may be a result of a significant dietary source of Hg(II), the preferential binding and sequestration of Hg(II) in liver, and/or a hepatic demethylation mechanism. Drevnick *et al.* (2008) reported that a major fraction of the Hg in northern pike livers was present as Hg(II). The authors speculated that the proportion of hepatic Hg(II) was not due to demethylation, but rather to due to the elevated concentrations of Hg(II) in the tissues of larval odonates, a common prey item of northern pike in that ecosystem (Drevnick *et al.*, 2008). However, differences in diet could not explain the observed differences in hepatic Hg speciation of largemouth bass (*Micropterus salmoides*) and spotted gar (*Lepisosteus oculatus*) from Caddo Lake, USA. In this lake, gar and bass feed at similar vertical trophic positions (as inferred from $\delta^{15}N$) yet have very different hepatic Hg speciation. Chumchal and colleagues reported that only 2% of the total Hg in spotted gar livers was present as MeHg, whereas in the livers of largemouth bass 74% of the total Hg was MeHg, suggesting these fish metabolize Hg differently (Barst *et al.*, 2011; Chumchal & Hambright, 2009; Chumchal *et al.*, 2011). Species-specific demethylation has been reported for waterbirds (Eagles-Smith *et al.*, 2009) and may also be species-dependent in fish. Recent research suggests that differences in hepatic Hg speciation in fish may be linked to phylogenetic differences in macrophage aggregates (Smith, 2012).

Macrophage aggregates are thought to represent primitive versions of the germinal centers of lymph nodes found in birds and mammals and are prevalent in hematopoietic tissues (Agius & Roberts, 2003). Hematopoiesis, or blood synthesis, takes place in the bone marrow of mammals, but fish do not have bone marrow (Moyle & Cech, 2004) and therefore, the creation of blood cells occurs mainly in spleen, kidney, and liver (Agius, 1980). The primary tissue where this occurs varies across phylogenetic groups of fish, and therefore so does the tissue distribution of MA. Evidence for this was provided by Agius (1980) who examined the hematopoietic tissues of 72 species of fish belonging to the groups Agnatha (jawless fish), Chondrichthyes (cartilaginous fish), and Osteichthyes

(bony fish) and found a pattern in the tissue distribution and degree of organization of MA based on phylogeny. In the tissues of representatives of Agnatha and Chondrichthyes, individual pigmented macrophages were found, which followed a random distribution. Macrophages appeared as organized centers in representatives of Osteichthyes, with the exception of salmonids which had MA that were not morphologically well defined. Furthermore, MA were primarily found in the livers of Agnatha, Chondrichthyes, and primitive Osteichthyes. Conversely, MA were identified mainly in the spleens and kidneys of advanced Osteichthyes (Agius, 1980). These results are consistent with the tissue distribution of hepatic MA in spotted gar and largemouth bass from Caddo Lake; gar livers are rich in MA, whereas MA are more prevalent in the spleens and livers of bass. It is not clear whether hepatic MA are directly responsible for MeHg demethylation or if they only serve to store Hg(II) after demethylation has occurred elsewhere in the liver. Support for their role as demethylators can be gleaned from studies with mammals, which show that MeHg is converted to Hg(II) in macrophage-rich lymph nodes (Havarinasab *et al.*, 2007). In addition, the conversion of MeHg to Hg(II) has been documented in other types of phagocytic cells of rats, humans, and rabbits, presumably as a result of free radicals produced within phagocytes during phagocytosis (Suda *et al.*, 1992). If MeHg demethylation proceeds in the same manner in fish, then MA may serve to both demethylate and store Hg.

8.7 **Analytical techniques to measure Hg speciation in fish tissues**

Current analytical techniques for the determination of Hg speciation in fish tissues include high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS) (Bushee, 1988), Hg-thiourea complex liquid chromatography cold vapor atomic fluorescence spectrometry (LC-CVAFS) (Shade, 2008), and probably the most popular, gas chromatography cold vapor atomic fluorescence spectrometry (GC-CVAFS) (Bloom, 1989). Although these techniques have low detection limits and enable the quantification of both Hg(II) and MeHg, instrumentation or outsourcing costs can be substantial. For this reason, studies may

rely more heavily on the more cost-effective total Hg analyses, while analyzing only a few samples for Hg speciation (Chumchal *et al.*, 2011). Recently, for the determination of total Hg, direct Hg analyzers have gained popularity due to their high sample throughput, ability to analyze both liquid and solid matrices, and relatively low detection limits and cost (Haynes *et al.*, 2006). Direct Hg analyzers, like Milestone's DMA-80, rely on thermal decomposition followed by gold amalgamation and detection with atomic absorption spectrometry. Former studies have also used direct Hg analysis to quantify MeHg in different matrices (Carbonell *et al.*, 2009; Maggi *et al.*, 2009; Nam & Basu, 2011; Scerbo & Barghigiani, 1998). As the direct Hg analyzer itself cannot discern between organic and inorganic forms of Hg, a procedural separation of Hg species is required before analysis. This is usually accomplished by chemically digesting the material, isolating MeHg from forms of Hg(II) by extraction with an organic solvent, back extracting MeHg into an aqueous cysteine solution, and measuring total Hg in the extract with a direct analyzer (Carbonell *et al.*, 2009; Maggi *et al.*, 2009; Nam & Basu, 2011; Scerbo & Barghigiani, 1998). Nam and Basu (2011) digested biological reference materials with a tris-buffered protease solution that was then treated with NaOH, cysteine, $CuSO_4$, and acidic NaBr, followed by organic Hg extraction with toluene and back extraction into an aqueous solution for analysis with a DMA-80. Recoveries of organic Hg from the reference materials were excellent and ranged between 86% and 107% relative to certified values for MeHg. Maggi and colleagues (2009) used a different digestion method with HBr, followed by toluene extraction, back extraction with an L-cysteine solution, and analysis by DMA-80 to analyze various certified reference materials as well as environmental samples. Using this approach, they recovered greater than 80% of MeHg from multiple reference materials, but recovery averaged only 74% from dogfish liver reference material (DOLT-3). Similarly, Scerbo and colleagues (1998) noted an increased degree of variation in the recovery of organic Hg from liver reference material DOLT-1, which they attributed to the relatively high lipid content of fish liver that affected quantitative back extraction with L-cysteine. Based on the results of these studies, low-cost methods which provide accurate Hg speciation data for lipid-rich fish tissues are needed.

9 GAPS IN KNOWLEDGE

Analytical techniques to determine Hg speciation in fish tissues

Mercury speciation analyses in fish tissues can be costly, due to either the price of instrumentation or the outsourcing of analyses. Previous studies have had various degrees of success in using direct Hg analyzers to determine Hg speciation in fish tissues. Accurate quantification of Hg speciation in lipid-rich tissues, like the liver, has been a particular problem in the past (Maggi *et al.*, 2009; Scerbo & Barghigiani, 1998). Therefore, low-cost methods which provide accurate Hg speciation for lipid-rich tissues are needed.

Hepatic Hg speciation and fish phylogeny

There is very little information regarding the relative proportions of MeHg and Hg(II) in the livers of wild fish and, therefore it is not well understood how hepatic Hg speciation might differ among species. With this in mind, the hepatic speciation of Hg should be evaluated for different species of fish. This information might provide insight into differences in species sensitivity to Hg and detoxification mechanisms.

Distribution of Hg in fish liver tissue

Previous studies have reported positive correlations between the numbers of hepatic melano-macrophage aggregates in fish and concentrations of Hg (Barst *et al.*, 2011; Batchelar *et al.*, 2013; Mela *et al.*, 2007; Raldúa *et al.*, 2007). Additionally, Hg and other metals have been shown to accumulate in these immune cells (Barst *et al.*, 2011; Pulsford *et al.*, 1992). However, it is not well understood to what extent Hg accumulates in the cells in relation to other essential and non-essential metals.

Sub-cellular partitioning of Hg in fish liver

Very few studies have determined the sub-cellular partitioning of Hg in fish. Even fewer studies have focused on the sub-cellular partitioning of Hg in the livers of fish (Araújo *et al.*, 2015), which is surprising given the role that this organ has in the detoxification of Hg. Therefore, more studies which focus on the sub-cellular partitioning of Hg in the livers of wild fish are needed. Studies, which aim to determine the sub-cellular partitioning of Hg in fish by using a differential centrifugation procedure, should also calculate mass balances to determine recovery of Hg.

Toxic effects of Hg in fish

Laboratory and field studies have demonstrated that dietary exposure to MeHg negatively affects the health of fish (Kidd & Batchelar, 2011; Sandheinrich & Wiener, 2011; Scheuhammer *et al.*, 2012). However it is not well understood how Hg exposure affects wild fish, especially in remote regions like the Arctic. This was made clear by a recent review on the biological effects of Hg in fish and wildlife of the Canadian Arctic which stated, "*there is an explicit need for Hg effects information for Arctic species themselves. Therefore, bioeffects studies should comprise a major focus of future Hg research in the Canadian Arctic.*"(Scheuhammer *et al.*, 2014). Research that goes beyond documenting Hg concentrations in fish and that will provide critical knowledge concerning fish health is warranted (Wiener *et al.*, 2003).

9.1 Study organisms

9.1.1 Yelloweye rockfish

Yelloweye rockfish (*Sebastes ruberrimus*) are native to the Pacific Coast of the United States and Canada, and are part of important recreational and commercial fisheries in the North Pacific (MacLellan & Station, 1997; Yamanaka *et al.*, 2006). Yelloweye were selected for study as they are extremely long-lived fish (oldest specimen >120 yrs) (MacLellan & Station, 1997) and were therefore likely to have elevated Hg

concentrations in their tissues. This assumption was supported by human health monitoring programs, which have recommended limited consumption of yelloweye rockfish by children and pregnant women due to the Hg concentrations in their tissues (McLaughlin & Gessner, 2007). Due to the life-history of yelloweye (long-lived with late sexual maturity) they may be particularly susceptible to Hg exposure. Yelloweye rockfish likely have small home ranges, as do other rockfish (Jorgensen *et al.*, 2006; Matthews, 1990; Yamanaka *et al.*, 2006), suggesting that their tissue concentrations of Hg reflect contamination of the food webs associated with where they were captured. They feed primarily on other fishes, such as other rockfish (*Sebastes sp.*) and herring (*Clupea pallasi*) (Yamanaka *et al.*, 2006).

9.1.2 Landlocked Arctic char

Landlocked Arctic char (*Salvelinus alpinus*) were chosen for study because they are often the only fish present in lakes of the Canadian High Arctic and are considered a keystone species (Gantner *et al.*, 2010; Power *et al.*, 2008). Lakes in the Canadian High Arctic have simple food webs, consisting of mainly Arctic char, chironomids, and chironomid food sources. Chironomids, are therefore a major food source and pathway for the uptake of Hg by Arctic char (Chételat *et al.*, 2008). Due to their circumpolar distribution, Arctic char have been targeted by monitoring programs which aim to document trends in contaminant concentrations in fish tissues, across a wide geographic region (AMAP, 2011). Previous results have shown that several populations of Arctic char from northern Canada and Greenland have Hg concentrations in their tissues at levels that exceed recently published toxicity thresholds for Hg (Drevnick, 2013), however very little is known about how Hg might affect the health of wild Arctic char.

9.1.3 Multiple species

The livers of yelloweye rockfish, landlocked Arctic char, and several other species of fish were analyzed to 1) help validate the Hg speciation method and 2) in order to understand if hepatic Hg speciation differs among groups of fish. Additional species included spotted gar (*Lepisosteus oculatus*), bowfin (*Amia calva*), channel catfish

(*Ictalurus punctatus*), northern pike (*Esox lucius*), largemouth bass (*Micropterus salmoides*), brook trout (*Salvelinus fontinalis*), and lake trout (*Salvelinus namaycush*). These species were selected because they represent several different taxanomic families, which range from primitive (Lepisosteidae and Amiidae) to more recently evolved (Centrarchidae and Salmonidae) bony fish. Brook trout were captured from eight lakes located in Parc national de la Mauricie, Québec. Lake trout and northern pike were sampled from lakes located in Isle Royale National Park, Michigan. Largemouth bass, channel catfish, bowfin, and spotted gar were sampled from Caddo Lake, Texas/Louisiana. Table 1 presents taxonomic information for these fish.

Table 1. Taxonomic information for the species of fish that were analyzed for hepatic mercury speciation.

Common Name	Genus and species	Family	Infraclass
Arctic char	*Salvelinus alpinus*	Salmonidae	Teleostei
Brook trout	*Salvelinus fontinalis*	Salmonidae	Teleostei
Lake trout	*Salvelinus namaycush*	Salmonidae	Teleostei
Northern pike	*Esox lucius*	Esocidae	Teleostei
Largemouth bass	*Micropterus salmoides*	Centrarchidae	Teleostei
Channel catfish	*Ictalurus punctatus*	Ictaluridae	Teleostei
Yelloweye rockfish	*Sebastes ruberrimus*	Scorpaenidae	Teleostei
Bowfin	*Amia calva*	Amiidae	Holostei
Spotted gar	*Lepisosteus oculatus*	Lepisosteidae	Holostei

10 OBJECTIVES AND HYPOTHESES

Given the previously outlined knowledge gaps, the research described in this dissertation was carried out to answer the following questions.

- Can direct Hg analyzers be used to generate Hg speciation data for fish tissues that are accurate and comparable to those obtained from an established technique?

- Does hepatic Hg speciation differ among species of wild fish?

- What is the relationship between Hg and macrophage aggregates in wild fish?

- How is Hg distributed at the tissue and sub-cellular levels in the livers of wild fish?

- Is the health of wild fish being negatively affected by Hg?

The principal objectives and their respective hypotheses are presented below. The first two objectives were met by studying multiple fish species. The remaining objectives are focused solely on yelloweye rockfish and Arctic char.

Objective 1: Develop a method to determine Hg speciation in fish tissues using a direct Hg analyzer.

Hypothesis: MeHg concentrations, calculated by the proposed method, will not differ significantly from the certified MeHg value in DOLT-4.

Hypothesis: MeHg concentrations, calculated from total Hg and inorganic Hg concentrations, will not differ significantly from direct MeHg measurements by GC-CVAFS.

Objective 2: Determine the hepatic Hg speciation in different species of bony fish.

Hypothesis: MeHg will comprise the majority of total Hg in bony fish livers.

Objective 3: Determine how Hg is related to liver pathologies in yelloweye rockfish and landlocked Arctic char.

Hypothesis: The number or area of MA will be positively correlated with Hg and other metals in the livers of yelloweye rockfish and Arctic char.

Hypothesis: Other liver pathologies will be positively correlated with Hg and other metals in the livers of yelloweye rockfish and landlocked Arctic char.

Objective 4: Determine how Hg and other metals are distributed at the tissue level in the livers of yelloweye rockfish.

Hypothesis: Relative concentrations of Hg and other metals will be higher in MA than the surrounding tissue.

Objective 5: Determine the sub-cellular partitioning of total Hg in the livers of landlocked Arctic char.

Hypothesis: Total Hg will be primarily associated with the heat-stable protein fraction (HSP).

Objective 6: Determine how Hg is related to the reproductive and general health of yelloweye rockfish and landlocked Arctic char.

Hypothesis: Hepatosomatic index will be negatively correlated with total Hg in the livers of yelloweye rockfish and Arctic char.

Hypothesis: Gonadosomatic index and body condition will be negatively correlated with total Hg in the muscle of Arctic char.

11 EXPERIMENTAL METHODS

The specific methods used for this research are outlined in detail in the articles attached to this dissertation. Therefore, only the main points of these methods are presented in the following paragraphs.

11.1 Study areas

11.1.1 Southeast Alaska

Prince of Wales Island (POW) is a relatively large island in the Alexander Archipelego in the Panhandle of Alaska, USA. Sampling of yelloweye rockfish took place at two sites, one of which was located to the east of POW in Ernest Sound (55°52'N, 132°13'W) and, one to the west between Coronation Island and Warren Island (55°52'N, 134°03'W) (Figure 2). This area of Alaska was chosen for study because it is remote without local industrial sources of Hg, yet the coastal waters are relatively easily accessible from the fishing village of Thorne Bay. Rapidly increasing Hg emissions in Asia (Fitzgerald & Lamborg, 2007; Selin, 2009), transported across the Pacific, may be causing increased Hg deposition in Alaska and Canada (Dan Engstrom, unpublished data). A recent modeling study reported that 20% of Hg deposited in northern British Colombia, Yukon, and Alaska is from East Asian anthropogenic sources (Jaffe & Strode, 2008). Mercury concentrations in fish of the North Pacific could increase with increasing emission and deposition of Asian Hg.

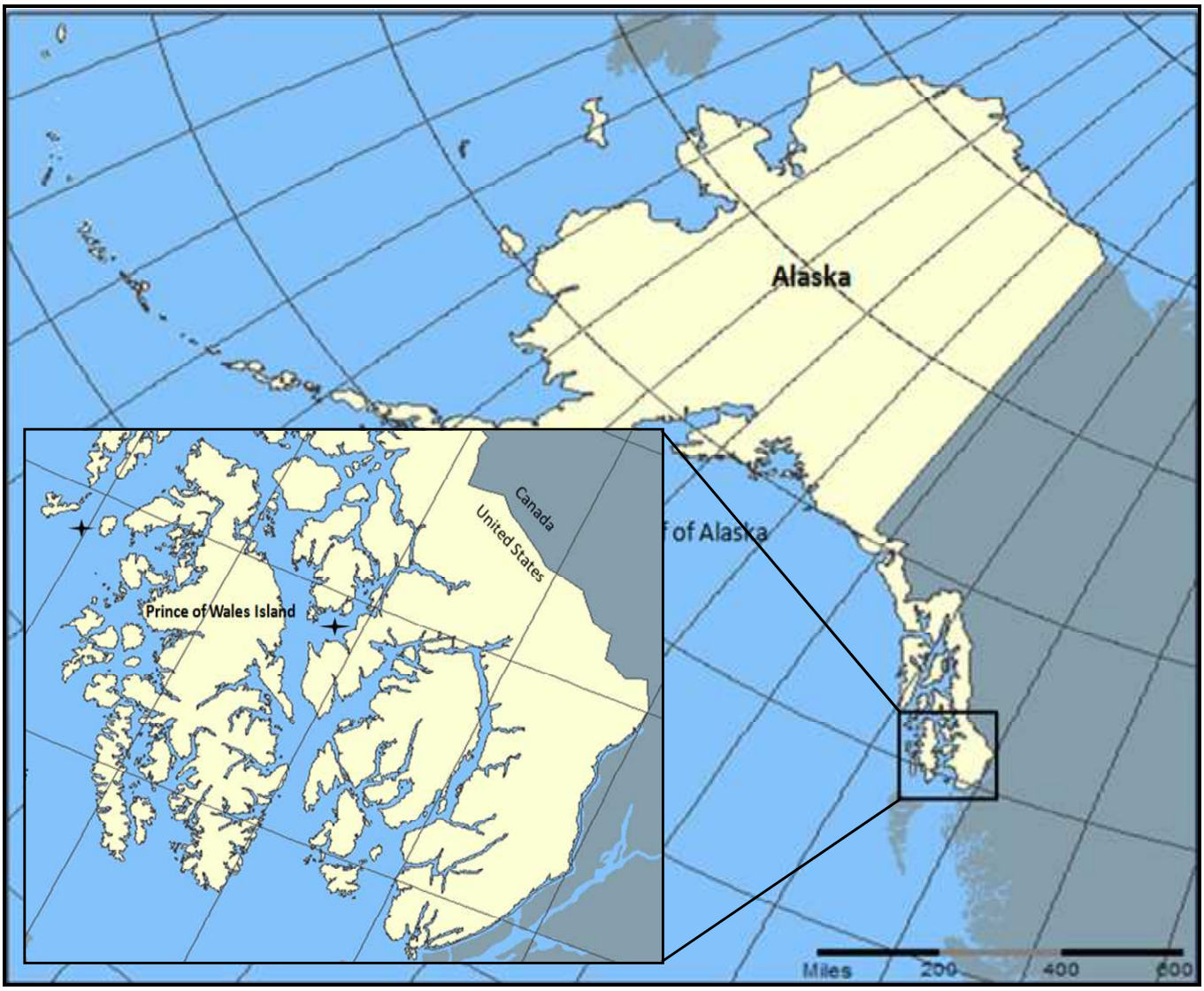

Figure 2. Location of Prince of Wales Island, Alaska. The black symbols in the inset image show the locations of the two sampling sites.

11.1.2 Canadian High Arctic

Arctic char were sampled from four lakes (Small, 9-Mile, North, and Amituk) located on Cornwallis Island, Nunavut in the Canadian High Arctic (75°08'N, 95°00'W). These lakes have been included in Environment Canada's "core" char monitoring project, which is designed to provide information on the temporal trends of Hg and persistent

organic pollutants (POPs), for several years (Amituk Lake since 1989). Based on information from the monitoring program, study lakes were selected to span a gradient of Hg contamination, and because POP concentrations are low in these lakes (Figure 3).

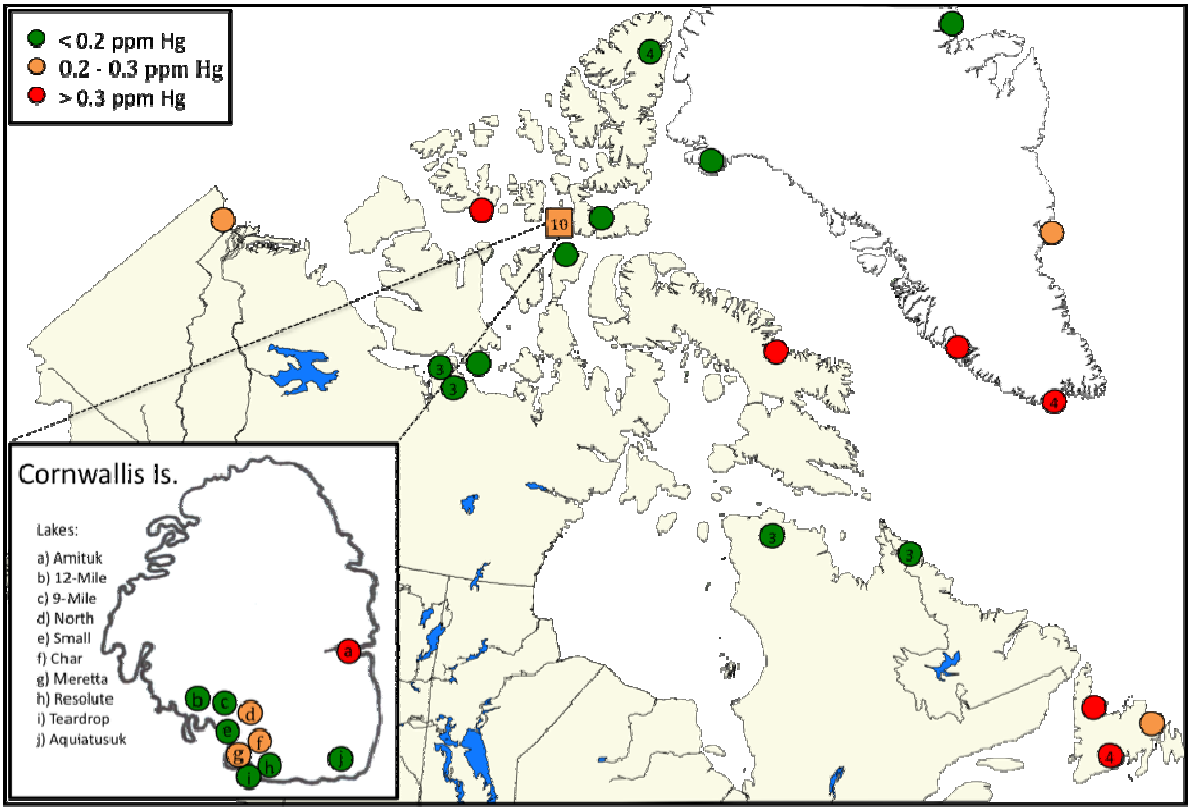

Figure 3. Mean Hg concentrations (µg·g^{-1} wet weight) in populations of Arctic char from Canada and Greenland. The orange and red dots represent populations which exceed the toxicity thresholds set by Beckvar *et al.* (2005) and Dillon *et al.* (2010) respectively. Figure from (Drevnick, 2013).

11.2 Metal analyses

11.2.1 Hg analyses

All tissue samples and sub-cellular fractions were freeze-dried and then analyzed for total Hg with a DMA-80 (Milestone Inc.), which uses thermal decomposition followed by gold amalgamation and detection with atomic absorption spectrometry according to United States Environmental Protection Agency (USEPA) method 7473. Specific

information on total Hg analyses is available in the three articles at the end of this dissertation.

The novel method for Hg(II) analysis of fish tissue is presented in article 1 at the end of this dissertation. Briefly, a homogenized subsample of lyophilized fish tissue is digested in HCl with microwave heating. Toluene is then added to the digest in order to separate organic Hg from Hg(II). After removal of the toluene layer, the remaining acidic aqueous fraction is diluted and analyzed for total Hg with a DMA-80. A separate subsample of the same fish tissue is analyzed for total Hg as described in the previous section. The concentration of Hg(II) is then subtracted from total Hg to obtain the concentration of organic Hg in the sample.

The accuracy of this method was validated by comparing calculated MeHg concentrations to certified MeHg concentrations in a reference material. Calculated MeHg concentrations were compared to measured MeHg concentrations in the same naturally-contaminated fish tissues. Measured MeHg was determined by GC-CVAFS, according to previously described methods (Bloom, 1989; Gill & Fitzgerald, 1987; Hammerschmidt & Fitzgerald, 2006; Tseng *et al.*, 2004). Further details are also provided in article 1.

11.2.2 Other metals

Other metals were measured by digesting freeze-dried subsamples of liver with concentrated HNO_3 (Aristar grade) followed by 30% (w/w) hydrogen peroxide (Trace Select Ultra grade). Ultrapure water was added to the digestates in order to reach a 10% HNO_3 concentration before analysis either by ICP-MS (Thermo Elemental X Series) or inductively coupled plasma atomic emission spectroscopy (ICP-AES; Vista AX). Further details are presented in articles 2 and 3.

11.3 Health indices

Hepatosomatic index (HSI), gonadosomatic index, and condition factor were calculated according to the following equations.

Equation 5. Hepatosomatic index

$$HSI = \left(\frac{W_L}{W_T}\right) \times 100$$

where W_L is the liver weight and W_T is the total weight of the fish.

Equation 6. Gonadosomatic index

$$GSI = \left(\frac{W_g}{W_T}\right) \times 100$$

where W_g is the gonad weight and W_T is the total weight of the fish.

Condition factor was calculated in one of two ways.

Equation 7. Fulton's condition factor

$$condition\ factor = \frac{1{,}000 \times W_T}{L^3}$$

where W_T is the total weight of the fish and L^3 is the total length of the fish cubed.

Equation 8. Relative condition factor

$$relative\ condition\ factor = \frac{W_T}{W'}$$

where W_T is the total weight of the fish and W' is the calculated weight for the observed length based on the length-weight relationship.

Fulton's condition factor assumes that the slope of the regression line between length and weight is equal to three and that growth is isometric. This was used to assess the condition of yelloweye rockfish because these assumptions were met. Relative condition factor was more appropriate for Arctic char because there was significant variation among slopes for the four char populations and not all of the slopes equaled three.

11.4 **Histology**

Fixed liver tissues, from yelloweye rockfish and Arctic char, were embedded in paraffin, sectioned, and mounted on glass slides. Liver sections were either stained, with hematoxylin and eosin (H&E) and evaluated by light microscopy, or left unstained and archived for metals imaging by laser ablation ICP-MS or X-ray fluorescence. For stained yelloweye rockfish tissue sections, each of three fields of view, at 100X magnification, were photographed. The mean percent coverage of MA, in relation to the total area of the field of view, was calculated for each photograph using Image J software (NIH http://rsbweb.nih.gov/ij/). A mean MA percent coverage was then calculated based on the three photographs. An example of this is presented in Figure 4.

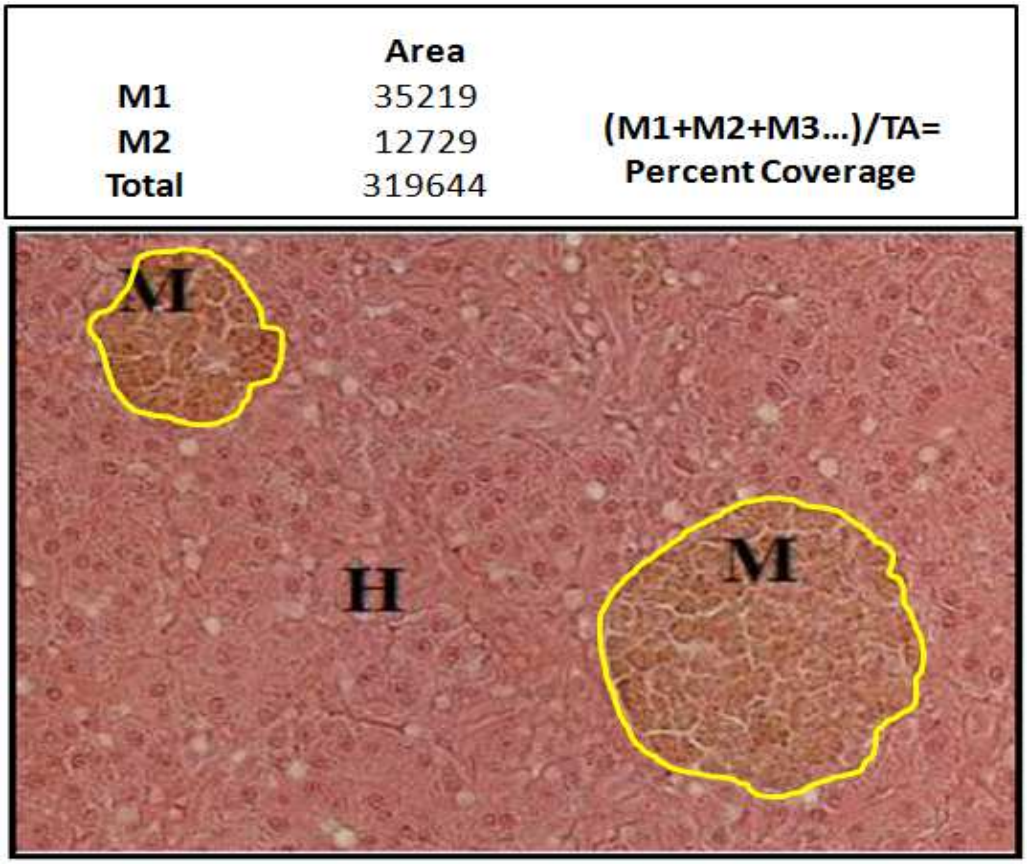

Figure 4. An example of how melano-macrophage percent coverage was determined for a microscopic field of view of a yelloweye rockfish liver section (H&E, 100X). In the image M=melano-macrophage aggregate, H=hepatocytes.

As noted by Agius (1980), MA in salmonids tend to be small and not well defined and because of this, it is difficult to determine their area in the same manner as above. Therefore, the numbers of MA in each of three fields of view at 100X magnification were averaged to determine a mean hepatic MA count for individual Arctic char.

11.5 Metal imaging in liver tissues

11.5.1 Laser ablation ICP-MS

Tissue sections were used to investigate the distribution of metals in yelloweye liver sections by LA-ICP-MS, according to the methods of Barst *et al.* (2011) with slight modification. Briefly, a microscope slide, with unstained, paraffin-embedded tissue, was placed into the chamber of a 213 nm Nd:YAG laser ablation source. Areas of normal hepatocytes and MA were chosen at random and ablated with a 55 µm beam diameter. An ICP-MS coupled to the laser source was used to monitor Hg, Se, Cd, Ni, Cu, and Zn in the tissue sections. The mean isotope counts of each element during laser warm-up were used as an estimate of background noise and subtracted from the mean counts for the remainder of the run to calculate a signal. We calculated the ratio of metal in MA relative to the surrounding normal tissue (M_{MA}:M_{Hep}) for Cu, Zn, Cd, Hg, and Se. After ablation, slides were viewed again under light microscopy to ensure MA had been accurately targeted.

11.5.2 X-ray fluorescence imaging

X-ray fluorescence images of a single yelloweye liver section were collected at the Advanced Photon Source, Argonne National Laboratory. According to methods previously described (Prince *et al.*, 2014). The intensities of the X-ray fluorescence lines: Hg Lα, Se Kα, Cu Kα, and Zn Kα were monitored. Unfortunately we were not able to determine the Cd concentration in the yelloweye liver section with X-ray fluorescence, as the energy of the Cd Lα overlaps with that of Ar Kα photons present in air.

11.6 Sub-cellular partitioning procedure

The sub-cellular partitioning procedure which was used has been described previously (Campbell & Hare, 2009; Giguère *et al.*, 2006; Rosabal *et al.*, 2012; Rosabal *et al.*, 2015). This separated char liver cells into six operationally defined fractions: (1) “Debris” (nuclei and cellular debris), (2) “Granules” (NaOH-resistant fraction/granule-like concretions), (3) Mitochondria, (4) “Organelles” (lysosomes and microsomes), (5) heat-denatured proteins (HDP) including enzymes, (6) peptides and heat-stable proteins (HSP) including metallothionein and GSH. These fractions were combined into sensitive (mitochondria + HDP + organelles) and detoxified (HSP + granules) groups in order to determine the potential for effects. The procedure involves a series of differential centrifugations as well as NaOH digestion and heat treatment steps. A flow-chart outlining these steps is presented in Figure 5. A more in-depth description of the methods is presented in article 3 at the end of this dissertation.

Figure 5. Sub-cellular partitioning procedure involving differential centrifugations, as well as NaOH and heat treatment steps. Figure adapted from (Giguère *et al.*, 2006).

12 RESULTS AND DISCUSSION

12.1 Determination of Hg speciation in fish tissue with by DMA-80

Measured concentrations of Hg(II) in the acidic fraction of DOLT-4 digestates were not significantly different from those estimated by difference between mean certified values for total Hg and MeHg. Recovery of Hg(II) from DOLT-4 averaged 99 ± 5% of the difference between mean certified values. Organic Hg can be estimated as the difference between measured concentrations of total Hg and Hg(II). With the proposed method, the mean estimated recovery of organic Hg from DOLT-4, by difference, was 100 ± 5% of the certified mean value for MeHg.

Estimates of MeHg, calculated by difference between measured concentrations of total Hg and Hg(II) with the proposed method, were in excellent agreement with direct speciation measurements of MeHg by established GC-CVAFS techniques (mean recovery = 99% ± 6%; Figure 6), for all liver and muscle samples, except spotted gar liver.

In contrast to the other fish livers analyzed, spotted gar had much greater concentrations of total Hg, of which only a small fraction was present as organic Hg (mean = 4.1%). Concentrations of organic Hg in gar livers, estimated by difference between measured Hg(II) and total Hg, averaged 183% greater than those determined by GC-CVAFS. These differences in estimated organic Hg versus measured MeHg are likely due to the increased uncertainty that results from estimating a third value (i.e., organic Hg) by difference between two large values (Hg(II) and total Hg).

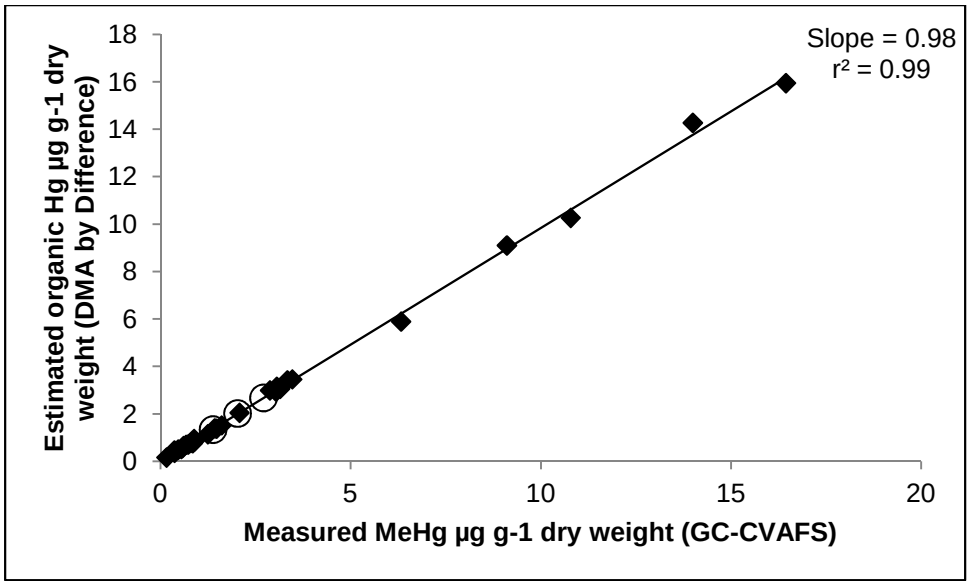

Figure 6. Comparison of MeHg measured by gas chromatographic cold vapor atomic fluorescence spectrometry (GC-CVAFS) and estimated organic Hg (by DMA-80 with proposed method) in fish liver (diamonds) and muscle tissue (circles).

By using the percent differences among duplicate samples as an estimate for error we were able to model percent recovery of MeHg versus percent Hg(II) for a group of hypothetical samples and determine the point at which our method no longer provides accurate estimates of MeHg. These hypothetical samples were assigned differing total Hg concentrations (0.5, 5, or 50 µg g^{-1}) and percent Hg(II) concentrations (between 0 and 100%) before their values were adjusted using our calculated percent differences. Both MeHg and adjusted MeHg values were calculated (by difference between total Hg and Hg(II)) and their percent recoveries are presented in Figure 7. Based on the hypothetical data and the data from our study, poor estimation of MeHg appears to be independent of total Hg concentration.

Thus, the proposed method is an excellent approach to directly quantify Hg(II) in fish liver and muscle and, from that, to estimate organic Hg in samples with less than 90% Hg(II). Estimates of either organic Hg or MeHg in samples with relatively high fractions of Hg(II) and elevated total Hg concentrations should be interpreted with caution and a more traditional direct measure of MeHg may be more appropriate.

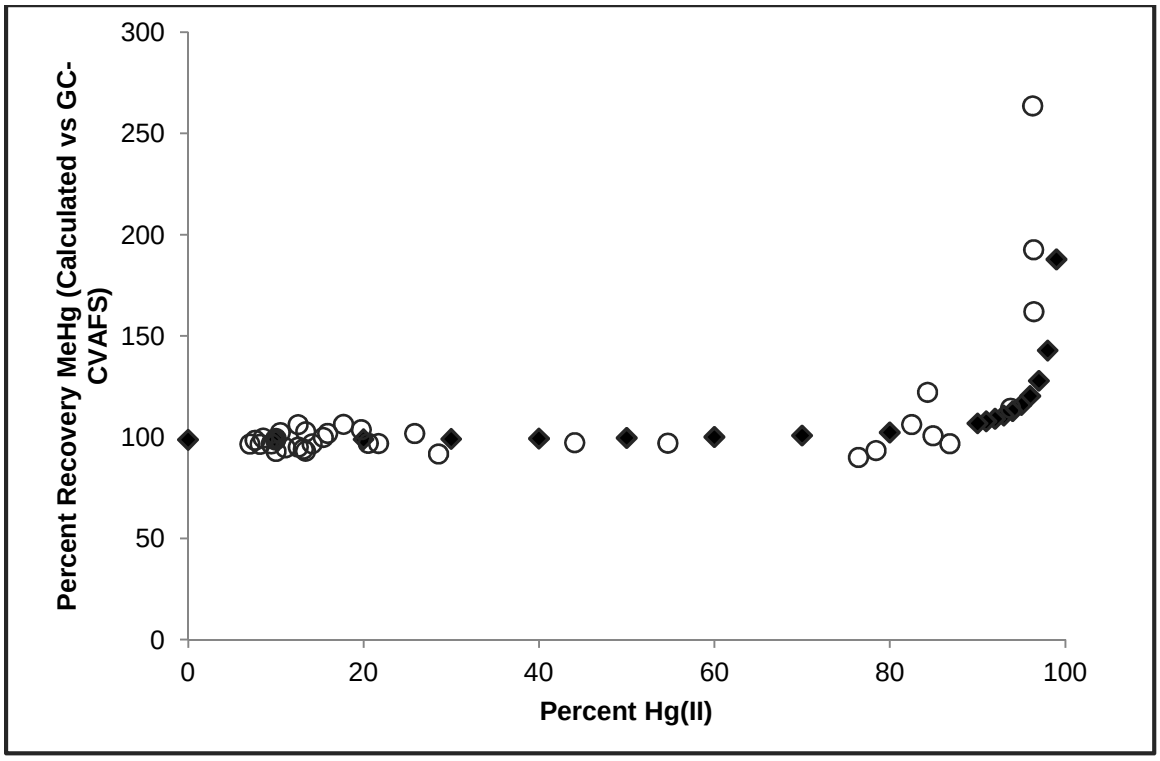

Figure 7. Comparison of percent recovery of MeHg (calculated vs. measured by gas chromatograpy cold vapor atomic fluorescence spectrometry [GC-CVAFS] and percent Hg(II). Diamonds represent hypothetical samples, and circles represent fish tissues that were analyzed for the present study.

12.2 Hepatic Hg speciation in bony fish

I used the previously described method to calculate MeHg concentrations in liver samples of nine species of bony fish. Due to the lack of confidence in calculated MeHg concentrations for fish with relatively high fractions (≥90%) of Hg(II) in their livers, MeHg was also measured in spotted gar and bowfin livers by GC-CVAFS. The mean proportion of MeHg for each species is presented in Table 2.

Table 2. Proportion of methylmercury in the livers of nine species of bony fish.

Common name	Genus and species	Family	Infraclass	Number of samples analyzed	Percentage (mean ± SD) of MeHg in Liver
Arctic char	*Salvelinus alpinus*	Salmonidae	Teleostei	143	80 ± 8.6%
Brook trout	*Salvelinus fontinalis*	Salmonidae	Teleostei	126	77 ± 12.8%
Lake trout	*Salvelinus namaycush*	Salmonidae	Teleostei	15	82 ± 10.0%
Northern pike	*Esox lucius*	Esocidae	Teleostei	10	44 ± 14.5%
Largemouth bass	*Micropterus salmoides*	Centrarchidae	Teleostei	9	58 ± 9.5%
Channel catfish	*Ictalurus punctatus*	Ictaluridae	Teleostei	10	20 ± 6.1%
Yelloweye rockfish	*Sebastes ruberrimus*	Scorpaenidae	Teleostei	51	41 ± 11.9%
Bowfin	*Amia calva*	Amiidae	Holostei	7	25 ± 11.5%
Spotted gar	*Lepisosteus oculatus*	Lepisosteidae	Holostei	8	18 ±11.3%

In the three salmonid species, the majority of hepatic total Hg was present as MeHg. This was in stark contrast to channel catfish and the more primitive spotted gar and bowfin, which had elevated proportions of Hg(II) in their livers. In the remaining species of fish, MeHg made up approximately half of the total hepatic Hg. These trends in hepatic Hg speciation are similar over a range of total hepatic Hg concentrations, as shown in Figure 8.

The differences in hepatic Hg speciation could be due to dietary differences among the species. For example, northern pike from Isle Royale, Michigan feed on larval odonates that are rich in Hg(II) (Drevnick *et al.*, 2008). Elevated proportions of Hg(II) could also be due to differences in how Hg is stored in the livers of different species of fish. Previous studies have shown that spotted gar have large numbers of MA in their livers which accumulate Hg (Barst *et al.*, 2011; Smith, 2012), however whether Hg is present as MeHg or Hg(II) is not known. Elevated proportions of Hg(II) in the livers of some species of fish could also be due to hepatic demethylation of MeHg.

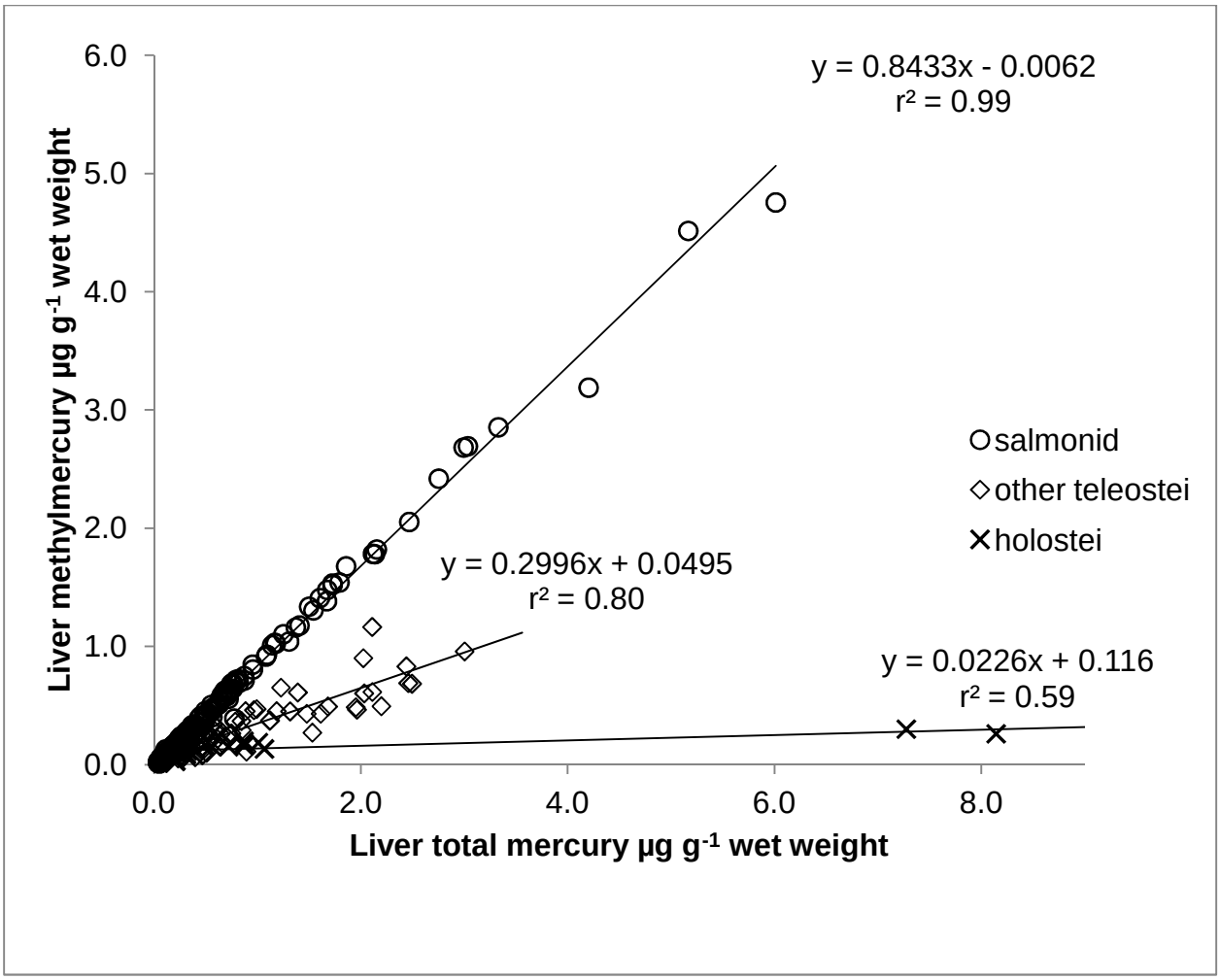

Figure 8. Comparison of the concentrations of total mercury and methylmercury in the livers of species of salmonids, other teleostei, and holostei.

12.3 Liver pathologies and metals in the livers of yelloweye rockfish and landlocked Arctic char

Concentrations of MeHg, Hg(II), Se, Cd, and Cu were positively correlated with the area of MA in the livers of yelloweye rockfish. Melano-macrophage aggregate area also increased with the age of the fish. Therefore, concentrations MeHg, Hg(II), Se, Cd, and Cu were incorporated, along with fish age, into a stepwise regression model to determine the best predictor of MA area. The results show that Hg(II) was the best predictor of MA area in the livers of yelloweye rockfish, suggesting that Hg(II) was the major contributor to the increased area of these pathologies.

The statistical results were further supported by the two metal imaging techniques, which demonstrated that Hg not only accumulates in the MA of yelloweye, but that it does so to a greater degree than most of the other metals. The one exception is Se, which accumulated in MA to approximately the same extent as Hg, relative to the surrounding hepatocytes. Neither LA-ICP-MS nor X-ray fluorescence provide information about the exact chemical form of Hg or Se in MA. However, the majority of the total Hg in yelloweye livers was present as Hg(II), the best predictor of MA area. Additionally, we noted significant correlations between both the Se and Hg concentrations in whole liver and the Se M_{MA}:M_{Hep} ratio and counts of Hg in MA. It is, therefore, possible that Hg(II) is present in MA bound to Se. In yelloweye rockfish, hepatic MA appear to be excellent biomarkers of Hg exposure.

In contrast to the MA in yelloweye rockfish livers, the hepatic MA of Arctic char were smaller and their edges were less well defined (Figure 9). This is consistent with previous reports on the MA of salmonids (Agius, 1980). The numbers of hepatic MA were positively correlated with the age of Arctic char. We also noted positive correlations between the numbers of MA and concentrations of MeHg and Hg(II) in the livers of Arctic char from Small, 9-Mile, and North lakes. However, in the livers of char from Amituk Lake, there were very few MA. When combining data for char from all lakes together, there was no relationship between concentrations of Hg and MA. Unfortunately, the MA of char were too small and the concentrations of Hg were too low (in Small and 9-Mile char) to determine if Hg and other metals were colocalized with MA by means of metal imaging techniques. Nevertheless, the statistical results do not suggest that MA increase in number in the livers of Arctic char as a result of Hg exposure.

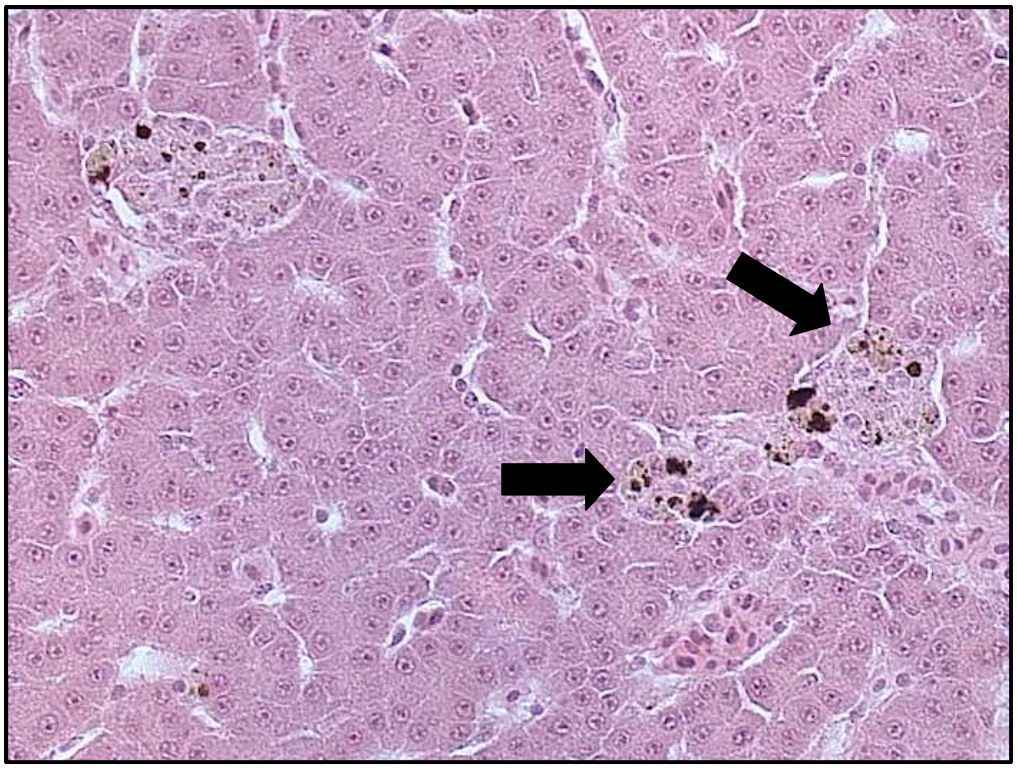

Figure 9. Micrograph of an Arctic char liver section. Arrows show the position of melano-macrophage aggregates (H&E stained, 200X).

Arctic char from Small and 9-Mile lakes had elevated concentrations of Fe in their livers, relative to char from the other two lakes, potentially as a result of feeding on Fe-rich larval chironomids. Concentrations of Fe were positively correlated with the numbers of hepatic MA, suggesting that Fe may play a role in the generation of these pathologies. By staining several Arctic char liver sections from Small and 9-Mile lakes with Prussian blue, we determined that Fe was present within hepatic MA as Fe(III). In salmonids, hematopoiesis mainly occurs in kidney and spleen and therefore, hepatic MA are less common,(Agius, 1980). However, it may be that a substantial dietary source of Fe, e.g. larval chironomids, can stimulate the formation of hepatic MA in these fish as a means of handling the elevated Fe burden.

The lack of correlation between Hg and MA across lakes was surprising given the excellent association of MA and Hg in yelloweye and the correlations previously reported by others (Barst *et al.*, 2011; Batchelar *et al.*, 2013). Given that MA tissue distribution differs with fish phylogeny, it may be that MA respond to Hg in the livers of

fish when MA are already present. This would explain the positive correlations between MA and Hg in Arctic char with elevated levels of Fe, and why MA were not abundant in Amituk char livers.

In addition to MA, yelloweye and Arctic char livers were assessed for general pathology. Yelloweye livers appeared to have normal morphology across the range of total Hg concentrations. This was not the case for Arctic char, whose livers ranged from those that appeared healthy (Figure 10) to livers that exhibited abnormal morphology. The most notable pathology in abnormal livers was fibrosis in the perisinusoidal area (Figure 11). A significantly greater number of individuals from Amituk Lake exhibited this abnormality than individuals from the other three study lakes. The presence of liver damage in Amituk Arctic char and not in yelloweye rockfish, may be due to differences in hepatic Hg speciation; most of the Hg in yelloweye livers was present as Hg(II), whereas in char livers MeHg predominated. Total Hg concentrations also tended to be higher in Amituk char livers than in yelloweye livers. It is also possible that yelloweye livers are better protected from Hg due to the presence of MA, which could sequester Hg and thus prevent further interaction of Hg with other cells of the liver. Lastly, hepatic fibrosis in Amituk Lake char livers may be a result of greater numbers of cestode parasites, which often increase in numbers in char that are piscivorous (Frandsen *et al.*, 1989).

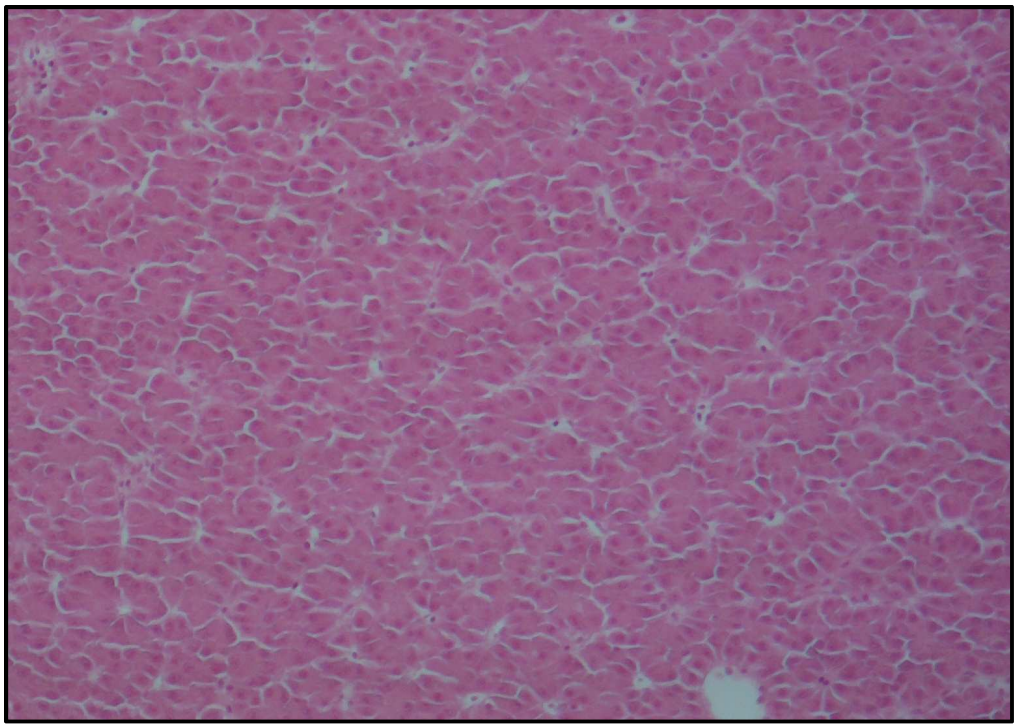

Figure 10. Micrograph of a liver section from an Arctic char from Small Lake showing normal liver morphology. Note the uniform hepatocytes and unremarkable sinusoids (H&E stained, 200X).

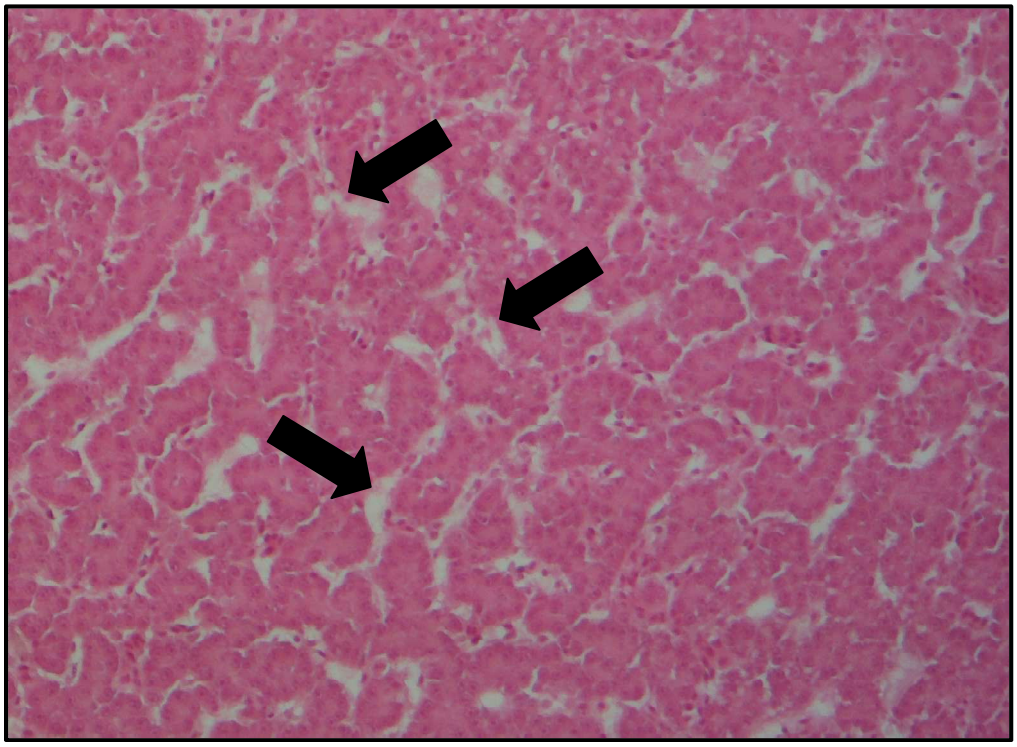

Figure 11. Micrograph of a liver section from an Arctic char from Amituk Lake showing abnormal perisinusoidal regions (black arrows) (H&E stained, 200X).

12.4 Sub-cellular distribution of Hg in the livers of Arctic char

Despite the significantly higher Hg concentrations in Amituk livers, compared to livers from Small lake char, Hg was distributed similarly in liver cells of char from both lakes; sensitive compartments contributed 73 and 61% of the Hg in Small and Amituk livers (Figure 12). This suggests that Hg is not well detoxified in the livers of Arctic char from either lake. The remaining Hg found in the detoxified compartments comprised 10 and 19% of the Hg contributions of Small and Amituk char respectively. Almost all of the Hg in the detoxified compartment was associated with the HSP fraction. presumably containing metallothionein, whereas very little was found in the granule-like fraction. In the potentially sensitive compartment, Hg accumulated mainly in mitochondria and the HDP fraction.

A low proportion of Hg was associated with the fraction containing HSP, and presumably metallothionein. Araújo and colleauges (2015) also found a low percentage of Hg in the HSP fraction of wild mullet livers, which they attributed to Hg concentrations which did not exceed the physiological threshold for metallothionein induction. Given the extreme Hg concentrations in Amituk char livers, it seems unlikely that a low proportion of Hg in the HSP fraction is a result of metallothionein not being induced. One possible explanation for a low percentage of Hg in this fraction may be related to Hg speciation. We did not measure Hg speciation in individual liver fractions and therefore do not know how MeHg and Hg(II) were distributed at the sub-cellular level. However, MeHg was the predominate form of total Hg in Arctic char liver homogenates. Inorganic Hg has been shown to induce and bind with metallothionein (Kidd & Batchelar, 2011). Conversely, MeHg is not thought to induce metallothionein synthesis (Kidd & Batchelar, 2011) and likely binds less strongly with its cysteine residues (Wang *et al.*, 2012). However, the assumption that a higher proportion of Hg(II) in the liver would lead to a greater proportion of Hg in the HSP fraction has not been tested in fish. Interestingly, aquatic invertebrates exposed to Hg(II) also accumulated very little Hg in the HSP fraction (Xie *et al.*, 2008). Therefore it is possible that neither form of Hg tends to associate with the HSP fraction of aquatic organisms.

The accumulation of Hg in the mitochondria and HDP fractions may have negative consequences for cellular respiration and enzymes of the redox defense system. Evidence for this comes from laboratory studies which have demonstrated that dietary MeHg exposure leads to structural abnormalities of mitochondria and inhibition of respiration (Cambier *et al.*, 2009), as well as decreased activity of GPx (Berntssen *et al.*, 2003).

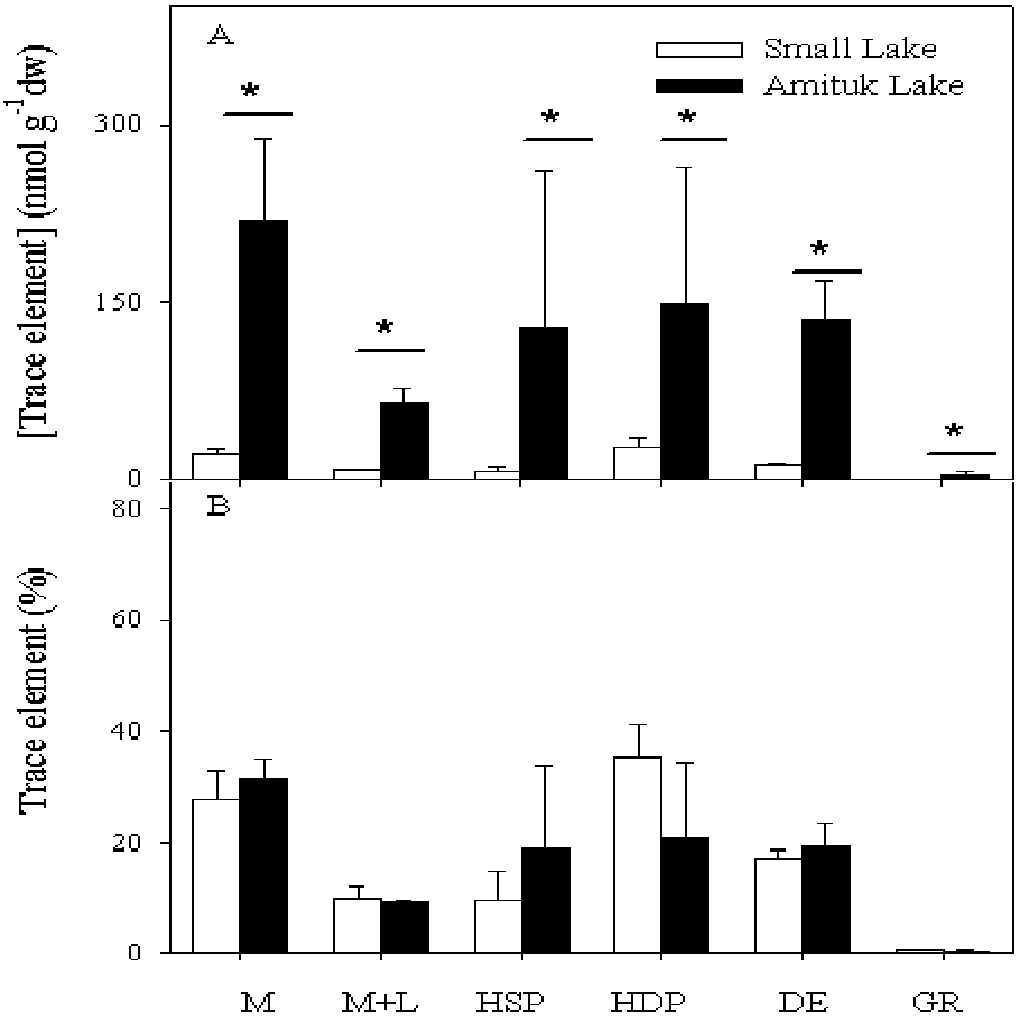

Figure 12. Sub-cellular partitioning of mercury in the livers of landlocked Arctic char collected from Small Lake (white bars) and Amituk Lake (black bars). The concentrations of Hg in sub-cellular fractions are represented in the upper panels (A) and the mercury contribution (%) in the lower panel (B). Significant differences between Small Lake and Amituk fractions are denoted by *. M = mitochondria, M+L = microsomes and lysosomes, HSP = heat-stable proteins, HDP = heat denatured proteins, DE = cellular debris and nuclei, GR = granule-like structures.

12.5 Health indices in relation to mercury concentrations in yelloweye rockfish and landlocked Arctic char

Both Fulton's condition factor and relative condition factor relate a fish's length with its weight, describing a fish's "plumpness". The HSI gives the weight of the liver relative to the total weight of the fish. Higher HSI and condition factor are generally interpreted as elevated fat or glycogen stores in the liver and body. How these indices relate to essential and non-essential metals varies throughout the literature as studies have documented positive, negative, and insignificant relationships as a result of metal exposure (Di Giulio & Hinton, 2008).

Condition factor and HSI were significantly higher in yelloweye rockfish from the western sampling area than in fish from the eastern area. Yelloweye from the west also had faster growth rates than the fish sampled from the east. Taken together, the elevated growth rate, HSI, and body condition of the yelloweye from the western site are likely a result of greater temperatures or food availability, both of which can influence growth rate. Yelloweye from the east likely had higher concentrations of Hg because they grew more slowly. For yelloweye rockfish, neither condition factor nor HSI were significantly correlated with concentrations of Hg.

HSI was positively correlated with Hg in the livers of male and female char. The HSI may increase after metal exposure due to hyperplasia (Adams *et al.*, 1990), which is an increase in the number of liver cells. HSI was also positively correlated with trophic position, which co-varied with Hg. Therefore, the increase in HSI in male and female char may be interpreted as a positive effect of feeding at a higher trophic position, i.e. char have greater fat reserves as a result of feeding on other char. This was supported by the positive correlation between HSI and C:N values. The condition factor of Arctic char was not correlated with trophic position, C:N values, or concentrations of Hg.

The GSI of Arctic char was a function of whether gonads were developed and body size, and did not differ significantly among lakes, for males or females. For mature females, GSI increased with total Hg concentrations. In regards to body size, as char grow bigger, they have proportionally bigger gonads, but also more Hg. If the "effect" of body size on GSI is removed (by ANCOVA), the results indicate that Hg is not related to

GSI. The four study lakes are very unproductive, meaning that Arctic char have restricted energy budgets. Since energy is allocated first for the maintenance of basic biological functions, then for growth and reproduction, char do not spawn every year. Even after sampling for two consecutive summers, only a few individuals were sexually mature. This makes it difficult to draw strong conclusions about the possible reproductive effects of Hg in Arctic char. After correcting for body size, by calculating relative fecundity (number of eggs / 100 g of body weight) we used quantile regression to determine if Hg may be limiting the reproductive output of Arctic char. The results in Figure 14 were not significant, which may have been a result of limited sample size.

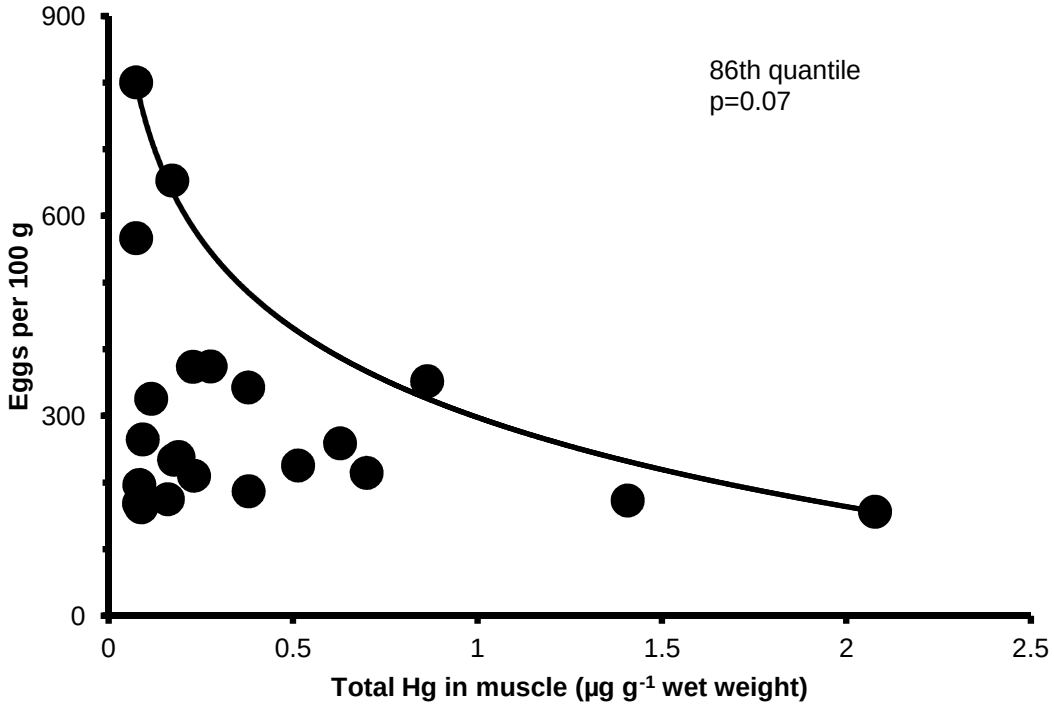

Figure 13. Total mercury in muscle versus relative fecundity of maturing female Arctic char from the four study lakes.

13 CONCLUSION

The method to determine Hg speciation in fish tissue with a direct mercury analyzer provides accurate MeHg concentrations, for samples with less than 90% Hg(II), over a range of total Hg concentrations. Inorganic Hg can be accurately measured in all samples, regardless of their percent Hg(II) content. In addition to providing accurate data, this method is also rapid and low-cost. This will hopefully facilitate the incorporation of Hg speciation in future studies on wild fish. Speciation is important to consider when studying the toxicity of Hg in wild fish, because it appears that not all species of fish have the same proportions of MeHg in their livers. This could result in differences in species sensitivity to Hg contamination. The majority of total Hg was present as Hg(II) in the livers of yelloweye rockfish. At the tissue level yelloweye store Hg, possibly as HgSe, in MA. This may serve to remove Hg from circulation and reduce Hg exposure to other cells, tissues, and organs. Conversely, Arctic char had elevated proportions of MeHg in their livers and their MA do not appear to respond to Hg in the same dose-response manner. In both Small Lake and Amituk Lake char, Hg was found predominately bound to potentially metal-sensitive sub-cellular sites, suggesting that Hg is not effectively detoxified in their livers. This may account for the observed damage in Amituk char livers. However, because feeding at higher trophic levels results in greater Hg exposure and may also lead to greater parasite burdens, one cannot point solely to Hg as the cause of hepatic fibrosis in Arctic char. Further work is needed to understand how char reproduction may be affected by Hg contamination. Finally, Hg does not appear to be negatively affecting the general health of yelloweye rockfish and Arctic char, based on the common health indices employed in this study.

14 FUTURE WORK

The research presented in this dissertation provides a starting point for several studies which could provide insight into how different groups of fish are affected by Hg contamination. In a recent review focused on the toxicity and fate of Hg in fish, Kidd and Batchelar (2011) called for comprehensive studies aimed at understanding the internal handling of MeHg and Hg(II) in fish, with particular importance placed on *in vivo* demethylation. I think that a study which involves the dietary exposure of several species of fish to an isotopically labeled source of MeHg could help determine if hepatic *in vivo* demethylation occurs and if differences exist among species. If certain species of fish are determined to be “demethylators” and others “non-demethylators”, then further studies could be designed to determine if hepatic demethylation protects other downstream organs such as the brain from MeHg toxicity. Research presented in this dissertation, and work by others, suggest that MA may be sites of *in vivo* MeHg demethylation. Imaging studies, using X-ray absorption near edge spectroscopy (XANES) combined with X-ray absorption fine structure spectroscopy (EXAFS) could be carried out to identify the form of Hg within MA. When combined with the previously mentioned laboratory study, this would demonstrate not only that fish demethylate MeHg but which cells are responsible.

It would also be interesting to compare the Hg speciation in sub-cellular fractions isolated from the livers of different species of fish. The sub-cellular research presented in this dissertation would be strengthened by information showing if Hg(II) and MeHg accumulate in different sub-cellular compartments. Additionally, analytical techniques like HPLC-ICP-MS could be used to better identify the Hg ligands in specific fractions. For example, HPLC-ICP-MS could be used to help confirm that Hg is actually bound with metallothioneins in the HSP fraction, rather than associated with other thermostable ligands.

Future work should also focus on how exposure to MeHg affects the reproductive success and sustainability of populations of wild fish. Environmentally-relevant concentrations of MeHg disrupt the reproduction of fish in the laboratory. Concentrations of Hg in many populations of wild fish exceed the Hg toxicity thresholds

derived from laboratory studies, suggesting wild fish may be suffering from reproductive impairment.

15 REFERENCES

Adams S, Shugart L, Southworth G & Hinton D (1990) Application of bioindicators in assessing the health of fish populations experiencing contaminant stress. *IN: Biomarkers of Environmental Contamination. Lewis Publishers, Chelsea, Michigan. 1990. p 333-353, 6 fig, 2 tab, 52 ref. DOE Contract DE-AC 05-84 OR 21400.*

Agius C (1980) Phylogenetic development of melano–macrophage centres in fish. *J. Zool.* 191(1):11-31.

Agius C & Roberts R (2003) Melano macrophage centres and their role in fish pathology. *J. Fish Dis.* 26(9):499-509.

AMAP (2011) Mercury in the Arctic. in *Arctic Monitoring and Assessment Programme (AMAP), Oslo, Norway. 42*).

Arai T, Ikemoto T, Hokura A, Terada Y, Kunito T, Tanabe S & Nakai I (2004) Chemical forms of mercury and cadmium accumulated in marine mammals and seabirds as determined by XAFS analysis. *Environ. Sci. Technol.* 38(24):6468-6474.

Araújo O, Pereira P, Cesário R, Pacheco M & Raimundo J (2015) The sub-cellular fate of mercury in the liver of wild mullets (*Liza aurata*)–Contribution to the understanding of metal-induced cellular toxicity. *Mar. Pollut. Bull.*

Barkay T, Miller SM & Summers AO (2003) Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiol. Rev.* 27(2-3):355-384.

Barst BD, Gevertz AK, Chumchal MM, Smith JD, Rainwater T, Drevnick P, Hudelson KE, Hart A, Verbeck GF & Roberts AP (2011) Laser Ablation ICP-MS Co-localization of Mercury and Immune Response in Fish. *Environ. Sci. Technol.*

Batchelar KL, Kidd KA, Drevnick PE, Munkittrick KR, Burgess NM, Roberts AP & Smith JD (2013) Evidence of impaired health in yellow perch (*Perca flavescens*) from a biological mercury hotspot in northeastern North America. *Environ. Toxicol. Chem.* 32(3):627-637.

Beckvar N, Dillon TM & Read LB (2005) Approaches for linking whole-body fish tissue residues of mercury or DDT to biological effects thresholds. *Environ. Toxicol. Chem.* 24(8):2094-2105.

Berntssen M, Aatland A & Handy R (2003) Chronic dietary mercury exposure causes oxidative stress, brain lesions, and altered behaviour in Atlantic salmon (*Salmo salar*) parr. *Aquat. Toxicol.* 65(1):55-72.

Berntssen M, Hylland K, Julshamn K, Lundebye AK & Waagbø R (2004) Maximum limits of organic and inorganic mercury in fish feed. *Aquacult. Nutr.* 10(2):83-97.

Bloom N (1989) Determination of picogram levels of methylmercury by aqueous phase ethylation, followed by cryogenic gas chromatography with cold vapour atomic fluorescence detection. *Can. J. Fish. Aquat. Sci.* 46(7):1131-1140.

Bloom N (1992) On the chemical form of mercury in edible fish and marine invertebrate tissue. *Can. J. Fish. Aquat. Sci.* 49(5):1010-1017.

Bridges CC, Bauch C, Verrey F & Zalups RK (2004) Mercuric conjugates of cysteine are transported by the amino acid transporter system b0,+: implications of molecular mimicry. *J. Am. Soc. Nephrol.* 15(3):663-673.

Bushee DS (1988) Speciation of mercury using liquid chromatography with detection by inductively coupled plasma mass spectrometry. *Analyst* 113(8):1167-1170.

Cambier S, Benard G, Mesmer-Dudons N, Gonzalez P, Rossignol R, Brethes D & Bourdineaud J-P (2009) At environmental doses, dietary methylmercury inhibits mitochondrial energy metabolism in skeletal muscles of the zebra fish (*Danio rerio*). *Intern. J. Biochem. & Cell Biol.* 41(4):791-799.

Campbell PG & Hare L (2009) Metal detoxification in freshwater animals. Roles of metallothioneins. *Metallothioneins and related chelators*:239-277.

Carbonell G, Bravo J, Fernández C & Tarazona J (2009) A new method for total mercury and methyl mercury analysis in muscle of seawater fish. *Bull. Environ. Contam. Toxicol.* 83(2):210-213.

Chételat J, Amyot M, Cloutier L & Poulain A (2008) Metamorphosis in chironomids, more than mercury supply, controls methylmercury transfer to fish in High Arctic lakes. *Environ. Sci. Technol.* 42(24):9110-9115.

Chumchal M & Hambright K (2009) Ecological factors regulating mercury contamination of fish from Caddo Lake, Texas, USA. *Environ. Toxicol. Chem.* 28(5):962-972.

Chumchal MM, Rainwater TR, Osborn SC, Roberts AP, Abel MT, Cobb GP, Smith PN & Bailey FC (2011) Mercury speciation and biomagnification in the food web of Caddo Lake (TX/LA, USA), a subtropical freshwater ecosystem. *Environ. Toxicol. Chem.*

Crump K & Trudeau V (2009) Mercury induced reproductive impairment in fish. *Environ. Toxicol. Chem.* 28(5):895-907.

Cuvin-Aralar MLA & Furness RW (1991) Mercury and selenium interaction: a review. *Ecotoxicol. Environ. Saf.* 21(3):348-364.

Depew DC, Basu N, Burgess NM, Campbell LM, Devlin EW, Drevnick PE, Hammerschmidt CR, Murphy CA, Sandheinrich MB & Wiener JG (2012) Toxicity of dietary methylmercury to fish: Derivation of ecologically meaningful threshold concentrations. *Environ. Toxicol. Chem.* 31(7):1536-1547.

Di Giulio RT & Hinton DE (2008) *The toxicology of fishes.* CRC,

Dillon T, Beckvar N & Kern J (2010) Residue based mercury dose response in fish: An analysis using lethality equivalent test endpoints. *Environ. Toxicol. Chem.*

Drevnick P (2013) Investigation of mercury toxicity in landlocked char in high Arctic lakes. Synopsis of Research Conducted under the 2012–2013 Northern Contaminants Program. (Aboriginal Affairs and Northern Development Canada, Ottawa), p 305-315.

Drevnick P, Roberts A, Otter R, Hammerschmidt C, Klaper R & Oris J (2008) Mercury toxicity in livers of northern pike (*Esox lucius*) from Isle Royale, USA. *Comp. Biochem. Phys. Part C: Toxicol. & Pharm.* 147(3):331-338.

Drevnick P & Sandheinrich M (2003) Effects of dietary methylmercury on reproductive endocrinology of fathead minnows. *Environ. Sci. Technol* 37(19):4390-4396.

Drevnick P, Sandheinrich M & Oris J (2006) Increased ovarian follicular apoptosis in fathead minnows (*Pimephales promelas*) exposed to dietary methylmercury. *Aquat. Toxicol.* 79(1):49-54.

Dutczak W & Ballatori N (1994) Transport of the glutathione-methylmercury complex across liver canalicular membranes on reduced glutathione carriers. *J. Biol. Chem.* 269(13):9746.

Dutczak WJ, Clarkson TW & Ballatori N (1991) *Biliary-hepatic recycling of a xenobiotic: gallbladder absorption of methyl mercury.* G873-G880 p. http://ajpgi.physiology.org/ajpgi/260/6/G873.full.pdf

Eagles-Smith C, Ackerman J, Yee J & Adelsbach T (2009) Mercury demethylation in waterbird livers: Dose-response thresholds and differences among species. *Environ. Toxicol. Chem.* 28(3):568-577.

El-Begearmi M, Sunde M & Ganther H (1977) A mutual protective effect of mercury and selenium in Japanese quail. *Poult. Sci.* 56(1):313-322.

Fitzgerald WF, Engstrom DR, Mason RP & Nater EA (1998) The case for atmospheric mercury contamination in remote areas. *Environ. Sci. Technol.* 32(1):1-7.

Fitzgerald WF & Lamborg CH (2007) Geochemistry of Mercury in the Environment. *Treatise on Geochemistry*, Editors in C, Xa, Heinrich Dh & Karl Kt (Édit.) Pergamon, Oxford. p 1-47.

Fitzgerald WF, Lamborg CH & Hammerschmidt CR (2007) Marine biogeochemical cycling of mercury. *Chem. Reviews* 107(2):641-662.

Frandsen F, Malmquist HJ & Snorrason SS (1989) Ecological parasitology of polymorphic Arctic charr, *Salvelinus alpinus* (L.), in Thingvallavatn, Iceland. *J. Fish Biol.* 34(2):281-297.

Friedmann A, Watzin M, Brinck-Johnsen T & Leiter J (1996) Low levels of dietary methylmercury inhibit growth and gonadal development in juvenile walleye (*Stizostedion vitreum*). *Aquat. Toxicol.* 35(3-4):265-278.

Gailer J, George GN, Pickering IJ, Madden S, Prince RC, Yu EY, Denton MB, Younis HS & Aposhian HV (2000) Structural basis of the antagonism between inorganic mercury and selenium in mammals. *Chem. Res. Toxicol.* 13(11):1135-1142.

Gantner N, Muir DC, Power M, Iqaluk D, Reist JD, Babaluk JA, Meili M, Borg H, Hammar J & Michaud W (2010) Mercury concentrations in landlocked Arctic char (*Salvelinus alpinus*) from the Canadian Arctic. Part II: Influence of lake biotic and abiotic characteristics on geographic trends in 27 populations. *Environ. Toxicol. Chem.* 29(3):633-643.

Giguère A, Campbell PG, Hare L & Couture P (2006) Sub-cellular partitioning of cadmium, copper, nickel and zinc in indigenous yellow perch (*Perca flavescens*) sampled along a polymetallic gradient. *Aquat. Toxicol.* 77(2):178-189.

Gill GA & Fitzgerald WF (1987) Picomolar mercury measurements in seawater and other materials using stannous chloride reduction and two-stage gold amalgamation with gas phase detection. *Mar. Chem.* 20(3):227-243.

Gutknecht J (1981) Inorganic mercury (Hg2+) transport through lipid bilayer membranes. *J. Membr. Biol.* 61(1):61-66.

Hall B, Bodaly R, Fudge R, Rudd J & Rosenberg D (1997) Food as the dominant pathway of methylmercury uptake by fish. *Water, Air, Soil Pollut.* 100(1-2):13-24.

Hammerschmidt CR & Fitzgerald WF (2006) Bioaccumulation and trophic transfer of methylmercury in Long Island Sound. *Arch. Environ. Contam. Toxicol.* 51(3):416-424.

Harada M (1995) Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. *Crit. Rev. Toxicol.* 25(1):1-24.

Harris HH, Pickering IJ & George GN (2003) The chemical form of mercury in fish. *Science* 301(5637):1203.

Havarinasab S, Björn E, Nielsen JB & Hultman P (2007) Mercury species in lymphoid and non-lymphoid tissues after exposure to methyl mercury: correlation with autoimmune parameters during and after treatment in susceptible mice. *Toxicol. Appl. Pharmacol.* 221(1):21-28.

Haynes S, Gragg RD, Johnson E, Robinson L & Orazio CE (2006) An evaluation of a reagentless method for the determination of total mercury in aquatic life. *Water, Air, Soil Pollut.* 172(1):359-374.

Jaffe D & Strode S (2008) Sources, fate and transport of atmospheric mercury from Asia. *Environ. Chem* 5(2):121-126.

Jorgensen SJ, Kaplan DM, Klimley A, Morgan SG, O'Farrell MR & Botsford LW (2006) Limited movement in blue rockfish *Sebastes mystinus*: internal structure of home range. *MARINE ECOLOGY-PROGRESS SERIES*- 327:157.

Khan MA & Wang F (2010) Chemical demethylation of methylmercury by selenoamino acids. *Chem. Res. Toxicol.* 23(7):1202-1206.

Kidd K & Batchelar K (2011) 5 - Mercury. *Fish Physiology*, Chris M. Wood Apf & Colin Jb (Édit.) Academic Press, Vol Volume 31, Part B. p 237-295.

Klaper R, Rees C, Drevnick P, Weber D, Sandheinrich M & Carvan M (2006) Gene expression changes related to endocrine function and decline in reproduction in fathead minnow (*Pimephales promelas*) after dietary methylmercury exposure. *Environ. Health Perspect.* 114(9):1337.

Korbas M, O'Donoghue JL, Watson GE, Pickering IJ, Singh SP, Myers GJ, Clarkson TW & George GN (2010) The chemical nature of mercury in human brain

following poisoning or environmental exposure. *ACS chemical neuroscience* 1(12):810-818.

Larose C, Canuel R, Lucotte M & Di Giulio RT (2008) Toxicological effects of methylmercury on walleye (*Sander vitreus*) and perch (*Perca flavescens*) from lakes of the boreal forest. *Comp. Biochem. Phys Part C: Toxicol. Pharm.* 147(2):139-149.

Leaner JJ & Mason RP (2002) Methylmercury accumulation and fluxes across the intestine of channel catfish, *Ictalurus punctatus*. *Comp. Biochem. Phys Part C: Toxicol. Pharm.* 132(2):247-259.

Leiva-Presa À, Capdevila M, Cols N, Atrian S & González-Duarte P (2004) Chemical foundation of the attenuation of methylmercury (II) cytotoxicity by metallothioneins. *Eur. J. Biochem.* 271(7):1323-1328.

Lemes M & Wang F (2009) Methylmercury speciation in fish muscle by HPLC-ICP-MS following enzymatic hydrolysis. *J. of Analyti. Atom. Spectr.* 24(5):663-668.

Lockhart W, Uthe J, Kenney A & Mehrle P (1972) Methylmercury in northern pike (*Esox lucius*): Distribution, elimination, and some biochemical characteristics of contaminated fish. *J. Fish. Res. Board Can.* 29(11):1519-1523.

MacLellan S & Station PB (1997) *How to Age Rockfish (Sebastes) Using S. Alutus as an Example, the Otolith Burnt Section Technique.* Nanaimo, BC: Fisheries and Oceans Canada,

Maggi C, Berducci M, Bianchi J, Giani M & Campanella L (2009) Methylmercury determination in marine sediment and organisms by Direct Mercury Analyser. *Anal. Chim. Acta* 641(1-2):32-36.

Mason A & Jenkins K (1995) Metal detoxification in aquatic organisms. *Metal speciation and bioavailability in aquatic systems* 3:479-608.

Matthews KR (1990) A telemetric study of the home ranges and homing routes of copper and quillback rockfishes on shallow rocky reefs. *Can. J. Zool.* 68(11):2243-2250.

McLaughlin J & Gessner BD (2007) Fish Consumption Advice for Alaskans: A Risk Management Strategy to Optimize the Public's Health.

Meinelt T, Krüger R, Pietrock M, Osten R & Steinberg C (1997) Mercury pollution and macrophage centres in pike (*Esox lucius*) tissues. *Environ. Sci. Poll. Res.* 4(1):32-36.

Mela M, Randi M, Ventura D, Carvalho C, Pelletier E & Oliveira Ribeiro C (2007) Effects of dietary methylmercury on liver and kidney histology in the neotropical fish *Hoplias malabaricus*. *Ecotoxicol. Environ. Saf.* 68(3):426-435.

Mergler D, Anderson HA, Chan LHM, Mahaffey KR, Murray M, Sakamoto M & Stern AH (2007) Methylmercury exposure and health effects in humans: a worldwide concern. *AMBIO: J. Hum. Environ.* 36(1):3-11.

Monteiro DA, Rantin FT & Kalinin AL (2010) Inorganic mercury exposure: toxicological effects, oxidative stress biomarkers and bioaccumulation in the tropical freshwater fish matrinxã, *Brycon amazonicus* (Spix and Agassiz, 1829). *Ecotox.* 19(1):105-123.

Moyle PB & Cech JJ (2004) *Fishes: an introduction to ichthyology.* Pearson Prentice Hall Upper Saddle River, NJ,

Muir D, Wang X, Yang F, Nguyen N, Jackson T, Evans M, Douglas M, Kock G, Lamoureux S & Pienitz R (2009) Spatial trends and historical deposition of mercury in eastern and northern Canada inferred from lake sediment cores. *Environ. Sci. Technol.* 43(13):4802-4809.

Müller A-K, Brinkmann M, Baumann L, Stoffel MH, Segner H, Kidd KA & Hollert H (2015) Morphological alterations in the liver of yellow perch (*Perca flavescens*) from a biological mercury hotspot. *Environ. Sci. Poll. Res.*:1-13.

Nam DH & Basu N (2011) Rapid methods to detect organic mercury and total selenium in biological samples. *Chem. Cent. J.* 5:3.

Onsanit S & Wang W-X (2011) Sequestration of total and methyl mercury in different subcellular pools in marine caged fish. *J. Hazard. Mater.* 198:113-122.

Pacyna EG, Pacyna JM, Steenhuisen F & Wilson S (2006) Global anthropogenic mercury emission inventory for 2000. *Atmos. Environ.* 40(22):4048-4063.

Palmisano F, Cardellicchio N & Zambonin P (1995) Speciation of mercury in dolphin liver: a two-stage mechanism for the demethylation accumulation process and role of selenium. *Mar. Environ. Res.* 40(2):109-121.

Pařízek J & Ošťádalová I (1967) The protective effect of small amounts of selenite in sublimate intoxication. *Experientia* 23(2):142-143.

Power M, Reist JD & Dempson JB (2008) Fish in high-latitude Arctic lakes. *Polar lakes and rivers*:249-267.

Prince L, Korbas M, Davidson P, Broberg K & Rand MD (2014) Target Organ Specific Activity of Drosophila MRP (ABCC1) Moderates Developmental Toxicity of Methylmercury. *Toxicol. Sci.*:kfu095.

Pulsford A, Ryan K & Nott J (1992) Metals and melanomacrophages in flounder, *Platichthys flesus*, spleen and kidney. *J. Mar. Biol. Assoc. U.K.* 72(02):483-498.

Raldúa D, Bayona J & Barceló D (2007) Mercury levels and liver pathology in feral fish living in the vicinity of a mercury cell chlor-alkali factory. *Chemosph.* 66(7):1217-1225.

Ralston NV & Raymond LJ (2010) Dietary selenium's protective effects against methylmercury toxicity. *Toxicol.* 278(1):112-123.

Roberts MS, Magnusson BM, Burczynski FJ & Weiss M (2002) Enterohepatic circulation. *Clin. Pharmaco.* 41(10):751-790.

Rosabal M, Hare L & Campbell PG (2012) Subcellular metal partitioning in larvae of the insect Chaoborus collected along an environmental metal exposure gradient (Cd, Cu, Ni and Zn). *Aquat. Toxicol.* 120:67-78.

Rosabal M, Pierron F, Couture P, Baudrimont M, Hare L & Campbell PG (2015) Subcellular partitioning of non-essential trace metals (Ag, As, Cd, Ni, Pb, Tl) in livers of American (*Anguilla rostrata*) and European (*Anguilla anguilla*) yellow eels. *Aquat. Toxicol.*

Sandheinrich M & Wiener J (2011) Methylmercury in freshwater fish: recent advances in assessing toxicity of environmentally relevant exposures. *Environmental Contaminants in Biota: Interpreting Tissue Concentrations* 2:169-190.

Sandheinrich MB & Miller KM (2006) Effects of dietary methylmercury on reproductive behavior of fathead minnows (*Pimephales promelas*). *Environ. Toxicol. Chem.* 25(11):3053-3057.

Scerbo R & Barghigiani C (1998) Organic mercury determination in fish samples using an automatic mercury analyser. *Environ. Technol.* 19(3):339-342.

Scheuhammer A, Braune B, Chan HM, Frouin H, Krey A, Letcher R, Loseto L, Noël M, Ostertag S & Ross P (2014) Recent progress on our understanding of the biological effects of mercury in fish and wildlife in the Canadian Arctic. *Sci. Total Environ.*

Scheuhammer AM, Basu N, Evers DC, Heinz GH, Sandheinrich MB & Bank MS (2012) Ecotoxicology of mercury in fish and wildlife: recent advances.

Scheuhammer AM, Meyer MW, Sandheinrich MB & Murray MW (2007) Effects of environmental methylmercury on the health of wild birds, mammals, and fish. *AMBIO: J. Hum. Environ.* 36(1):12-19.

Schwindt AR, Fournie JW, Landers DH, Schreck CB & Kent ML (2008) Mercury concentrations in salmonids from western US national parks and relationships with age and macrophage aggregates. *Environ. Sci. Technol.* 42(4):1365-1370.

Selin NE (2009) Global biogeochemical cycling of mercury: a review. *Ann. Rev. Environ. and Resour.* 34:43-63.

Shade CW (2008) Automated simultaneous analysis of monomethyl and mercuric Hg in biotic samples by Hg-thiourea complex liquid chromatography following acidic thiourea leaching. *Environ. Sci. Technol.* 42(17):6604-6610.

Sigel A, Sigel H & Sigel RK (2009) *Metallothioneins and related chelators.* Royal Society of Chemistry,

Simmons-Willis T, Koh A, Clarkson T & Ballatori N (2002) Transport of a neurotoxicant by molecular mimicry: the methylmercury–L-cysteine complex is a substrate for human L-type large neutral amino acid transporter (LAT) 1 and LAT2. *Biochem. J* 367:239-246.

Smith JD (2012) *A Comparison of Mercury Localization, Speciation, and Histology in Multiple Fish Species from Caddo Lake, a Fresh Water Wetland*. (University of North Texas).

Suda I, Totoki S, Uchida T & Takahashi H (1992) Degradation of methyl and ethyl mercury into inorganic mercury by various phagocytic cells. *Arch. Toxicol.* 66(1):40-44.

Terman A & Brunk UT (2004) Lipofuscin. *Intern. J. Biochem. Cell Biol.* 36(8):1400-1404.

Tseng CM, Hammerschmidt CR & Fitzgerald WF (2004) Determination of methylmercury in environmental matrixes by on-line flow injection and atomic fluorescence spectrometry. *Analy. Chem.* 76(23):7131-7136.

UNEP (2013) Global Mercury Assessment: Sources, Emissions, Releases and Environmental Transport. Geneva, Switzerland).

Wallace WG, Lee B-G & Luoma SN (2003) Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar. Ecol. Prog. Ser.* 249:183-197.

Wang F, Lemes M & Khan M (2012) Metallomics of mercury: Role of thiol-and selenol-containing biomolecules. *Environmental Chemistry and Toxicology of Mercury*:517.

Wang W-X & Wong RSK (2003) Bioaccumulation kinetics and exposure pathways of inorganic mercury and methylmercury in a marine fish, the sweetlips *Plectorhinchus gibbosus*. *Mar. Ecol. Prog. Ser.* 261:257-268.

Webb MAH, Feist G, Fitzpatrick M, Foster E, Schreck C, Plumlee M, Wong C & Gundersen D (2006) Mercury concentrations in gonad, liver, and muscle of white sturgeon *Acipenser transmontanus* in the lower Columbia River. *Arch. Environ. Contam. Toxicol.* 50(3):443-451.

Wiener J & Spry D (1996) Toxicological significance of mercury in freshwater fish.

Wiener JG, Krabbenhoft DP, Heinz GH & Scheuhammer AM (2003) Ecotoxicology of mercury. *Handbook of ecotoxicology* 2:409-443.

Xie L, Flippin JL, Deighton N, Funk DH, Dickey DA & Buchwalter DB (2008) Mercury (II) bioaccumulation and antioxidant physiology in four aquatic insects. *Environ. Sci. Technol.* 43(3):934-940.

Yamanaka KL, Lacko L, Withler R, Grandin C, Lochead J, Martin J, Olsen N & Wallace S (2006) *A review of yelloweye rockfish Sebastes ruberrimus along the Pacific coast of Canada: biology, distribution and abundance trends.* Fisheries and Oceans Canada, Science,

Yang H, Engstrom DR & Rose NL (2010) Recent changes in atmospheric mercury deposition recorded in the sediments of remote equatorial lakes in the Rwenzori Mountains, Uganda. *Environ. Sci. Technol.*

Zalups RK, Barfuss DW & Lash LH (1999) Relationships between alterations in glutathione metabolism and the disposition of inorganic mercury in rats: effects of biliary ligation and chemically induced modulation of glutathione status. *Chem.-Biol. Interact.* 123(3):171-195.

DEUXIÈME PARTIE: ARTICLES

16 ARTICLE 1

Determination of Hg speciation in fish tissue with a direct mercury analyzer

Benjamin D. Barst[*†], Chad R. Hammerschmidt[‡], Matthew M. Chumchal[§], Derek C. G. Muir[‖], James D. Smith[#], Aaron P. Roberts[#], Thomas R. Rainwater[††**], and Paul E. Drevnick[†]

[†]INRS-ETE, Québec, QC, Canada

[‡]Department of Earth and Environmental Sciences, Wright State University, Dayton, OH, USA

[§]Department of Biology, Texas Christian University, Fort Worth, TX, USA

[‖]Aquatic Ecosystem Protection Research Division, Environment Canada, Burlington, ON, CAN

[#]Department of Biological Sciences & Institute of Applied Science, University of North Texas, Denton, TX, USA

[††]Department of Obstetrics and Gynecology, Medical University of South Carolina, Charleston, SC, USA

**Current address: U.S. Fish and Wildlife Service, Ecological Services Field Office, Charleston, SC, 29407, USA

Article published in the journal *Environmental Toxicology and Chemistry* (DOI: 10.1002/etc.2184)

Abstract- Knowledge of mercury speciation in tissue is valuable for assessing potential toxicological effects in fish. Direct mercury analyzers, which use thermal decomposition and atomic absorption spectrometry, have recently gained popularity for determining organic mercury after procedural solvent extraction from some environmental media, although quantitative recovery from lipid-rich materials, such as fish liver, has been problematic. We developed a new method by which organic mercury in fish liver and muscle is estimated by difference from direct measurements of inorganic mercury in an acid extract and total mercury in whole tissue. The method was validated by analysis of a certified reference material (DOLT-4 dogfish liver) and naturally contaminated fish tissues with comparison to an established mercury speciation method (gas chromatography cold vapor atomic fluorescence spectrometry). Recovery of organic mercury from DOLT-4, estimated by difference, averaged 99± 5% of the mean certified value for methylmercury. In the majority of liver samples and all muscle samples, estimates of organic mercury from the proposed method were indiscernible from direct speciation measurements of methylmercury (99± 6%). Estimation of organic mercury by difference between total mercury and inorganic mercury was less accurate in liver samples with high percent inorganic mercury (90%). This was due to the increased uncertainty that results from estimating a third value (i.e., organic mercury) by difference between two large concentrations (inorganic mercury and total mercury). The proposed method is a useful tool for examining the speciation of mercury in fish muscle and liver, and by extension, potentially other tissues and environmental media.

Keywords: Mercury speciation, Direct Mercury Analyzer, Fish tissue

INTRODUCTION

Mercury (Hg) is an environmental contaminant that poses health risks to humans and wildlife [1-2]. In aquatic ecosystems, the conversion of inorganic Hg (Hg(II)) to methylmercury (MeHg) and the subsequent biomagnification of MeHg in food webs results in high concentrations in fish [3]. Humans are primarily exposed to MeHg through the consumption of fish and shellfish, accordingly, risk assessments and monitoring programs are based upon the concentration of MeHg in fish muscle or whole body [4]. MeHg contamination of aquatic environments is extensive and negatively affects fish health; a recent study, for example, estimated that more than 40% of walleye (*Sander vitreus*) populations in the Great Lakes region of North America are at risk of MeHg toxicity [5]. MeHg has been shown to negatively impact survival, growth, behavior, and reproduction of fish [6-10]. Additional effects have been observed in the liver of fish, where Hg causes damage through oxidative stress [11]. Ingested MeHg passes through the intestinal wall to blood, in which it is transported by the portal vein to the liver. The liver therefore has a “first pass” at either accumulating or detoxifying MeHg before it is circulated to other tissues. Primary detoxification of MeHg involves complexation with glutathione before excretion into bile and transfer to the intestine [12-13]. Hepatic demethylation of MeHg, which has been shown to occur in marine mammals, may also be carried out in fish [14-15]. Although Hg(II) is less likely than MeHg to bioaccumulate and biomagnify, several studies have observed greater occurrence of liver damage with increasing Hg(II) in fish [11, 16-17]. Thus, knowledge of Hg speciation is valuable for assessing potential toxicological effects in fish.

There are multiple analytical techniques for Hg speciation analysis in tissues, including, for example, high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS; [18]), Hg-thiourea complex liquid chromatography cold vapor atomic fluorescence spectrometry (LC-CVAFS; [19]), and gas chromatography cold vapor atomic fluorescence spectrometry (GC-CVAFS; [20]). Recently, direct mercury analyzers, such as the Milestone DMA-80, have gained popularity for analysis of total Hg due to their 1) ability to analyze both liquid and solid matrices effectively, 2) high sample throughput, and 3) relatively low detection limits and cost [21-22]. DMA-80 analysis involves thermal decomposition followed by gold amalgamation and detection with atomic absorption spectrometry. Several studies also have used direct mercury analysis to quantify MeHg (or organic Hg) in various sample matrices [23-26]. Often, this is done by chemically digesting the material, procedurally isolating MeHg from forms of Hg(II) by extraction with an organic solvent, back extracting MeHg into aqueous solution, and measuring total Hg in the extract with a direct analyzer [23-26]. Measured total Hg in the final extract is interpreted as either MeHg or organic Hg.

Prior studies have had varying degrees of success with quantitative extraction of organic Hg from various matrices for direct mercury analysis. Nam and Basu [25] digested biological reference materials with a tris-buffered protease solution that was then treated with NaOH, cysteine, $CuSO_4$, and acidic NaBr, followed by organic Hg extraction with toluene and back extraction into an aqueous solution for analysis with a DMA-80. Recoveries of organic Hg from the reference materials were excellent and ranged between 86% and 107% relative to certified values for MeHg [25]. Maggi and colleagues [24] used a different digestion method with HBr, followed by toluene

extraction, back extraction with an L-cysteine solution, and analysis by DMA-80 to analyze various certified reference materials as well as environmental samples. Using this approach, they recovered greater than 80% of MeHg from multiple reference materials, but recovery averaged only 74% from dogfish liver reference material (DOLT-3). Similarly, Scerbo and colleagues [23] noted an increased degree of variation in the recovery of organic Hg from liver reference material DOLT-1, which they attributed to the relatively high lipid content of fish liver that affected quantitative back extraction with L-cysteine [23].

Here, we propose and validate a new method for the quantification of Hg(II) in fish muscle and liver with a direct mercury analyzer. Tissue is digested in acid with microwave heating and organic Hg is extracted with toluene. The novel aspect of this approach is that the remaining acidic fraction, containing Hg(II), is analyzed instead of organic Hg in the nonpolar fraction. This approach, in contrast to prior methodologies, does not involve an additional step of analyte back extraction into an aqueous phase, and it thereby minimizes both random errors and the potential for procedural biases associated with lipid-rich matrices from which back extraction may not be quantitative, as observed by others [23,24]. Measured Hg(II) concentrations are subtracted from those of total Hg, determined with the same instrument, to estimate MeHg by difference. Also in contrast to prior studies, this method was validated with naturally contaminated fish muscle and liver tissue that were analyzed with the proposed technique and an established speciation method (GC-CVAFS), in addition to analysis of a certified reference material.

MATERIALS AND METHODS

Reagents and standards

Reagents used for digestion and extraction included high-purity HCl (J.T. Baker, A.C.S. grade) and toluene (Fisher, A.C.S. grade). Calibration standards and known additions of Hg were made with a solution traceable to the U.S. National Institute of Standards and Technology (NIST). All dilutions were made with reagent-grade water (resistivity ≥ 18 MΩ-cm).

Sampling and sample preparation

Arctic char (*Salvelinus alpinus)* livers were collected from five lakes on Cornwallis Island (Nunavut, Canada) in July 2009 (Amituk Lake) and 2010 (Small, 9 Mile, North, and Char lakes). Spotted gar (*Lepisosteus oculatus*), largemouth bass (*Micropterus salmoides)*, bowfin (*Amia calva*), and channel catfish (*Ictalurus punctatus*) were collected from Caddo Lake (TX/LA, USA) in June and July of 2007 and 2011. Muscle and liver samples were dried and homogenized prior to Hg analysis. Extractions and analyses of certified reference material DOLT-4 (dogfish liver), which has a lipid content of 6.3% by mass, also were used to validate the proposed method.

Mercury analyses

DMA-80 total Hg analyses. All tissue samples were analyzed for total Hg at INRS-ETE with a DMA-80 (Milestone Inc.). Our quality assurance protocol included calibration with NIST-traceable standards, analyses of certified reference materials from the National Research Council of Canada (MESS-3 marine sediment, TORT-2 lobster

hepatopancreas, and DOLT-4 dogfish liver), and duplicate samples. All analyses of total Hg in reference materials were within certified ranges and the relative difference between duplicate samples averaged 1.2% (*n* = 15).

DMA-80 Hg(II) analyses of fish muscle and liver. Our sample digestion method for Hg speciation analysis was modified from that of Ashoka and colleagues [27]. Briefly, between 50 and 100 mg of dried sample was weighed accurately into a 50-mL centrifuge tube to which was added 1 mL of 12 M HCl. Samples in capped tubes were irradiated in a conventional 1 100 W microwave oven at 30% power for 30 sec and then allowed to cool for 5 min and irradiated again at 30% power for 30 sec. After cooling, 5 mL of toluene were added to each sample, the tubes capped and shaken with a wrist-action shaker for 20 min, and then centrifuged at 2 400 rpm for 15 min. Supernatant toluene was transferred to a separate 50-mL tube. The acidic fraction remaining after toluene extraction of the original digestate was diluted 5-fold with reagent-grade water and an aliquot was added to a quartz analytical vessel for quantification of ionic Hg with a DMA-80. Continual analysis of samples containing the maximum allowable HCl concentration (10%) can increase corrosion of the instrument. We chose to reduce the HCl concentration of our samples, by dilution with reagent-grade water, to approximately 7% to reduce the risk of corrosion to instrument components. Quality assurance analyses included reagent blanks, known additions to reagent blanks (10, 20, 100, and 1 000 ng), and duplicate samples. Recovery of known additions (*n* = 10) averaged 92% relative to calibration standards, reagent blanks had no detectable amount of Hg, and the relative difference between duplicate samples averaged 2.1% (*n*

=10). The method detection limit (MDL) was 0.41 ng Hg and was estimated by analyzing seven replicates of known Hg additions to reagent solutions (20 ng Hg) and multiplying the standard deviation among replicates by 3.14, the *t*-value for a 99% confidence interval.

MeHg and total Hg analyses of fish muscle and liver by CVAFS. Subsamples of Arctic char liver (*n* = 20), spotted gar liver (*n* = 4), bowfin liver (n=3), catfish liver (*n* = 3), largemouth bass liver (*n* = 3) and muscle (*n* = 3) were digested with 7 mL of 4.57 N HNO_3 for 12 h in a 60° C water bath [28]. Digestates we re analyzed for MeHg after derivatization with sodium tetraethylborate by flow-injection GC-CVAFS [20, 29]. Analyses were calibrated with MeHg standards and blanks taken through the digestion procedure. MeHg standards were calibrated against Hg^0 [30] and a NIST-traceable Hg(II) solution. Relative difference between duplicate digestates of the same sample (*n* = 13) averaged 3.2%. All analyses of two standard reference materials (TORT-2 and DORM-3 fish protein; *n* = 9 each) were within the certified ranges.

Total Hg was also measured in digestates of all fish tissues by dual-Au amalgamation CVAFS [31] after HNO_3 digestion and BrCl oxidation [28]. Analyses were calibrated with an Hg(II) solution traceable to U.S. NIST. All analyses of total Hg in TORT-2 and DORM-3 (n=9 each) were within certified ranges, and the relative difference between duplicate digestates of the same sample (*n* = 18) averaged 2.5%.

RESULTS AND DISCUSSION

Method validation with certified reference material

DOLT-4 has lipid (6.3% by mass), MeHg (1 330 ng/g), and total Hg (2 580 ng/g) contents that are similar to those in livers of many other fish species and should therefore pose a representative analytical challenge. Measured concentrations of Hg(II) in the acidic fraction of DOLT-4 digestates (1 243 ± 66 ng/g) were not significantly different from those estimated by difference between mean certified values for total Hg (2 580 ± 220 ng/g) and MeHg (1 330 ± 120 ng/g) (paired *t*-test, $p = 0.69$). Recovery of Hg(II) from DOLT-4 averaged 99 ± 5% of the difference between mean certified values ($n = 8$). Organic Hg can be estimated as the difference between measured concentrations of total Hg and Hg(II). With the proposed method, the mean estimated recovery of organic Hg from DOLT-4, by difference, was 100 ± 5% of the certified mean value for MeHg. This is a substantial improvement over our own attempts (70 ± 2%), and those of others [23-24], to determine organic Hg directly in organic solvent extracts of liver.

Hg in naturally contaminated fish tissues

Organic Hg in char, catfish, bowfin, and largemouth bass livers and muscle was estimated by difference between measured concentrations of total Hg and Hg(II) and compared to direct speciation analysis of MeHg by GC-CVAFS. In these samples, concentrations of total Hg ranged from 460 to 40 700 ng/g dry weight and Hg(II) from 49 to 36 700 ng/g. Hg(II) comprised a relatively minor fraction of total Hg in Arctic char

livers (mean = 14%), largemouth bass livers (mean = 42%) and largemouth bass muscle (mean = 10%). In contrast, Hg(II) was the major Hg species in livers of bowfin (mean = 81%), channel catfish (mean = 83%), and spotted gar (mean = 96%). Variation in mercury speciation (inorganic and organic) has been observed in fish livers both across and within species [32-35], and the reason for this variation is the subject of current research. Estimates of MeHg, calculated by difference between measured concentrations of total Hg and Hg(II) with the proposed method, were in excellent agreement with direct speciation measurements of MeHg by established GC-CVAFS techniques (mean recovery = 99% ± 6%; Fig. 1), for all liver and muscle samples, except spotted gar liver.

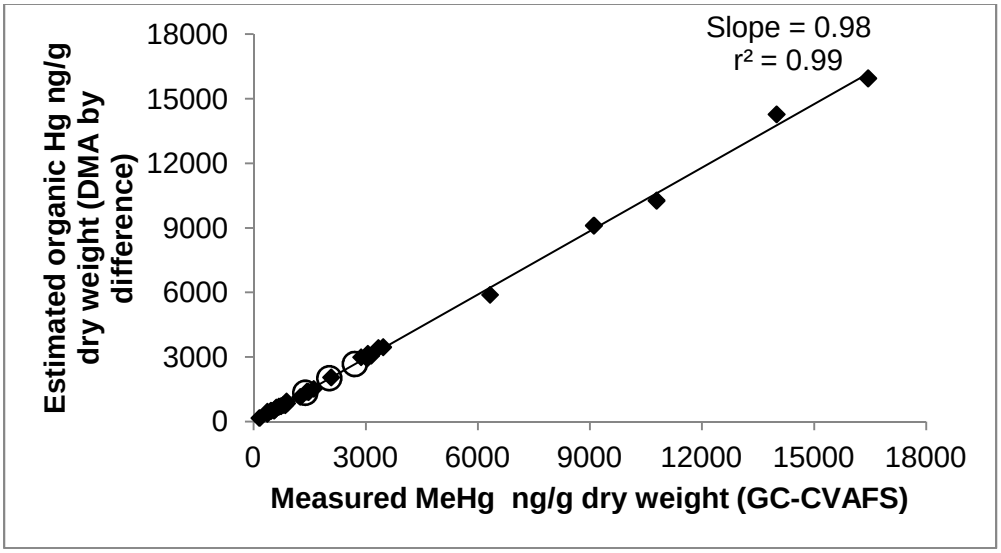

Figure 1. Comparison of methylmercury measured by gas chromatographic cold-vapor atomic fluorescence spectrometry (GC-CVAFS) and estimated organic mercury (by DMA-80 with proposed method) in fish liver (diamonds) and muscle tissue (circles).

Hg in spotted gar livers

In contrast to the other fish livers analyzed, spotted gar had much greater concentrations of total Hg (mean = 28 200 ± 12 100 ng/g dw), of which only a small fraction was as organic mercury (mean = 4.1%; Table 1).

Table 1. Comparison of mercury speciation determined with the proposed DMA-80 method and gas chromatographic cold-vapor atomic fluorescence spectrometry (GC-CVAFS) technique in livers of four spotted gar from Caddo Lake (TX/LA).

	DMA-80			GC-CVAFS	
Fish	Total Hg (ng/g dw)	Hg(II) (ng/g dw)	Estimated MeHg[a] (ng/g dw)	Measured MeHg (ng/g dw)	MeHg Percent Recovery DMA vs. GC-CVAFS
Spotted gar 1	16229	15287	942	581	162.1
Spotted gar 2	19661	18259	1402	1226	114.4
Spotted gar 3	36374	33851	2524	1310	192.6
Spotted gar 4	40716	36735	3982	1510	263.7

[a]Methylmercury estimated by subtracting inorganic mercury from total mercury

In spotted gar livers, concentrations of total Hg measured by DMA-80 and CVAFS differed by less than 3%, which, as noted, was comparable to the procedural variability between duplicate samples for both methods. However, concentrations of organic Hg in gar livers, estimated by difference between measured Hg(II) and total Hg, averaged 183% greater than those determined by GC-CVAFS. These differences in estimated organic mercury versus measured MeHg are likely due to the increased uncertainty that results from estimating a third value (i.e., organic Hg) by difference between two large values (Hg(II) and total Hg). The proposed method was developed to measure Hg(II), and despite the difficulties in estimating organic Hg in gar livers, the method accurately

quantifies inorganic Hg. Interestingly, calculated Hg(II) (calculated by subtracting MeHg from total Hg measured by GC-CVAFS) and measured Hg(II) were within 6% of one another, suggesting that Hg(II) was accurately measured in these samples.

In order to avoid analysis of a complete fish liver or muscle, samples are routinely homogenized and subsampled for Hg analysis. Analysis of homogenized subsamples provides a way to estimate the true Hg concentration of a complete tissue. We analyzed duplicate samples of fish liver and muscle tissue for both total Hg and Hg(II). The percent difference between duplicate samples was 1.2% for total Hg and 2.1% for Hg(II) analyses. These small percent differences between duplicate samples demonstrate that our tissues were well homogenized and all subsamples taken were representative of the complete tissue. Some variation in Hg concentration is expected, but prevents the accurate estimation of MeHg in high percent Hg(II) samples (>90%) like spotted gar. By using the percent differences between duplicate samples as an estimate for error we were able to model percent recovery of MeHg versus percent Hg(II) for a group of hypothetical samples and determine the point at which our method no longer provides accurate estimates of MeHg (Fig. 2). These hypothetical samples were assigned differing total Hg concentrations (500, 5 000, or 50 000 ng/g) and percent Hg(II) concentrations (between 0 and 100%) before their values were adjusted using our calculated percent differences. Both MeHg and adjusted MeHg values were calculated (by difference between total Hg and Hg(II))and their percent recoveries are presented in Figure 2. Based on the hypothetical data and the data from our study, poor estimation of MeHg appears to be independent of total Hg concentration.

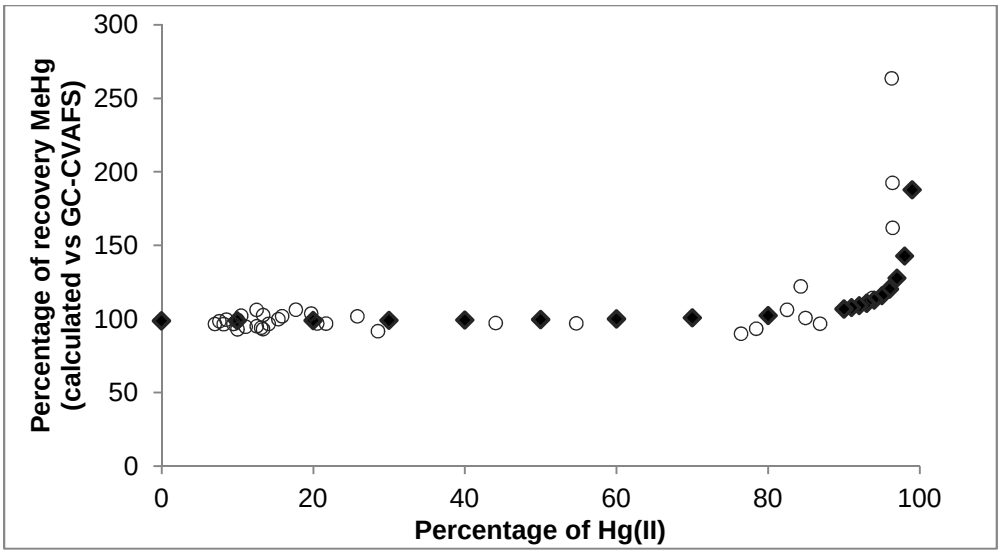

Figure 2. Comparison of percent recovery of methylmercury (Calculated versus measured by GC-CVAFS) and percent inorganic mercury. Diamonds represent hypothetical samples and circles represent samples from this study.

Thus, the proposed method is an excellent approach to directly quantify Hg(II) in fish liver and muscle and, from that, to estimate organic mercury in samples with less than 90% Hg(II). The robustness of this method is supported by the accurate estimation of organic Hg across a range of organic and total Hg concentrations and widely varying percent organic Hg values. Greater uncertainty of organic Hg estimates is likely to occur when Hg(II) is the major fraction of a large total Hg concentration, as in the case of spotted gar liver. A recent study by Chumchal and colleagues [35] also reported elevated total Hg concentrations and high percent Hg(II) in spotted gar livers from Caddo Lake. Aside from these two studies, there are no reported cases of such elevated total Hg concentrations coupled with high percent Hg(II) in fish liver. Additionally, other studies, as well as this one, have noted that MeHg is the predominant form of mercury in fish muscle tissue [11, 36]. Thus, the total Hg concentrations and high relative Hg(II) concentrations found in spotted gar livers are not

common to most fish, meaning the proposed method is appropriate for estimating organic Hg in the majority of fish liver and muscle samples. The lack of certainty in estimated organic Hg concentrations in spotted gar livers is not overwhelmingly significant from an ecotoxicological perspective, as the toxicological threat of organic Hg in gar liver is likely outweighed by the much more abundant inorganic Hg species. Nevertheless, estimates of either organic Hg or MeHg in samples with relatively high fractions of Hg(II) and elevated total Hg concentrations should be interpreted with caution and a more traditional direct measure of MeHg may be more appropriate.

CONCLUSION

We have described a new low-cost, and rapid (~60 min) procedure for the determination of Hg(II) and organic Hg in fish liver and muscle tissue by direct mercury analysis. It is important to note that cost estimates of the proposed method should include the requirement of two analyses (total Hg and Hg(II)) to obtain organic Hg by difference. Additionally, the method allows for the generation of percent MeHg data that can be of assistance in biomagnification and demethylation studies. Other studies have used the DMA-80 to measure MeHg in fish tissues [23-26], but these techniques use many hazardous and/or greater quantities of chemicals that contribute to the cost of analysis and waste disposal. Moreover, some prior techniques appear to have shortcomings related to quantitative extraction of organic Hg from lipid-rich biological materials such as fish liver. The proposed method was validated with analyses of reference material DOLT-4 and naturally contaminated fish liver and muscle that were compared with direct speciation measurements by GC-CVAFS. Greater uncertainty of organic Hg, and

perhaps a positive bias, was observed for livers in which Hg(II) comprised a majority of elevated total Hg concentrations. Therefore, estimates of either organic Hg or MeHg in samples with greater than 90% Hg(II) should be interpreted with caution and evaluated for methodological bias.

With further development, this method should be useful to determine Hg(II) and estimate organic Hg in other tissues, organisms, and environmental media. Application of the method should be limited to materials with Hg concentrations that, for a given mass or volume, will exceed the MDL of about 0.4 ng Hg.

Acknowledgements- Funding was provided by Natural Sciences and Engineering Research Council Discovery Grant (Drevnick), AANDC Northern Contaminants Program (Drevnick and Muir), and Caddo Lake Institute (Chumchal). We thank Barney J. Venables (University of North Texas) for his help with this project.

Conflict of interest- The authors state no conflict of interest.

References

1. Mergler D, Anderson HA, Chan LHM, Mahaffey KR, Murray M, Sakamoto M, Stern AH. 2007. Methylmercury exposure and health effects in humans: a worldwide concern. *AMBIO: A Journal of the Human Environment* 36:3-11.
2. Selin NE. 2009. Global biogeochemical cycling of mercury: a review. *Annual Review of Environment and Resources* 34:43-63.
3. Fitzgerald WF, Lamborg CH. 2007. Geochemistry of Mercury in the Environment. In Editors in C, xA, Heinrich DH, Karl KT, eds, *Treatise on Geochemistry*. Pergamon, Oxford, pp 1-47.

4. Wiener J, Bodaly R, Brown S, Lucotte M, Newman M, Porcella D, Reash R, Swain E. 2007. Monitoring and evaluating trends in methylmercury accumulation in aquatic biota. *Ecosystem responses to mercury contamination: Indicators of change*:87-122.
5. Sandheinrich MB, Weiner JG. 2011. Mercury in Freshwater Fish: Recent Advances in Assessing Toxicity of Environmentally Relevant Exposures. In Beyer W, Meador J, eds, *Environmental Contaminants in Biota: Interpreting Tissue Concentrations*, 2nd edition ed. Taylor and Francis, Boca Raton.
6. Friedmann A, Watzin M, Brinck-Johnsen T, Leiter J. 1996. Low levels of dietary methylmercury inhibit growth and gonadal development in juvenile walleye (Stizostedion vitreum). *Aquat Toxicol* 35:265-278.
7. Scheuhammer AM, Meyer MW, Sandheinrich MB, Murray MW. 2007. Effects of environmental methylmercury on the health of wild birds, mammals, and fish. *AMBIO: A Journal of the Human Environment* 36:12-19.
8. Hammerschmidt C, Sandheinrich M, Wiener J, Rada R. 2002. Effects of dietary methylmercury on reproduction of fathead minnows. *Environ Sci Technol* 36:877-883.
9. Drevnick P, Sandheinrich M. 2003. Effects of dietary methylmercury on reproductive endocrinology of fathead minnows. *Environ Sci Technol* 37:4390-4396.
10. Webber HM, Haines TA. 2003. Mercury effects on predator avoidance behavior of a forage fish, golden shiner (Notemigonus crysoleucas). *Environ Toxicol Chem* 22:1556-1561.
11. Drevnick P, Roberts A, Otter R, Hammerschmidt C, Klaper R, Oris J. 2008. Mercury toxicity in livers of northern pike (Esox lucius) from Isle Royale, USA. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 147:331-338.
12. Dutczak W, Ballatori N. 1994. Transport of the glutathione-methylmercury complex across liver canalicular membranes on reduced glutathione carriers. *J Biol Chem* 269:9746.

13. Mugesh G, Singh HB. 2000. Synthetic organoselenium compounds as antioxidants: glutathione peroxidase activity. *Chem Soc Rev* 29:347-357.
14. Palmisano F, Cardellicchio N, Zambonin P. 1995. Speciation of mercury in dolphin liver: a two-stage mechanism for the demethylation accumulation process and role of selenium. *Mar Environ Res* 40:109-121.
15. Martoja R, Berry JP. 1980. Identification of tiemannite as a probable product of demethylation of mercury by selenium in cetaceans. A complement to the scheme of the biological cycle of mercury. *Vie Milieu* 30:7-10.
16. Raldúa D, Bayona J, Barceló D. 2007. Mercury levels and liver pathology in feral fish living in the vicinity of a mercury cell chlor-alkali factory. *Chemosphere* 66:1217-1225.
17. Barst BD, Gevertz AK, Chumchal MM, Smith JD, Rainwater T, Drevnick P, Hudelson KE, Hart A, Verbeck GF, Roberts AP. 2011. Laser Ablation ICP-MS Co-localization of Mercury and Immune Response in Fish. *Environ Sci Technol*.
18. Bushee DS. 1988. Speciation of mercury using liquid chromatography with detection by inductively coupled plasma mass spectrometry. *Analyst* 113:1167-1170.
19. Shade CW. 2008. Automated simultaneous analysis of monomethyl and mercuric Hg in biotic samples by Hg-thiourea complex liquid chromatography following acidic thiourea leaching. *Environ Sci Technol* 42:6604-6610.
20. Bloom N. 1989. Determination of picogram levels of methylmercury by aqueous phase ethylation, followed by cryogenic gas chromatography with cold vapour atomic fluorescence detection. *Can J Fish Aquat Sci* 46:1131-1140.
21. Haynes S, Gragg RD, Johnson E, Robinson L, Orazio CE. 2006. An evaluation of a reagentless method for the determination of total mercury in aquatic life. *Water, Air, Soil Pollut* 172:359-374.
22. US EPA Method 7473: Mercury in solids and solutions by thermal decomposition, amalgamation, and atomic absorption spectrophotometry.
23. Scerbo R, Barghigiani C. 1998. Organic mercury determination in fish samples using an automatic mercury analyser. *Environ Technol* 19:339-342.

24. Maggi C, Berducci M, Bianchi J, Giani M, Campanella L. 2009. Methylmercury determination in marine sediment and organisms by Direct Mercury Analyser. *Anal Chim Acta* 641:32-36.
25. Nam DH, Basu N. 2011. Rapid methods to detect organic mercury and total selenium in biological samples. *Chemistry Central Journal* 5:3.
26. Carbonell G, Bravo J, Fernández C, Tarazona J. 2009. A new method for total mercury and methyl mercury analysis in muscle of seawater fish. *Bull Environ Contam Toxicol* 83:210-213.
27. Ashoka S, Peake BM, Bremner G, Hageman KJ, Reid MR. 2009. Comparison of digestion methods for ICP-MS determination of trace elements in fish tissues. *Anal Chim Acta* 653:191-199.
28. Hammerschmidt CR, Fitzgerald WF. 2006. Bioaccumulation and trophic transfer of methylmercury in Long Island Sound. *Arch Environ Contam Toxicol* 51:416-424.
29. Tseng CM, Hammerschmidt CR, Fitzgerald WF. 2004. Determination of methylmercury in environmental matrixes by on-line flow injection and atomic fluorescence spectrometry. *Analytical chemistry* 76:7131-7136.
30. Gill GA, Fitzgerald WF. 1987. Picomolar mercury measurements in seawater and other materials using stannous chloride reduction and two-stage gold amalgamation with gas phase detection. *Mar Chem* 20:227-243.
31. Bloom N, Fitzgerald WF. 1988. Determination of volatile mercury species at the picogram level by low-temperature gas chromatography with cold-vapour atomic fluorescence detection. *Anal Chim Acta* 208:151-161.
32. Baeyens W, Leermakers M, Papina T, Saprykin A, Brion N, Noyen J, De Gieter M, Elskens M, Goeyens L. 2003. Bioconcentration and biomagnification of mercury and methylmercury in North Sea and Scheldt estuary fish. *Arch Environ Contam Toxicol* 45:498-508.
33. Storelli M, Marcotrigiano G. 2002. Mercury speciation and relationship between mercury and selenium in liver of Galeus melastomus from the Mediterranean Sea. *Bull Environ Contam Toxicol* 69:516-522.

34. Yang DY, Chen YW, Belzile N. 2010. Inverse relationships between selenium and mercury in tissues of young walleye (Stizosedion vitreum) from Canadian boreal lakes. *Sci Total Environ* 408:1676-1683.
35. Chumchal MM, Rainwater TR, Osborn SC, Roberts AP, Abel MT, Cobb GP, Smith PN, Bailey FC. 2011. Mercury speciation and biomagnification in the food web of Caddo Lake (TX/LA, USA), a subtropical freshwater ecosystem. *Environ Toxicol Chem*.
36. Bloom N. 1992. On the chemical form of mercury in edible fish and marine invertebrate tissue. *Can J Fish Aquat Sci* 49:1010-1017.

17 ARTICLE 2

The role of melano-macrophage aggregates in the storage of mercury and other metals: an example from yelloweye rockfish (*Sebastes ruberrimus*)

Benjamin D. Barst[†], Kristin Bridges[‡], Malgorzata Korbas[§,‖], Aaron P. Roberts[‡], Kray Van Kirk[#], Kevin McNeel[#], and Paul E. Drevnick[†a*]

[†]INRS-ETE, 490 de la Couronne, Québec, QC G1K 9A9, Canada

[‡]Department of Biological Sciences & Institute of Applied Sciences, University of North Texas, Denton, TX, USA

[§]Canadian Light Source, Saskatoon, SK, Canada

[‖]Department of Anatomy and Cell Biology, University of Saskatchewan, Saskatoon, SK, Canada

[#]Division of Commercial Fisheries, Alaska Department of Fish and Game, Douglas, AK, USA

[a]University of Michigan Biological Station and School of Natural Resources and Environment, Ann Arbor, MI, USA

Article published in the journal *Environmental Toxicology and Chemistry (*DOI: 10.1002/etc.3009)

Abstract- Melano-macrophage aggregates, collections of specialized cells of the innate immune system of fish, are considered a general biomarker for contaminant toxicity. In order to elucidate further the relationship between macrophage aggregates and metals exposure, we sampled yelloweye rockfish (*Sebastes ruberrimus*), a long-lived species, from the east and west coasts of Prince of Wales Island, Alaska. Metals concentrations in livers (inorganic Hg, methyl Hg, Se, Ni, Cd, Cu, Zn) and spleens (inorganic Hg and methyl Hg) were determined as well as their correlations with melano-macrophage aggregate area. Sections of liver tissue were analyzed by laser ablation inductively coupled plasma mass spectrometry to determine how metals were spatially distributed between hepatocytes and macrophage aggregates. The concentration of inorganic Hg in whole tissue was the best predictor of macrophage area in yelloweye livers and spleens. Macrophage aggregates had higher relative concentrations of most metals compared to the surrounding hepatocytes. However, not all metals were accumulated to the same degree as evidenced by differences in the ratios of metals in macrophages compared to hepatocytes. Laser ablation data were corroborated with the results of X-ray synchrotron fluorescence imaging of a yelloweye liver section. Hepatic macrophage aggregates in yelloweye rockfish may play an important role in the detoxification and storage of Hg and other metals.

Keywords: Melano-macrophage aggregates, mercury, metals, LA-ICP-MS, X-ray fluorescence imaging

INTRODUCTION

Histopathological biomarkers, such as melano-macrophage aggregates (MA), have been used widely as indicators of contaminant exposure in fish [1]. These aggregates, composed of specialized cells of the innate immune system, can be found in the spleens, kidneys, and livers of teleost fish, and often contain pigments such as ceroid, lipofuscin, hemosiderin, and melanin. They serve as repositories for the remains of damaged cells and are thought to be primitive forms of the lymph nodes found in higher vertebrates [2]. Both laboratory and field studies have demonstrated an increased occurrence of MA in fish exposed to various contaminants. They are therefore considered a general biomarker for contaminant toxicity [3]. For example, in a laboratory study, perchlorate was shown to induce MA in the trunk kidneys of zebrafish (*Danio rerio*) [4], while several other studies have demonstrated that metals can lead to increased MA formation [5-6]. Additional evidence that MA are suitable biomarkers of contaminant exposure has been provided by recent field studies. Khan and colleagues noted increased MA in flatfish inhabiting an environment contaminated with both petroleum and polychlorinated biphenyls (PCBs) [7]. Mercury (Hg), a ubiquitous environmental contaminant deposited from the atmosphere to even the most remote aquatic environments, has also been linked to MA formation. Mela et al. [5] reported increased MA in *Hoplias malabaricus* fed contaminated methylmercury (MeHg) diets for 70 days. Additionally, Raldúa and colleagues [8] found that feral fish downstream of a Hg cell chlor-alkali plant had increased numbers of hepatic MA. An extensive study by Schwindt and colleagues [9] determined that whole-body MeHg concentrations were positively correlated with MA in liver and spleens of salmonids from eight U.S. national

parks. The authors also measured over 90 pesticides and other compounds in 100 brook trout from seven lakes and determined that Hg explained over one-third of the variation in MA. Further evidence of the link between Hg and MA in fish was provided by Barst and colleagues [10] who demonstrated that Hg and MA were co-localized by analyzing histological sections of spotted gar liver tissue by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). This study went beyond identifying statistical correlations by demonstrating that contaminant (Hg) and biomarker (MA) spatially overlapped. Similar findings have been noted for hepatic MA in yellow perch (*Perca flavescens*) from a Hg hotspot [11]. This information is especially useful when attempting to assign cause and effect in wild fish, which are exposed to mixtures of contaminants rather than a single chemical.

The main objective of this study was to determine the relationship between metals and macrophage aggregates in the livers of two populations of yelloweye rockfish, a long-lived species, inhabiting the relatively pristine coastal waters of Prince of Wales Island (POW), Alaska. We demonstrate that the concentration of inorganic Hg in whole tissue is highly correlated with macrophage area in yelloweye livers and spleens and that these immune cells accumulate higher relative concentrations of most metals compared to the surrounding hepatocytes. However, not all metals were accumulated to the same degree as evidenced by two in situ imaging techniques (LA-ICP-MS and X-ray Fluorescence).

MATERIALS AND METHODS

Study Sites and Sampling

Yelloweye rockfish are native to the Pacific Coast of the U.S. and Canada and are part of important recreational and commercial fisheries in the North Pacific [12-13]. Yelloweye were selected for study as they are extremely long-lived fish (oldest specimen >120 yrs) [12] and likely have small home ranges, as do other rockfish [13-15].

During June and July of 2011, 2012, and 2013, yelloweye rockfish were captured off the east (n=28) and west (n=25) coasts of Prince of Wales Island (POW) Alaska (Figure 1), using rod and reel.

Figure 1. Location of Prince of Wales Island, Alaska. The black symbols in the inset image show the locations of the two sampling sites.

Fish were euthanized immediately after capture. Total length and wet weights (total and liver) were recorded and samples of axial muscle, liver, and spleen tissues were collected. Length (cm), weight (g), and liver weight (g) were used to calculate condition factor and hepatosomatic index (HSI) according to the following formulas.

$$\text{condition factor} = \frac{1{,}000 \times \text{weight}}{\text{length}^3}$$

$$\text{hepatosomatic index} = \left(\frac{\text{liver weight}}{\text{weight}}\right) \times 100$$

Subsamples of tissues for elemental analysis were placed in individual plastic bags and frozen at -20℃. Subsamples of liver and spleens fo r histological analyses were fixed in neutral buffered formalin and later transferred to 70% ethanol for storage. Sagittal otoliths were collected for age analysis which was performed at the Alaska Department of Fish and Game (ADF&G) Age Determination Unit using the break-and-burn method [12].

Elemental analysis

Frozen tissue samples were lyophilized to constant weight and homogenized. Total Hg was analyzed in the homogenized samples with a direct Hg analyzer (DMA-80, Milestone Inc.). Quality assurance included reference and duplicate samples. The Hg concentrations in reference materials were within certified ranges and the mean relative

percent difference of duplicate samples of muscle, liver, and spleen were 0.58, 1.43, and 0.97% respectively.

A more in depth description of the method of Hg(II) analysis is presented in Barst et al. [16]. Briefly, tissues were digested in HCl with microwave heating and organic Hg was extracted with toluene. The acidic fraction remaining after toluene extraction of the original digestate was diluted 5-fold with reagent-grade water and an aliquot was added to a quartz analytical vessel for quantification of ionic Hg with a DMA-80. MeHg was calculated by subtracting Hg(II) from total Hg of the same sample. The mean recovery of a digested reference material (DOLT-4) was 99.14% (n=7), and the mean relative percent difference of duplicate samples of liver and spleen were 0.74 and 1.22% respectively.

Metals (selenium (Se), nickel (Ni), cadmium (Cd), copper (Cu), and zinc (Zn)) were measured by digesting lyophilized subsamples of liver with concentrated HNO_3 (Aristar grade) followed by 30% (w/w) hydrogen peroxide (Trace Select Ultra grade). Ultrapure water was added to the digestates in order to reach a 10% HNO_3 concentration before analysis by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Elemental X Series). Certified reference material (lobster hepatopancreas, TORT-3, National Research Council of Canada) was subjected to the same digestion procedure and analysis. Recoveries of metals in the reference material were within certified ranges. Spleens and muscle samples were not analyzed for metals other than Hg.

Melano-macrophage aggregates

Both spleen and fixed liver tissues were embedded in paraffin, sectioned, and mounted on glass slides. Seven-µm sections were stained with eosin and hematoxylin (H&E) and

viewed under light microscopy (Axiovert 200, Zeiss Microscopy) to evaluate general pathology. Each of three fields of view, at 100X magnification, were photographed (Axiocam HRc, Zeiss Microscopy) for each H&E stained liver and spleen. The mean percent coverage of MA, in relation to the total area of the field of view, was calculated using Image J software (NIH http://rsbweb.nih.gov/ij/) [17]. Alternate unstained sections were archived for LA-ICP-MS (20 µm) analysis and X-ray fluorescence imaging (7µm).

LA-ICP-MS and X-ray fluorescence imaging

Tissue sections were used to investigate the distribution of metals in yelloweye liver sections by LA-ICP-MS, according to the methods of Barst et al. [10] with slight modification. Briefly, a microscope slide, with unstained, paraffin-embedded tissue, was placed into the chamber of a 213 nm Nd:YAG laser ablation source (New Wave Research). A CCD camera enabled zooming and scanning of the slide to locate the tissue. Three areas of normal liver tissue and three MA were chosen at random and ablated from sections representing individual yelloweye (n=21) with a 55 µm beam diameter. A Varian 820 ICP-MS coupled to the laser source was used to monitor Hg, Se, Cd, Ni, Cu, and Zn. The mean isotope counts of each element during laser warm-up were used as an estimate of background noise and subtracted from the mean counts for the remainder of the run to calculate a signal. After ablation, slides were viewed again under light microscopy to ensure MA were accurately targeted [10]. Table S1 in the Supplemental Data describes the instrument parameters used for analysis.

Table S1. Instrument parameters for laser ablation ICP-MS analyses.

Mass flow	1000
Output	70%
Spot size	55 microns
Dwell time	8 seconds
Repetition rate	10 Hz
Replicates	5
Noise delay	7 seconds

X-ray fluorescence images of a yelloweye liver section (mounted in paraffin on a Thermanox plastic coverslip) were collected at the Advanced Photon Source, Argonne National Laboratory with beamline 20-ID-BD (PNC/XOR). The incident X-ray energy was kept at 13.45keV and the 7.0 GeV storage ring was operated in continuous top-up mode at 102 mA. Beam focusing and harmonic rejection were accomplished with a Si(111) double crystal monochromator and Rh-coated silicon mirrors. Incident X-rays were measured by using the intensity of the nitrogen-filled ion chambers. Kilpatrick-Baez Rh-coated focusing mirrors produced a microfocused beam of 5 µm diameter. The sample was mounted at 45° to the incident X-ray bea m and was spatially rastered in the microbeam with a step size of 5 µm and a beam exposure of 0.5 s per step. The intensities of the X-ray fluorescence lines (Hg Lα, Se Kα, Cu Kα, and Zn Kα) at each step were monitored using a four-element silicon-drift Vortex detector (SII NanoTechnology USA, Inc.) Data were processed using SMAK software (http://home.comcast.net/~sam_webb/smak.html). Windowed X-ray fluorescence counts

were normalized by the incident X-ray intensity and background corrected by subtracting the average intensity obtained from pixels outside the tissue. Quantities of Se and Zn per pixel were directly calibrated by measuring the respective X-ray fluorescence calibration standard which was deposited as a thin layer on 6.3 µm-thick Mylar film (56.7 µg/cm^2 of Se and 20 µg/cm^2 of ZnTe). The quantity of Hg per pixel was calibrated with two X-ray fluorescence thin standards (50.8 µg/cm^2 of Au and 53.1 µg/cm^2 of TlCl on 6.3 µm-thick Mylar film (Micromatter). Elemental Hg is volatile and therefore Tl and Au, elements adjacent to Hg in the periodic table, were used for calibration. Average background intensities for windowed fluorescence from the standards were estimated from the X-ray fluorescence image of the 6.3 µm-thick Mylar film. The background-corrected Au and Tl Lα fluorescence intensities were used to interpolate the Hg Lα fluorescence intensity, which was then applied to the background-corrected Hg distribution map to obtain the quantity of Hg per pixel in µg/cm^2 [18]. Unfortunately we were not able to determine the Cd concentration in the yelloweye liver section with X-ray fluorescence, as the energy of the Cd Lα overlaps with that of Ar K α photons present in air. Additionally, a Cu standard was not available, so Cu data are presented as normalized Cu K α fluorescence counts. Nickel was not detected by X-ray fluorescence in the yelloweye liver section due to the low concentration of this metal in the tissue.

Statistical Analyses

Statistics were performed with JMP 9 Statistical Analysis Software (SAS Institute). Data transformations, such as log or arcsine, were employed if necessary, to meet the assumptions of tests. Linear regression models were used to describe relationships

between variables. The residuals of the linear regression models were tested for normality and tested in order to determine whether outliers had biased the coefficients of determination. Levene's test was used to determine unequal variances. Concentrations of total Hg (in muscle, liver, and spleen) were compared between sampling sites after correcting for fish length. This was carried out by comparing the residuals obtained from linear regressions between log total Hg and fork length with two-sample *t* tests. Two-sample *t* tests were also used to determine differences in hepatic concentrations of Se, Ni, Cd, Cu, and Zn between sites. The residuals, after regression of liver MA percent coverage and fish age, were included with metal concentrations in a stepwise linear regression model. As only Hg was measured in spleens, a stepwise regression was carried out only for data obtained from yellloweye livers. Yelloweye rockfish growth curves were compared with Tukey's honest significance difference test. Significance of statistical tests was determined with a type I error (α) of 0.05.

RESULTS

Metal concentrations in fish tissues

Mean concentrations of metals in yelloweye tissues are presented in Table 1. All concentrations are reported as mean values in µg/g wet weight ± SD. Total Hg concentrations, in all fish, were highest in liver samples (0.87 ± 0.75), intermediate in spleen (0.71 ± 0.60), and lowest in muscle (0.52 ± 0.30). Hg(II) was the predominant form of Hg in both livers and spleens comprising 58 and 73% of the total Hg concentrations in these organs respectively. Fish length was positively correlated with concentrations of total Hg in liver (r^2=0.27, $p<0.0001$), spleen (r^2=0.35, $p<0.0001$), and

muscle (r^2=0.36, p<0.0001). Fish from the eastern sampling site had significantly higher Hg concentrations in muscle (two-sample *t* test, p<0.0001), liver (p=0.0001), and spleen (p<0.0001).

Table 1. Mean metal concentrations ± SD (µg/g wet weight) in tissues of yelloweye rockfish (*Sebastes ruberrimus*) from Prince of Wales Island, Alaska. Asterisks note significant differences between sampling sites.

	Muscle	Spleen	Liver					
Sampling area	Total Hg	Total Hg	Total Hg	Se	Ni	Cd	Cu	Zn
East	0.60 ± 0.32***	0.85 ± 0.67***	1.1 ± 0.87**	3.0 ± 1.16	0.03 ± 0.023	3.9 ± 3.04*	7.3 ± 4.81***	24.1 ± 4.89**
West	0.44 ± 0.24***	0.56 ± 0.47***	0.7 ± 0.55**	2.7 ± 0.83	0.04 ± 0.023	2.0 ± 0.99*	3.2 ± 1.38***	20.0 ± 2.57**

*p<0.05

**p<0.001

***p<0.0001

Liver Se was positively correlated with total Hg (r^2=0.60, p<0.0001, Se:Hg mean molar ratio 2.2 ± 1.54) and concentrations were similar between east and west coast fish. Nickel concentrations were low (0.035 ± 0.023) in all yelloweye livers, and did not differ significantly between the populations. However, the mean liver concentrations of Cd, Cu, and Zn were significantly higher in the livers of yelloweye sampled from the east coast of POW (Table 1). Of the metals measured in yelloweye livers only Hg (r^2=0.55, p<0.0001), Se (r^2=0.27, p<0.0001), and Cd (r^2=0.33, p<0.0001) were positively correlated with fish age.

Melano-macrophage aggregate area

Melano-macrophage aggregates were identified by fluorescence and light microscopy in both the livers and spleens of yelloweye rockfish and contained lipofuscin, hemosiderin, and melanin pigments (Figure 2).

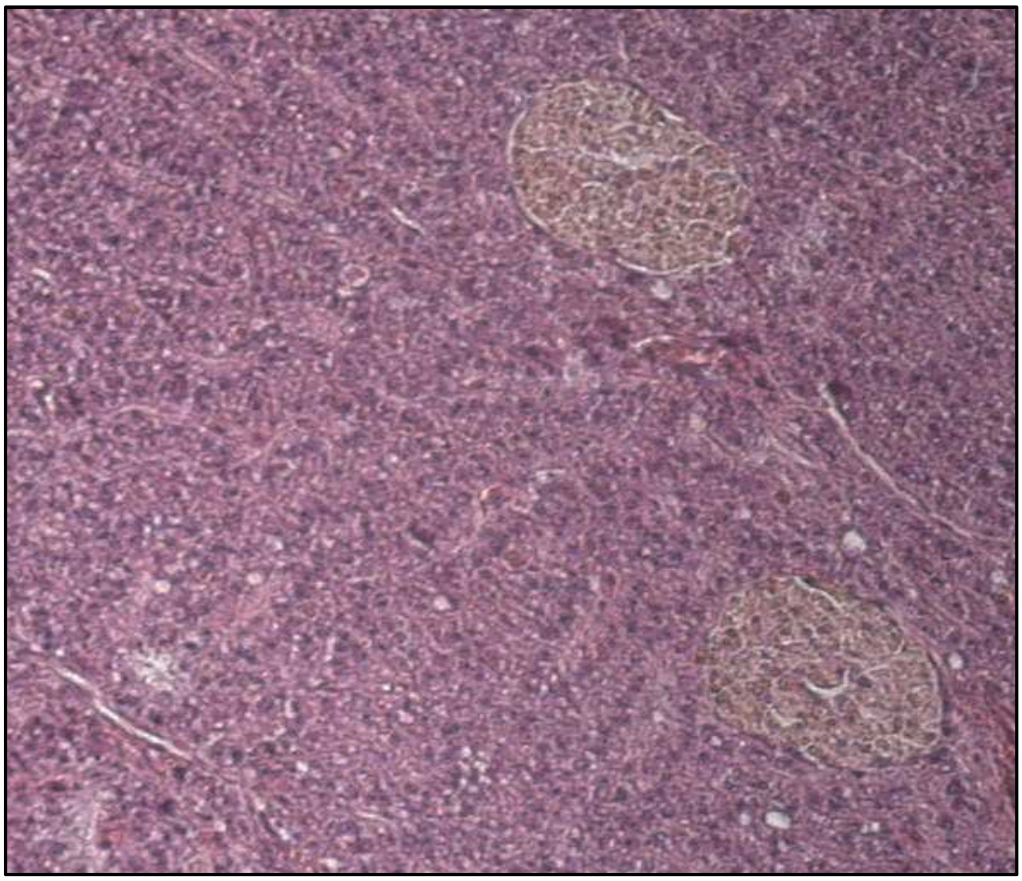

Figure 2. Micrograph of a yelloweye liver section at 100X magnification showing pink hepatocytes and brown melano-macrophage aggregates.

During preliminary histological analyses a qualitative difference in MA size was noted which led to the proposed use of imaging software to quantify macrophage area. Image analysis software for the quantification of fish MA has been performed previously by Schwindt and colleagues [19], who used ImagePro Plus, and Mizuno and colleagues [20], who used NIH Image (Ver 1.57). We opted to use Image J software which has

replaced NIH Image and is free to download from the National Institutes of Health (NIH http://rsbweb.nih.gov/ij/). In general, MA were larger and more prevalent in spleens (mean coverage = 6.2%) than in liver (mean coverage = 1.1%), which could be due to the increased turnover of erythrocytes in the organ [21]. Fish age was positively correlated with MA area in both liver (r^2=0.49, p<0.0001) and spleen (r^2=0.41, p<0.0001). The metal explaining most of the variance in hepatic MA was Hg(II) (r^2=0.64, p<0.0001). Positive correlations were also noted between hepatic MA area and concentrations of total Hg (r^2=0.62, p<0.0001), MeHg (r^2=0.51, p<0.0001), Se (r^2=0.35, p<0.0001), Cd (r^2=0.41, p<0.0001), and Cu (r^2=0.09, p=0.044). Hg(II), also better explained MA area (r^2=0.59, p<0.0001) than MeHg in the spleens of yelloweye. There was no significant difference in MA area between the two sampling sites in either liver (two-sample *t* test, p=0.57) or spleen (two-sample *t* test, p=0.97). An increase in MA size due to aging has been previously reported [2] and presumably occurs due to increased phagocytosis of effete cells. It is therefore, important to account for fish age when determining the association between MA area and concentrations of metals. By including the effects of fish age and concentrations of metals in a model, we determined that Hg(II) was still the best predictor of MA area (stepwise regression, r^2=0.33, p<0.0001).

Metals in hepatic melano-macrophage aggregates

Counts of Hg, Se, Cd, and Cu were consistently higher in MA than adjacent hepatocytes, indicating that MA accumulate these metals to a greater degree than the surrounding tissue. In 95% of the ablated MA, Hg counts were higher than the surrounding hepatocytes. Selenium, Cd, and Cu were higher in 67%, 76%, and 71% of

the ablated MA compared to nearby hepatocytes. Counts of Ni were low in all ablated regions, barely rising above background in the majority of cases. Counts of Zn were higher in MA than hepatocytes for less than half (43%) of the ablations. We calculated the ratio of metal in MA relative to the surrounding normal tissue (M_{MA}:M_{Hep}) for Cu, Zn, Cd, Hg, and Se. Mercury had the highest mean M_{MA}:M_{Hep} ratio at 2.73 followed by Se (2.71), Cu (1.87), Cd (1.53), and Zn (0.98). The order of these ratios, determined by LA-ICP-MS, is supported by X-ray fluorescence data gathered from the analysis of a yelloweye liver (Figure 3).

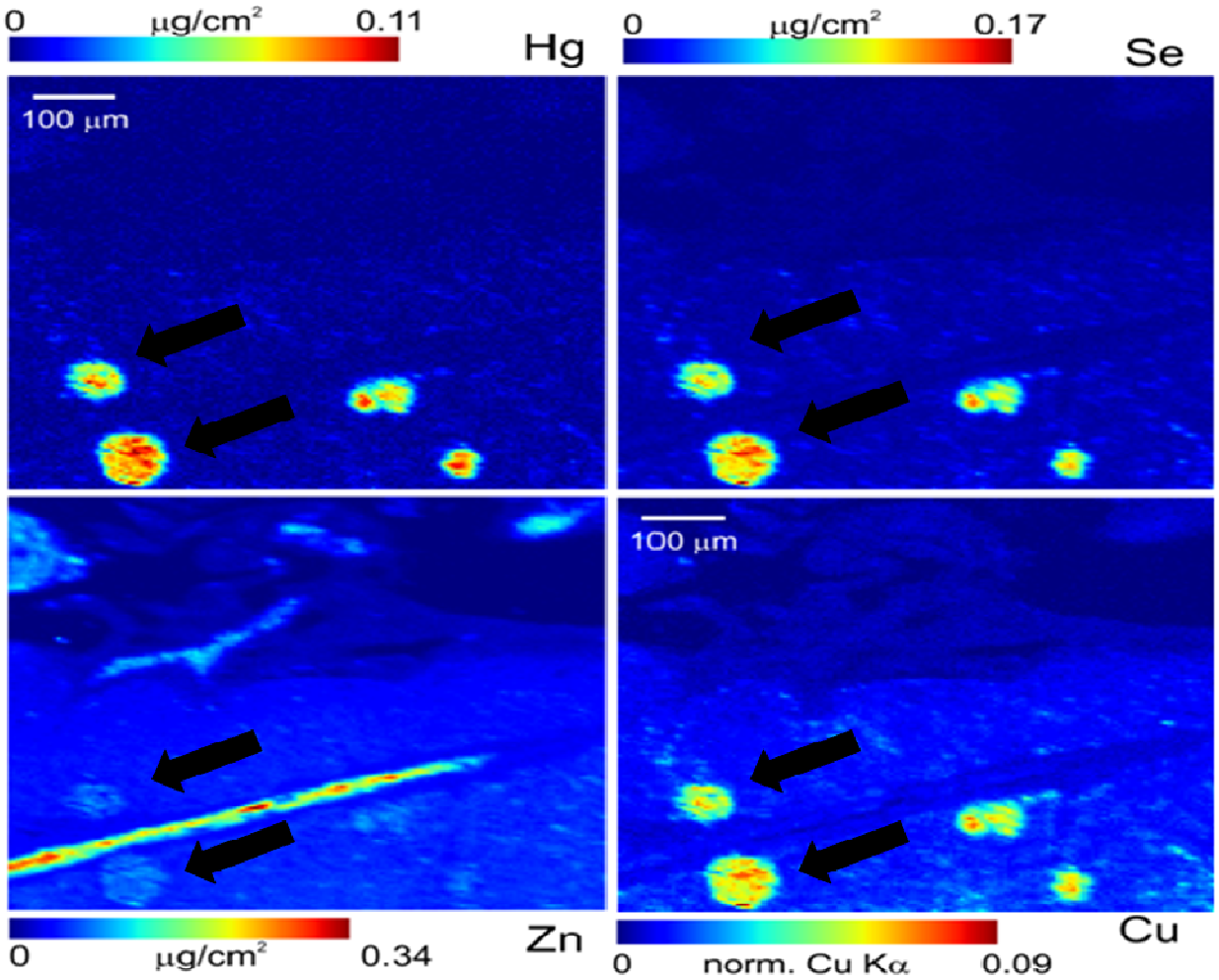

Figure 3. X-ray fluorescence images of a yelloweye rockfish liver section. The black arrows mark the positions of the melano-macrophage aggregates.

Condition Indices

We assessed the physical condition of yelloweye in this study by calculating two indices. These were the condition factor, which relates body length and weight, and the hepatosomatic index, which gives the weight of the liver relative to the total weight of the fish. In this study, body condition was not significantly correlated with concentrations of any of the metals and did not differ between males (mean = 1.60) and females (mean = 1.72). However, condition factor was significantly higher in fish from the western sampling area (mean = 1.71) than in fish from the eastern area (mean = 1.55) (two-sample *t* test, p=0.003).

Mean HSI was significantly lower in female (mean = 0.83) than male (mean = 1.35) yelloweye (two-sample *t* test, p=0.003). HSI is sensitive to differences in the relative weights of male and female gonads. There is an energetic trade-off between liver investment and gonad investment (e.g., Merrill and Collins [22]), and ovaries require a bigger investment than testes. Therefore, a larger relative gonad weight for female yelloweye is the most likely cause for the lower HSI in female fish. Like body condition index, HSI was significantly greater in fish from the western sampling area (mean = 1.42) than in those from the eastern area (mean = 1.10) (two-sample *t* test, p=0.034). When regressing metal concentrations with HSI, all male fish were pooled together and then analyzed separately based on sampling site. For all male fish, HSI was negatively correlated with concentrations of Cu (r^2=0.29; p=0.0004), Zn (r^2=0.37; p<0.0001), Cd (r^2=0.20; p=0.005), and Se (r^2=0.21; p=0.003). Only Se (r^2=0.60; p=0.008) was

significantly negatively correlated with HSI in female fish, though significance of the other relationships may have been hampered by a low sample size (n=11).

For males from the western sampling site, HSI significantly decreased with increasing concentrations of Cu (r^2=0.65; p<0.0001), Zn (r^2=0.68; p<0.0001), and Se (r^2=0.34; p=0.012). Only Cd was negatively correlated with HSI in males from the eastern sampling location (r^2=0.22; p=0.03). HSI was negatively correlated with Zn (r^2=0.95; p=0.0010) for female fish from the eastern sampling site, however there were no significant correlations between metals and HSI for females from the west.

DISCUSSION

Metal concentrations in fish tissues

The reason for higher concentrations of hepatic Hg, Cd, Cu, and Zn in yelloweye from the eastern sampling site is unknown, but may be linked to higher exposure concentrations, differences in growth rate, and/or differences in fish condition. Although we did not measure concentrations of metals in water, Ernest Sound may receive greater terrestrial inputs of metals resulting in higher exposure concentrations. Geologic deposits of Cu and Zn, which contain deposits of Cd [23], are present on Prince of Wales Island and in other parts of Alaska's Inside Passage [24] and have been historically mined in the area [25]. In contrast to the sampling site in Ernest Sound, the western sampling site is not surrounded by land, and would therefore not be expected to receive as much terrestrial runoff. Hepatic metals contents may also vary due to differences in growth rate between the two samples. Although, there was no significant difference in mean fish age between the two sites (two-sample *t* test, p=0.37), the

growth rate, determined by length and age distributions, differed between groups. Measurements of length and age are routinely collected by ADF&G from yelloweye caught by commercial fishers in Alaskan waters. Length versus age plots for yelloweye captured by commercial fishers from both the inside (n=5,504) and outside (n=7,790) waters of Southeastern Alaska are presented in Figure 4. The 25 individuals from the eastern (inside) waters and the 23 individuals from the western (outside) waters are also plotted in this graphic. The von Bertalanffy growth curves for the two larger datasets are not significantly different suggesting that overall fish from the outside and inside populations have similar growth rates. However, the two growth curves based solely on the fish sampled in this study are significantly different ($p= 0.005$). The 25 fish from the eastern sampling site fall within the age/length distribution of the ADF&G inside waters dataset. However, the 23 fish sampled from the western site near Coronation Island exhibit a localized size grouping which is not representative of the entire population of yelloweye from the outside waters. Size and growth rates of yelloweye rockfish may vary greatly between localized sampling sites based on differences in temperature and nutrient availability across their range. An increase in growth rate has been linked to decreased Hg concentrations by means of somatic growth dilution in aquatic invertebrates and fish [26-27].

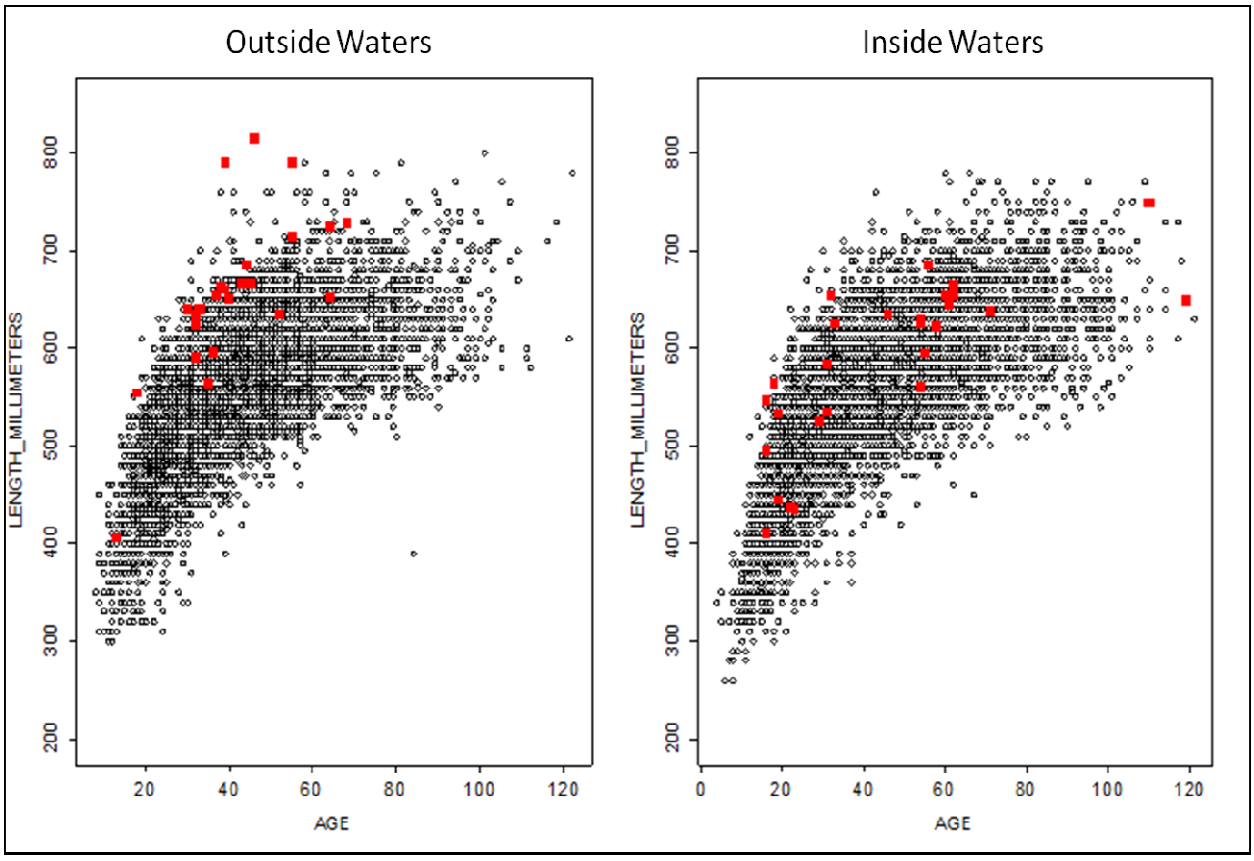

Figure 4. Length and age distributions for yelloweye rockfish captured from the outside (left) and inside (right) waters of Southeast Alaska. The red markers indicate fish from the western (left) and eastern (right) sampling sites for this study.

We tested whether Hg concentrations in yelloweye tissues decreased due to increased growth rate by regressing the residuals of linear regressions between length and age and length and log Hg concentrations. The lack of any significant negative relationships resulting from these regressions indicates that differences in Hg concentrations between the two sampled populations are unlikely due to growth dilution.

Condition indices

Correlations between condition factor and metal concentrations in fish tissues vary throughout the literature. For example, Rajotte et al. [28] documented lower condition in

yellow perch from metal-contaminated lakes near Sudbury, Ontario. No differences in condition factor were reported for rainbow trout exposed to run-off from copper mine tailings [29] or for lake whitefish (*Coreogonus clupeaformis*) exposed to dietary nickel [30]. Drevnick et al. [31] noted decreased condition factor of northern pike (*Esox lucius*) with higher Hg concentrations. In wild fish, multiple factors may contribute to changes in body condition including food availability, temperature, and contaminants [32]. Importantly, yelloweye body condition is unlikely to be linked to differences in metal body burdens as none of the metals were correlated with fish condition.

HSI is a more specific index than body condition, as it may demonstrate an effect on the liver, which is often a target of contaminants because of its role as a detoxification organ. Changes in HSI indicate decreases in energy reserves and altered metabolism in fish, both of which may be the targets of toxic chemicals [33]. Similar to body condition, reports of how HSI correlates to metals exposure differ. HSI decreased with increasing Cd concentration in the livers of winter flounder (*Pleuronectes americanus*) after an aqueous exposure for 71 days [34]. Decreases in HSI have also been reported for grey mullet (*Chelon labrosus*) fed Cu containing diets [35], and for wild walleye (*Sander vitreus*) with elevated Hg concentrations [36]. HSI has also been shown to increase in fish from contaminated sites, presumably resulting from cellular hypertrophy [32, 37]. Other studies have documented non-significant correlations between HSI and metal tissue concentrations [38-39]. Differences in the results regarding HSI and body condition are likely dependent on the types of contaminants present, the species of fish, and exposure duration/pathway as well as other environmental factors including food availability.

Taken together, the elevated growth rate, HSI, and body condition of the yelloweye from the western site are likely a result of greater temperatures or food availability. Increased food availability boosts hepatic reserves of lipids and glycogen thus increasing the relative weight of the liver and overall body condition. Yelloweye with fewer energy stores in the liver would likely have higher metal contents due to the concentrating effect of lipid removal. This has been reported in other studies [31, 40] and may explain the elevated hepatic metal contents in yelloweye from the eastern sampling site.

Melano-macrophage aggregates

The co-localization of MA and metals has been documented in previous studies. Pulsford et al. [41] noted the presence of 19 metals in MA in kidney and spleen of flounder (*Platichthys flesus*) by x-ray microanalysis. Woshner et al. [42] used autometallography to show that in the livers of beluga whales (*Delphinapterus leucas*), Hg was within stellate macrophages. Barst et al. [10] noted elevated relative concentrations of both Hg and Se in the hepatic MA of spotted gar from Caddo Lake. Most recently, Hg was also demonstrated to accumulate in the MA of yellow perch by Batchelar et al. [11]. In this study, the authors noted that Hg was on average 2.45 times higher in MA than in hepatocytes. Similar to Batchelar et al. [11], we calculated the ratio of metal in MA relative to the surrounding normal tissue for Cu, Zn, Cd, Hg, and Se. Mercury had the highest mean M_{MA}:M_{Hep} ratio at 2.73, which was similar to the ratio reported by Batchelar et al. [11]. The mean Se M_{MA}:M_{Norm} ratio was 2.71, followed by Cu (1.87), Cd (1.53), and Zn (0.98). The order of these ratios, determined by LA-ICP-MS, is supported by X-ray fluorescence data gathered from the analysis of a yelloweye liver (Figure 3).

The accumulation of metals in MA may be due to the phagocytosis of cells which are dead or dying as a result of metal toxicity or because some metals are needed for the normal functioning of these immune cells. Copper, Zn, and Se are micronutrients, often incorporated into the active sites of enzymes, however, in excess, these metals may be toxic [43]. Both Cu and Zn are found in CuZn superoxide dismutase (SOD), an important antioxidant enzyme which accelerates the conversion of superoxide radicals to hydrogen peroxide and oxygen [32]. SOD has been shown to increase phagocytic activity of macrophages in mice [44] and may also be upregulated in the MA of fish. Furthermore, impaired respiratory burst has been documented in Cu deficient rats [45] and been linked to decreased CuZn-SOD activity in bovine macrophages [46], suggesting that these metals are necessary for the normal functioning of these cells. Conversely, neither Hg nor Cd have any known biological function, therefore their presence in MA is more likely a result of metal-induced cellular damage.

Selenium is an essential nutrient which is incorporated into selenocysteine and selenoproteins, including glutathione peroxidase (GPx), which along with glutathione and glutathione reductase, make up an extremely important cellular antioxidant defense system [47]. Like CuZn-SOD, the glutathione system plays an important role in the protection of macrophages from self-induced oxidative injury. Selenium's protective effect from Hg toxicity has been previously reported in the literature [48-50] and a 1:1 molar ratio between the two is assumed to result in an inert HgSe compound [50]. In excess, Hg may result in a deficiency of Se needed to produce selenoenzymes [51]. Neither LA-ICP-MS nor X-ray fluorescence provide information about the exact chemical form of Hg or Se in MA. However, 58% of the total Hg in yelloweye livers was

Hg(II), the best predictor of MA area. Additionally, we noted significant correlations between both the Se and Hg concentrations in whole liver and the Se M_{MA}:M_{Hep} ratio and counts of Hg in MA. It is, therefore, possible that Hg(II) is present in MA bound to Se. HgSe is considered to be an inert end-product of Hg detoxification in marine mammals [42, 52], however the end-product has not been identified in fish. As we did not analyze other metals in yelloweye spleens, future work could focus on how metals are distributed in this organ. Further research is warranted to determine the role MA play in the storage of Hg and other metals.

CONCLUSION

We noted increased area of MA, a general biomarker of contaminant exposure, in the livers and spleens of yelloweye rockfish from Prince of Wales Island, Alaska. The variation in hepatic MA was best described by concentrations of Hg(II) even after accounting for other metals and the age of the fish, demonstrating that MA are excellent biomarkers of Hg exposure. The positive relationship between MA area and Hg concentration implies a dose-response relationship whereby cell damage increases with metal exposure. Higher relative concentrations of most metals were detected in MA, though Hg and Se tended to accumulate to a greater extent relative to surrounding hepatocytes. We hypothesize that Hg may be bound to Se in hepatic MA resulting in the formation of an inert HgSe compound. Although we noted changes in tissues, we found no changes in HSI or body condition that were related to Hg. Storage in hepatic MA removes Hg from circulation, reducing Hg exposure to other cells, tissues, and organs, and thus appears to protect against further Hg toxicity. Differences in metal

concentrations between the two sampled populations could be due to differences in metal inputs to the two environments or due to differences in food availability between the two groups of fish.

SUPPLEMENTAL DATA

Includes one table (Table S1).

Acknowledgements- Funding was provided by **Natural Sciences and Engineering Research Council** Discovery Grant (Drevnick). We greatly appreciate the assistance provided by the Griggers family and R. and M. Holley at TreeTops Lodge. We thank R. Gordon for assistance at the 20-ID beamline at the Advanced Photon Source and B. Bewer and K. Nienaber for synchrotron data collection. PNC/XSD facilities at the Advanced Photon Source, and research at these facilities, are supported by the US Department of Energy - Basic Energy Sciences, a Major Resources Support grant from NSERC, the University of Washington, the Canadian Light Source and the Advanced Photon Source. Use of the Advanced Photon Source, an Office of Science User Facility operated for the U.S. Department of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the U.S. DOE under Contract No. DE-AC02-06CH11357. The synchrotron part of this work was supported by the Canadian Foundation of Innovation through funding for BioXAS: Life Science Beamline for X-ray Absorption Spectroscopy at the Canadian Light Source Inc.

Conflict of interest- The authors state no conflict of interest.

REFERENCES

[1] Wolke R. 1992. Piscine macrophage aggregates: a review. *Annu Rev Fish Dis* 2:91-108.

[2] Agius C, Roberts R. 2003. Melano macrophage centres and their role in fish pathology. *J Fish Dis* 26:499-509.

[3] McCarthy J, Shugart L. 1990. Biomarkers of environmental contamination.

[4] Capps T, Mukhi S, Rinchard JJ, Theodorakis CW, Blazer VS, Patiño R. 2004. Exposure to perchlorate induces the formation of macrophage aggregates in the trunk kidney of zebrafish and mosquitofish. *J Aquat Anim Health* 16:145-151.

[5] Mela M, Randi M, Ventura D, Carvalho C, Pelletier E, Oliveira Ribeiro C. 2007. Effects of dietary methylmercury on liver and kidney histology in the neotropical fish Hoplias malabaricus. *Ecotoxicol Environ Saf* 68:426-435.

[6] Giari L, Manera M, Simoni E, Dezfuli B. 2007. Cellular alterations in different organs of European sea bass< i> Dicentrarchus labrax</i>(L.) exposed to cadmium. *Chemosphere* 67:1171-1181.

[7] Khan R. 2003. Health of flatfish from localities in Placentia Bay, Newfoundland, contaminated with petroleum and PCBs. *Arch Environ Contam Toxicol* 44:485-492.

[8] Raldúa D, Bayona J, Barceló D. 2007. Mercury levels and liver pathology in feral fish living in the vicinity of a mercury cell chlor-alkali factory. *Chemosphere* 66:1217-1225.

[9] Schwindt AR, Fournie JW, Landers DH, Schreck CB, Kent ML. 2008. Mercury concentrations in salmonids from western US national parks and relationships with age and macrophage aggregates. *Environ Sci Technol* 42:1365-1370.

[10] Barst BD, Gevertz AK, Chumchal MM, Smith JD, Rainwater T, Drevnick P, Hudelson KE, Hart A, Verbeck GF, Roberts AP. 2011. Laser Ablation ICP-MS Co-localization of Mercury and Immune Response in Fish. *Environ Sci Technol*.

[11] Batchelar KL, Kidd KA, Drevnick PE, Munkittrick KR, Burgess NM, Roberts AP, Smith JD. 2013. Evidence of impaired health in yellow perch (Perca flavescens) from a biological mercury hotspot in northeastern North America. *Environ Toxicol Chem* 32:627-637.

[12] Fisheries CDo, Oceans, MacLellan S, Station PB. 1997. *How to Age Rockfish (Sebastes) Using S. Alutus as an Example, the Otolith Burnt Section Technique*. Nanaimo, BC: Fisheries and Oceans Canada.

[13] Yamanaka KL, Lacko L, Withler R, Grandin C, Lochead J, Martin J, Olsen N, Wallace S. 2006. *A review of yelloweye rockfish Sebastes ruberrimus along the Pacific coast of Canada: biology, distribution and abundance trends*. Fisheries and Oceans Canada, Science.

[14] Matthews KR. 1990. A telemetric study of the home ranges and homing routes of copper and quillback rockfishes on shallow rocky reefs. *Canadian Journal of Zoology* 68:2243-2250.

[15] Jorgensen SJ, Kaplan DM, Klimley A, Morgan SG, O'Farrell MR, Botsford LW. 2006. Limited movement in blue rockfish Sebastes mystinus: internal structure of home range. *MARINE ECOLOGY-PROGRESS SERIES*- 327:157.

[16] Barst BD, Hammerschmidt CR, Chumchal MM, Muir DC, Smith JD, Roberts AP, Rainwater TR, Drevnick PE. 2013. Determination of mercury speciation in fish tissue with a direct mercury analyzer. *Environ Toxicol Chem* 32:1237-1241.

[17] Beaudin L, Johannessen SC, Macdonald RW. 2010. Coupling Laser Ablation and Atomic Fluorescence Spectrophotometry: An Example Using Mercury Analysis of Small Sections of Fish Scales. *Analytical chemistry*.

[18] Prince L, Korbas M, Davidson P, Broberg K, Rand MD. 2014. Target Organ Specific Activity of Drosophila MRP (ABCC1) Moderates Developmental Toxicity of Methylmercury. *Toxicol Sci*:kfu095.

[19] Schwindt AR, Truelove N, Schreck CB, Fournie JW, Landers DH, Kent ML. 2006. Quantitative evaluation of macrophage aggregates in brook trout Salvelinus fontinalis and rainbow trout Oncorhynchus mykiss. *Dis Aquat Org* 68:101.

[20] Mizuno S, Misaka N, Miyakoshi Y, Takeuchi K, Kasahara N. 2002. Effects of starvation on melano-macrophages in the kidney of masu salmon (< i> Oncorhynchus masou</i>). *Aquaculture* 209:247-255.

[21] Fänge R, Nilsson S. 1985. The fish spleen: structure and function. *Experientia* 41:152-158.

[22] Merrill L, Collins PM. 2015. Environment and sex-specific allocation strategies among gonadal, somatic and immune indices in a marine fish. *Canadian Journal of Zoology*.

[23] Ripley EA, Redmann RE. 1995. *Environmental effects of mining*. CRC Press.

[24] Robinson G, Twenhofel W. 1953. Some lead-zinc and zinc-copper deposits of the Ketchikan and Wales districts. *Alaska: US Geological Survey Bulletin*:59-84.

[25] Wright CW, Wright C. 1907. *Lode mining in southeastern Alaska*. US Government Printing Office.

[26] Karimi R, Chen CY, Pickhardt PC, Fisher NS, Folt CL. 2007. Stoichiometric controls of mercury dilution by growth. *Proceedings of the National Academy of Sciences* 104:7477-7482.

[27] Ward DM, Nislow KH, Chen CY, Folt CL. 2010. Rapid, efficient growth reduces mercury concentrations in stream-dwelling Atlantic salmon. *Trans Am Fish Soc* 139:1-10.

[28] Rajotte JW, Couture P. 2002. Effects of environmental metal contamination on the condition, swimming performance, and tissue metabolic capacities of wild yellow perch (Perca flavescens). *Can J Fish Aquat Sci* 59:1296-1304.

[29] Dethloff G, Bailey H, Maier K. 2001. Effects of dissolved copper on select hematological, biochemical, and immunological parameters of wild rainbow trout (Oncorhynchus mykiss). *Arch Environ Contam Toxicol* 40:371-380.

[30] Ptashynski M, Pedlar R, Evans R, Baron C, Klaverkamp J. 2002. Toxicology of dietary nickel in lake whitefish (< i> Coregonus clupeaformis</i>). *Aquat Toxicol* 58:229-247.

[31] Drevnick P, Roberts A, Otter R, Hammerschmidt C, Klaper R, Oris J. 2008. Mercury toxicity in livers of northern pike (Esox lucius) from Isle Royale, USA. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 147:331-338.

[32] Di Giulio RT, Hinton DE. 2008. *The toxicology of fishes*. CRC.

[33] Van der Oost R, Beyer J, Vermeulen NP. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ Toxicol Pharmacol* 13:57-149.

[34] Pereira J, Mercaldo-Allen R, Kuropat C, Luedke D, Sennefelder G. 1993. Effect of cadmium accumulation on serum vitellogenin levels and hepatosomatic and gonadosomatic indices of winter flounder (Pleuronectes americanus). *Arch Environ Contam Toxicol* 24:427-431.

[35] Baker R, Handy R, Davies S, Snook J. 1998. Chronic dietary exposure to copper affects growth, tissue lipid peroxidation, and metal composition of the grey mullet,< i> Chelon labrosus</i>. *Mar Environ Res* 45:357-365.

[36] Larose C, Canuel R, Lucotte M, Di Giulio RT. 2008. Toxicological effects of methylmercury on walleye (Sander vitreus) and perch (Perca flavescens) from lakes of the boreal forest. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 147:139-149.

[37] Van Dyk J, Cochrane M, Wagenaar G. 2012. Liver histopathology of the sharptooth catfish< i> Clarias gariepinus</i> as a biomarker of aquatic pollution. *Chemosphere* 87:301-311.

[38] Giguère A, Campbell PG, Hare L, McDonald DG, Rasmussen JB. 2004. Influence of lake chemistry and fish age on cadmium, copper, and zinc concentrations in various organs of indigenous yellow perch (Perca flavescens). *Can J Fish Aquat Sci* 61:1702-1716.

[39] Berntssen M, Hylland K, Julshamn K, Lundebye AK, Waagbø R. 2004. Maximum limits of organic and inorganic mercury in fish feed. *Aquacult Nutr* 10:83-97.

[40] Cizdziel J, Hinners T, Cross C, Pollard J. 2003. Distribution of mercury in the tissues of five species of freshwater fish from Lake Mead, USA. *J Environ Monit* 5:802-807.

[41] Pulsford A, Ryan K, Nott J. 1992. Metals and melanomacrophages in flounder, Platichthys flesus, spleen and kidney. *J Mar Biol Assoc UK* 72:483-498.

[42] Woshner V, O'Hara T, Eurell J, Wallig M, Bratton G, Suydam R, Beasley V. 2002. Distribution of inorganic mercury in liver and kidney of beluga and bowhead whales through autometallographic development of light microscopic tissue sections. *Toxicol Pathol* 30:209.

[43] Bertini I. 2007. *Biological inorganic chemistry: structure and reactivity*. University Science Books.

[44] Manni ML, Tomai LP, Norris CA, Thomas LM, Kelley EE, Salter RD, Crapo JD, Chang L-YL, Watkins SC, Piganelli JD. 2011. Extracellular superoxide dismutase in macrophages augments bacterial killing by promoting phagocytosis. *The American journal of pathology* 178:2752-2759.

[45] Babu U, Failla ML. 1990. Respiratory burst and candidacidal activity of peritoneal macrophages are impaired in copper-deficient rats. *The Journal of nutrition* 120:1692-1699.

[46] Cerone S, Sansinanea A, Streitenberger S, Garcia C, Auza N. 2000. Bovine monocyte-derived macrophage function in induced copper deficiency. *General physiology and biophysics* 19:49-58.

[47] Rotruck J, Pope A, Ganther H, Swanson A, Hafeman DG, Hoekstra W. 1973. Selenium: biochemical role as a component of glutathione peroxidase. *Science* 179:588-590.

[48] Pařízek J, Ošťádalová I. 1967. The protective effect of small amounts of selenite in sublimate intoxication. *Experientia* 23:142-143.

[49] Ganther H, Goudie C, Sunde M, Kopecky M, Wanger P, Hoh S, Hoekstra W. 1972. Selenium: relation to decreased toxicity of methylmercury added to diets containing tuna. *Science,(Wash)* 175:1122-1124.

[50] Khan MAK, Wang F. 2009. Mercury selenium compounds and their toxicological significance: Toward a molecular understanding of the mercury selenium antagonism. *Environ Toxicol Chem* 28:1567-1577.

[51] Ralston NV, Blackwell III JL, Raymond LJ. 2007. Importance of molar ratios in selenium-dependent protection against methylmercury toxicity. *Biol Trace Elem Res* 119:255-268.

[52] Martoja R, Berry JP. 1980. Identification of tiemannite as a probable product of demethylation of mercury by selenium in cetaceans. A complement to the scheme of the biological cycle of mercury. *Vie Milieu* 30:7-10.

18 ARTICLE 3

Sub-cellular distribution of trace elements and liver histology in landlocked Arctic char (*Salvelinus alpinus*) sampled along a mercury contamination gradient

Benjamin D. Barst[a], Maikel Rosabal[a], Peter G.C. Campbell[a], Derek G.C. Muir[c], Xioawa Wang[c], Günter Köck[d], and Paul E. Drevnick[ab,*]

[a]Institut national de la recherche scientifique, Centre Eau Terre Environnement (INRS-ETE), 490 de la Couronne, Québec, Québec, G1K 9A9, Canada

[b]University of Michigan Biological Station and School of Natural Resources and Environment, 440 Church St., Ann Arbor, Michigan, 48109, USA

[c]Aquatic Contaminants Research Division, Environment Canada, Burlington, Ontario, L7R 4A6, Canada

[d]Austrian Academy of Sciences, International research programmes, Dr. Ignaz Seipel-Platz 2, 1010 Vienna, Austria

Article in preparation.

ABSTRACT

We sampled landlocked Arctic char (*Salvelinus alpinus*) from four lakes (Small, 9-Mile, North, Amituk) in the Canadian High Arctic which span a gradient of mercury contamination. Metals (Hg, Se, Tl, and Fe) were measured in char tissues to determine their relationships with health indices (condition factor, hepatosomatic index), stable nitrogen isotope ratios, and liver histology. A sub-cellular partitioning procedure was employed to determine how metals were distributed between potentially sensitive and detoxified compartments of Arctic char livers from a low- and high-mercury lake (Small Lake and Amituk Lake, respectively). Differences in health indices and metal concentrations among char populations were likely related to differences in feeding ecology. Concentrations of Hg, Se, and Tl were highest in the livers of Amituk char, while concentrations of Fe were highest in Small and 9-Mile char. At the sub-cellular level we found that although Amituk char had higher concentrations of Tl in whole liver than Small Lake char, they maintained a greater proportion of this metal in detoxified fractions, suggesting an attempt at detoxification. Mercury was found mainly in potentially sensitive fractions of both Small and Amituk Lake char, indicating that Arctic

char are not effectively detoxifying this metal. Histological changes in char livers, mainly in the form of melano-macrophage aggregates and hepatic fibrosis, could be a result of the concentrations and sub-cellular distributions of essential or non-essential metals.

Keywords: Arctic char, mercury, sub-cellular distribution, metals

INTRODUCTION

Anthropogenic emissions of Hg to the atmosphere, notably from fossil fuel combustion, have led to increased atmospheric Hg deposition in even the most remote ecosystems such as the Canadian High Arctic.[1] This occurs through long-range transport of gaseous elemental Hg, which is then converted to inorganic Hg (Hg(II)) before removal from the atmosphere by wet and dry deposition. During summer months Hg and other elements, stored in watersheds, may be transported to Arctic lakes in snowmelt. Once in aquatic environments Hg(II) can be converted by microbes to methylmercury (MeHg), which readily bioaccumulates and biomagnifies resulting in elevated concentrations of Hg in predatory fish.[2] Mercury concentrations in edible muscle of predatory fish from lakes in northern Canada usually exceed the Health Canada guideline of 0.2 µg g-1 wet weight for subsistence consumption.[3]

Mercury concentrations in predatory fish may also be toxic to the fish themselves. Recent analyses of Hg toxicity data indicate that effects in fish are likely to occur at whole-body concentrations above 0.2 µg g^{-1} [4], or 0.3 µg g^{-1} wet weight[5-6] (equivalent concentrations in edible muscle are 0.33 and 0.5 µg g^{-1} wet weight, respectively), or for

dietary concentrations above 0.2 µg g^{-1} wet weight.[7] Landlocked Arctic char (*Salvelinus alpinus*) have a circumpolar distribution and are often the only species of fish present in Arctic lakes. For these reasons, Arctic char populations have been targeted extensively by monitoring programs which aim to describe spatial and temporal trends of Hg and other contaminants in the Arctic.[8] A comparison of the available Hg data in landlocked char with the aforementioned thresholds indicates that several of the fish populations sampled in northern Canada may be at risk.[9] Though monitoring programs, which have been established to track changes in the levels of Hg and other metals in landlocked Arctic char and other Arctic species, will remain an integral part of Arctic contaminant research, more studies on how Hg affects Arctic fish and wildlife are necessary.[10]

Several studies have documented correlations between altered liver histology and Hg concentrations in wild fish. For example, Drevnick and colleagues[11] found evidence of liver damage, as a result of oxidative stress, in northern pike (*Esox lucius*) with elevated Hg concentrations. Melano-macrophage aggregates, which scavenge cells damaged by oxidation, have been shown to increase in size and number with increasing Hg concentrations in several fish species.[12-14] A dietary exposure performed in the laboratory supports findings from the field that show Hg exposure alters liver histology.[15] With chronic Hg toxicity and the liver's continued but ineffective attempt at Hg detoxification, fish can be in poor condition.[11, 16]

As a defense mechanism, hepatocytes, the main cell type of the liver, are equipped with high levels of intracellular binding proteins and peptides which aid in the sequestration of non-essential metals, thus preventing their interaction with potentially sensitive sites.[17-18] To determine the intracellular distribution of metals, prior studies have employed partitioning procedures which isolate sub-cellular fractions so that their metal concentrations may be determined.[19-22] Non-essential metals found in potentially sensitive sub-cellular compartments may indicate the potential for toxicological effects.[18] However, studies combining evidence of effects with the sub-cellular distribution of non-essential metals are lacking.

To address these issues, we sampled Arctic char (*Salvelinus alpinus*) from four lakes in the Canadian High Arctic which have been the focus of routine monitoring for several

years and span a gradient of Hg contamination. A previous study[23] on Arctic char from the lake representing the high end of the Hg gradient (Amituk Lake), also reported elevated levels of thallium (Tl) in char muscle. Therefore, we measured Hg, Tl, as well as several essential metals in char tissues and evaluated liver histology of individuals from the four lakes. We also determined essential and non-essential metal partitioning in isolated sub-cellular liver fractions of char from a low and a high Hg lake to determine how Hg and other metals (Se, Tl, and Fe) were distributed within liver cells.

EXPERIMENTAL SECTION

Study sites, sampling methods, and measures of condition. The four study lakes (Small, North, 9-Mile, and Amituk) are located on Cornwallis Island, NU (75°08′N 95°00′W), in the Canadian High Arctic. The lakes are connected to the sea by small streams that do not allow migration and thus, char are landlocked. The four lakes are ultra-oligotrophic and char are the only fish species present, feeding mainly on chironomids (Diptera: Chironomidae); however some large char switch to cannibalism as an alternate feeding strategy.[23-24] Lakes were selected to represent a Hg contamination gradient based on Hg data presented in previous studies.[23, 25] Lake chemistry, Hg biomagnification, and catchment areas have been described elsewhere.[23, 26]

Arctic char were sampled from the four lakes in July/August of 2011 and 2012 with gill nets. Char (n=114) were measured for length and weight before being sacrificed. Axial muscle was collected, bagged, and frozen for analysis of total Hg and stable isotopes of carbon and nitrogen. Otoliths were collected for aging by John Babaluk (Winnipeg MB) using a break-burn method or thin-section method.[27] Relative condition factor (CF) was calculated for each individual according to the following equation:

$$CF = \frac{W_T}{W'}$$

Where W_T is the total weight of the fish and W' is the calculated weight for the observed length based on the length-weight relationship for all char.[28]

Livers were dissected and weighed. Subsamples were preserved in buffered formalin for 48 hours, and then transferred to 70 % ethanol for histology. Additional, subsamples

of livers from Small and Amituk Lake char were placed in liquid nitrogen for sub-cellular partitioning. The remaining liver tissues were placed in food-grade plastic bags and frozen at -20 ℃ for metal determinations. A hepato somatic index (HSI) was calculated for these fish according to the following equations:

$$HSI = \left(\frac{W_L}{W_T}\right) \times 100$$

where W_L is the liver wet weight and W_T is the total wet weight of the fish.

Stable isotope analyses. A few milligrams of freeze-dried muscle from each fish were ground to a fine powder and analyzed for stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) at the Environmental Isotope Laboratory, University of Waterloo, Waterloo ON. Stable isotope analyses were performed on a Micromass VG Isochrom continuous-flow isotope ratio mass spectrometer connected to a Carlo Erba elemental analyzer.[25] The ratio of C to N was calculated for individual char as a proxy for lipid content, similar to a previous study.[29]

Sub-cellular Trace-Element Partitioning. Subsamples of Arctic char livers (~50 mg wet weight; n=4) were combined to form composite samples (n=3 Small Lake and n=3 Amituk Lake). We used a sub-cellular trace-element partitioning procedure that separated char liver cells into six operationally defined fractions: (1) “Debris” (nuclei and cellular debris), (2) “Granules” (NaOH-resistant fraction/granule-like concretions), (3) Mitochondria, (4) “Organelles” (lysosomes and microsomes), (5) heat-denatured proteins (HDP) including enzymes, (6) peptides and heat-stable proteins (HSP) including metallothionein and glutathione (GSH). The procedure (SI Methods) involves a series of differential centrifugations as well as NaOH digestion and heat treatment steps.[21]

Hg analyses. Liver and axial muscle subsamples for Hg analyses were freeze-dried to constant weight and homogenized. Total Hg was determined by direct mercury analyzer (DMA-80, Milestone Inc., Monroe, CT). Additional subsamples of liver tissue were analyzed for Hg(II) according to Barst et al.[30] MeHg was calculated by subtracting the Hg(II) concentration from the total Hg concentration in the same sample. Freeze-dried liver homogenates and sub-cellular fractions (n=6 composites) of Small and Amituk char

were solubilized with 7% HCl to facilitate their transfer to quartz analytical vessels where they were analyzed for total Hg by DMA-80. Quality assurance included the analysis of certified reference materials (MESS-3, marine sediment and DOLT-4, dogfish liver, National Research Council of Canada) and duplicate samples.

Elemental analyses. All labware used for the trace-element partitioning procedure was soaked in 15% nitric acid (v/v; Omnitrace grade, Fisher Scientific), rinsed with ultrapure water (18 MΩ cm) and dried under a laminar-flow hood to prevent trace-element contamination. Freeze-dried homogenate samples and sub-cellular fractions were digested in 100 µL of nitric acid (Optima grade, Fisher Scientific) per mg dry weight for two days. Samples were then heated for 2 h at 65 ℃. Samples were allowed to cool to room temperature before 40 µL per mg dry weight of hydrogen peroxide were added (Optima grade, Fisher Scientific). After 1 d ultrapure water, 1 mL per mg dry weight, was added to the digestates.

Metal concentrations in 1) homogenate samples and associated sub-cellular fractions (n=6 composites), and 2) subsamples of individual char livers (n=7 to 9 per lake), were measured either by inductively coupled plasma mass spectrometry (ICP-MS; Elemental X Series; selenium (Se), and Tl) or by inductively coupled atomic emission spectroscopy (ICP-AES; Vista AX; iron (Fe)). Samples of certified reference material (TORT-3, lobster hepatopancreas, National Research Council of Canada) were digested using the same digestion procedure and analyzed. A mass balance calculation was used to compare elemental burdens in a 100 µL aliquot of the liver tissue homogenate to the sum of the elemental burdens in each fraction.

QA/QC. For the analysis of total Hg in liver, Hg recovery from certified reference materials (MESS-3, DOLT-4, TORT-2) averaged (mean ± standard deviation) 95 ± 3.7 % (n=16) and the mean relative percent difference of duplicate samples was 0.9% (n=9). For muscle mean total Hg recovery from DOLT-4, TORT-2, and NIST SRM 2976 was 96 ± 2.6% (n=28). The calculated concentrations of MeHg were within 100 ± 7.8 % (n=10) of the certified value of DOLT-4 and the mean relative percent difference of duplicate samples was 1.6% (n=13). Recoveries of Se and Fe from TORT-3 were 90 ± 6 % and 92 ± 3.2 % (n=3). Note that we did not find an appropriate reference material

for Tl. Recoveries of metals in sub-cellular fractions, relative to the liver homogenate were: Fe 94 ± 26 %, Hg 93 ± 5.4 %, Se 75 ± 11 %, and Tl 99 ± 26 % (n=6).

Histology. Fixed subsamples of liver were embedded in paraffin, sectioned at seven µm, and mounted on glass slides. Sections were stained with eosin and hematoxylin (H&E) and viewed with light microscopy (Axiovert 200, Zeiss Microscopy) to evaluate pathology. Melano-macrophage aggregates (MA), if present, were counted at low magnification (100X) in each of three microscope fields to generate a mean hepatic MA count per fish. Additional sections were stained with Prussian blue to detect deposits of Fe (as Fe(III)) in liver sections.

Statistical Analyses. Statistics were performed with JMP 9 Statistical Analysis Software (SAS Institute, Cary, NC, USA). Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) and were $\log_{10}$ transformed if necessary. When the previous assumptions were not met, the nonparametric Kruskall-Wallis test was used to compare metal concentrations among lakes; if assumptions were met, one way analysis of variance (ANOVA) was used. Multiple comparisons were examined with the Tukey-Kramer HSD test or an appropriate non-parametric test. Linear regression models were used to describe relationships among variables (metals, length, weight, age, HSI, CF, $\delta^{15}N$, C:N, MA). The residuals of the linear regression models were tested for normality and tested in order to determine whether outliers had biased the coefficients of determination. Elemental concentrations in sub-cellular fractions prepared from Small and Amituk char were compared using Wilcoxon signed-rank tests. Histological abnormalities were compared among lakes using Fisher's Exact tests which were Bonferroni-corrected. An $\alpha=0.05$ was used for all other statistical analyses.

RESULTS

Arctic char size and age distribution. Summary data for Arctic char are presented in Table 1. Arctic char consisted of males (n=36), females (n=61), and immature fish (n=17) whose sex could not be determined. The mean lengths of Arctic char were similar among lakes, however their mean weights differed significantly ($p<0.02$), as char from North Lake were significantly heavier than those from Small. Char ages also

differed significantly among lakes, as fish from 9-Mile and North Lake were significantly older than fish captured from Small Lake ($p<0.0005$). Ages and lengths were positively correlated in Small ($r^2=0.60$, $p<0.0001$), 9-Mile ($r^2=0.65$, $p<0.0001$), North ($r^2=0.47$, $p<0.0001$), and Amituk char ($r^2=0.30$, $p=0.004$). Values of $\delta^{15}N$ increased with length, in char from 9-Mile ($r^2=0.85$, $p<0.0001$) and Amituk ($r^2=0.53$, $p<0.0001$), but not for char from Small ($r^2=0.12$, $p=0.06$) and North ($r^2=0.22$, $p=0.22$). Similar relationships existed between $\delta^{15}N$ and the weights of char from 9-Mile ($r^2=0.77$, $p<0.0001$), Amituk ($r^2=0.41$, $p<0.0006$), North ($r^2=0.07$, $p=0.17$), and Small ($r^2=0.07$, $p=0.13$).

Table 1. Summary statistics for lengths, weights, ages, stable isotope ratios, and concentrations of metals (µg g^{-1} wet weight) measured in landlocked Arctic char from four lakes on Cornwallis Island, Nunavut. All metal concentrations were measured in liver tissue unless noted otherwise. Different letters in the same column note significant differences ($p<0.05$).

Lake	Stat	Total Length (mm)	Weight (g)	Age (yr)	Condition	HSI	$\delta^{13}C$	$\delta^{15}N$	Hg in muscle	Hg	Se	Fe	Tl
Small	Mean	358	274^{a}	14^{a}	0.91^{a}	0.82^{a}	-23.22	9.30^{a}	0.095^{a}	0.208^{a}	1.80^{a}	1219^{a}	0.008^{a}
n=8*	min	280	140	8	0.69	0.48	-24.12	7.73	0.040	0.090	1.29	707.9	0.004
n=30**	max	402	360	19	1.24	1.86	-22.50	11.49	0.189	0.450	2.43	1681.5	0.018
9-Mile	Mean	366	356^{ab}	18^{c}	1.0^{b}	1.11^{b}	-22.19	10.10^{b}	0.154^{b}	0.335^{ab}	2.38^{a}	735.5^{ab}	0.030^{a}
n=7*	min	290	150	12	0.77	0.68	-24.09	8.82	0.058	0.080	1.18	158.3	0.014
n=30**	max	530	1170	29	1.31	1.62	-21.41	12.64	0.394	1.100	3.00	1012.1	0.042
North	Mean	393	439^{b}	17^{bc}	1.04^{b}	1.31^{bc}	-23.75	11.09^{c}	0.255^{c}	0.555^{b}	1.76^{a}	640.5^{b}	0.023^{a}
n=8*	min	330	270	11	0.85	0.51	-25.99	9.77	0.092	0.160	0.93	69.7	0.006
n=28**	max	467	830	25	1.22	2.43	-22.50	12.94	0.699	1.370	2.29	1289.7	0.059
Amituk	Mean	364	367^{ab}	15^{ab}	0.98^{b}	1.58^{c}	-23.22	11.54^{c}	0.828^{d}	1.78^{c}	4.37^{b}	120.8^{c}	0.113^{b}
n=9*	min	215	60	8	0.58	1.01	-25.20	8.17	0.139	0.260	3.64	29.3	0.058
n=25**	max	536	1220	24	1.25	3.37	-21.98	13.09	2.077	6.460	6.46	289.8	0.259

*sample size of livers analyzed for Se, Fe, and Tl

**sample size for all other endpoints

We found no significant differences in CF among male, female, or immature char ($p=0.37$), and therefore all fish were used for comparison among lakes. Arctic char from North had significantly higher CFs than char from Small Lake ($p=0.002$). Females had significantly higher HSI than male char ($p=0.0027$), therefore among lake comparisons were made for males and females separately. For male char, mean HSI was significantly higher for fish from Amituk and 9-Mile than those collected from Small Lake ($p=0.0002$). For females, mean HSI was significantly higher for North and Amituk Lake char than for Small Lake char ($p<0.0001$). The C:N values, an indicator of lipid content,[29] increased with $\delta^{15}N$ ($r^2=0.89$, $p<0.0001$). This ratio differed significantly between lakes ($p<0.0001$), with mean values which were significantly higher for Amituk and North Lake char than for those from Small Lake. The relative weight of the liver was positively correlated with C:N in both female ($r^2=0.29$, $p<0.0001$) and male ($r^2=0.37$, $p<0.0001$) Arctic char. However, C:N values were not correlated with the CF of Arctic char.

Metal concentrations in Arctic char tissues. Summary data for metals in char tissues are presented in Table 1. For all char, muscle total Hg was positively correlated with length ($r^2=0.19$, $p<0.0001$) and age ($r^2=0.0.06$, $p<0.0001$), therefore the largest and oldest char collected from each of the four study lakes tended to have the highest Hg concentrations. Before comparison among lakes, log transformed Hg concentrations were length corrected by comparing the residuals obtained from linear regressions between log total Hg and char length. Char from Amituk Lake had the highest total Hg concentrations in muscle with 73% and 77% of the individuals exceeding the toxicity thresholds reported by Dillon et al.[5] and Beckvar et al.[4], respectively. Fewer individuals, 11% and 21%, from North Lake exceeded these thresholds, while only 6% of the char from 9-mile had concentrations above the threshold presented by Beckvar et al.[4] None of the char from Small Lake had total Hg concentrations in muscle above 0.33 µg g^{-1} wet wt. Concentrations of total Hg in liver were 2 to 5 times greater than in muscle, with a maximum of 6.5 µg g^{-1} wet wt for an individual from Amituk Lake. In char from all lakes the proportion of MeHg ranged from 51 to 90% of the total Hg in livers, and increased exponentially with $\delta^{15}N$ values ($r^2=0.70$, $p<0.0001$). Muscle total Hg was positively correlated with $\delta^{15}N$ values in Small, 9-Mile, North, and Amituk Lake. Char

sampled from Amituk Lake had the highest mean $\delta^{15}N$ values followed by char from North, 9-Mile, and Small. In Arctic char livers, from all four lakes, Tl ranged from 0.004 to 0.26 µg g^{-1} wet wt. Char from Amituk Lake had significantly greater hepatic concentrations of Tl than char from the remaining three lakes ($p<0.0001$). Like Hg, concentrations of Tl increased significantly with $\delta^{15}N$ values ($r^2=0.61$, $p<0.0001$). In liver samples, Se ranged from 0.9 to 6.5 µg g^{-1} wet wt. Concentrations of hepatic Se were significantly higher in char from Amituk Lake than in fish from the other study lakes ($p<0.0001$) and were positively correlated with total Hg ($r^2=0.48$, $p<0.0001$). In terms of molar concentration, there was a greater amount of Se than Hg (mean molar ratio Hg:Se = 0.15) in all char livers, though the stoichiometry approached unity with increasing Hg concentration to a maximum ratio of 0.7 in a fish from Amituk Lake. Hepatic Fe ranged from 29 to 1680 µg g^{-1}wet wt across lakes and was significantly greater in Small Lake than in North and Amituk char livers ($p<0.0001$).

For all char, CF was not related to total Hg in muscle or liver. Char CF was negatively correlated with Fe ($r^2=0.32$, $p=0.0008$), and positively correlated with Tl ($r^2=0.19$, $p=0.01$). Similar relationships existed for HSI and Fe ($r^2=0.28$, $p=0.044$), and Tl ($r^2=0.39$, $p=0.012$) in female char. For male char, HSI decreased with increasing Fe ($r^2=0.58$, $p=0.0006$), and increased with Tl ($r^2=0.46$, $p=0.004$). Female HSI was also weakly correlated with total Hg in liver ($r^2=0.12$, $p=0.006$) and muscle ($r^2=0.21$, $p=0.0002$). In male char HSI was positively correlated with total Hg in liver ($r^2=0.39$, $p<0.0001$) and muscle ($r^2=0.41$, $p<0.0001$).

Sub-cellular partitioning. We measured essential and non-essential metals in operationally defined sub-cellular fractions in order to determine how they were distributed in Arctic char liver cells and if differences existed in their sub-cellular distributions for Small and Amituk Lake char. Sub-cellular fractions were combined into sensitive (mitochondria + HDP + organelles) and detoxified (HSP + granule-like) groups in order to determine the potential for effects. The “debris” fraction, containing cellular debris and nuclei, cannot be assigned to either of these groups as the toxicological importance of metals in this fraction is difficult to interpret.[31] Thus, we have focused on the presence of metals in the remaining fractions. For each metal, we determined the relative contribution (i.e. percentage) of each fraction to the total metal concentration in

the liver homogenate as well as the concentrations of metals in each fraction, based on liver dry weight. Results for individual metals are described below and are presented in Figure 1.

Despite the significantly higher Hg concentrations in Amituk livers, Hg was distributed similarly in liver cells of char from both lakes; sensitive compartments contributed 73 and 61% of the contributions in Small and Amituk livers. The remaining Hg was found in the detoxified compartments, which comprised 10 and 19% of the Hg contributions of Small and Amituk char respectively. Almost all of the Hg in the detoxified compartment was associated with the HSP fraction, presumably containing metallothionein, whereas very little was found in the granule-like fraction. In the potentially sensitive compartment, Hg accumulated mainly in the mitochondria and HDP fraction.

We found significantly greater contributions of Tl in all of the isolated fractions from Amituk char than from Small Lake char. The majority of Tl was associated with sensitive fractions of Small Lake char (53%). Amituk char, however, had lower Tl proportions in sensitive fractions (42%). The majority of Tl in the detoxified compartment was found in the HSP fraction, though Tl was also found in the fraction containing granule-like structures. The greatest contribution of Tl to the potentially sensitive compartment was found in the fraction containing mitochondria.

Even though Se concentrations were greater in Amituk char livers than in those from Small Lake, Se was distributed similarly between sensitive and detoxified compartments in char from both lakes. The sensitive fractions contributed 58 and 55% of the Se in Small and Amituk char livers, respectively. In the sensitive compartment, Se was found predominately in the fraction containing mitochondria, followed by the HDP fraction and the organelles. In the detoxified compartment Se was found mainly in the HSP fraction and to a lesser extent the granule-like fraction.

Though Fe concentrations were significantly higher in the livers of char from Small Lake, Fe was predominately found in the sensitive fractions of both Small (63%) and Amituk (70%) livers. A significantly greater contribution of Fe was found in the fraction containing microsomes and lysosomes in fish from Small Lake compared with Amituk

Lake char. Amituk char had significantly higher Fe contributions in the fractions containing mitochondria and debris than in Small Lake char.

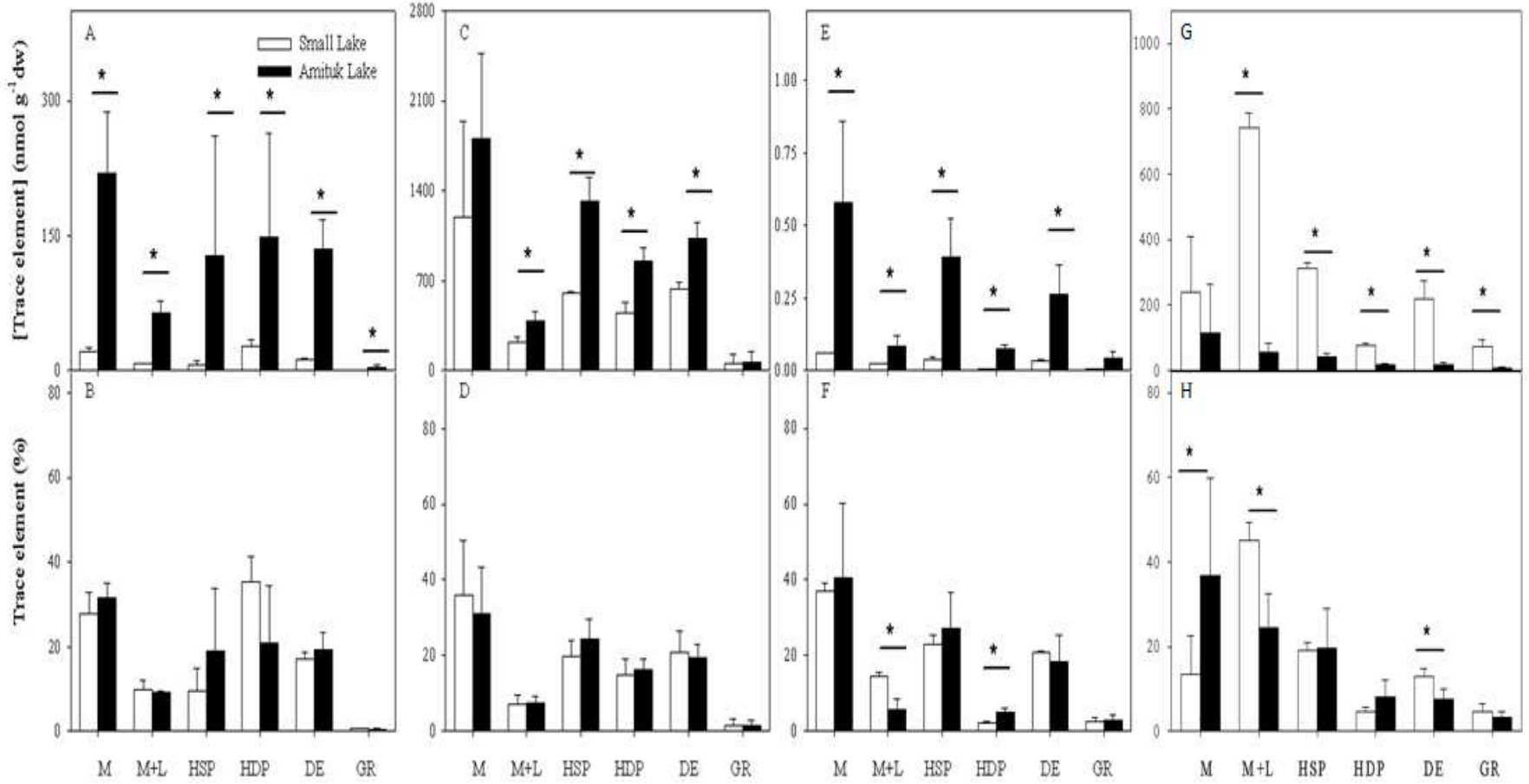

Figure 1. Sub-cellular partitioning of Hg (panels A and B), Se (panels C and D), Tl (panels E and F), and Fe (panels G and H) in the livers of landlocked Arctic char collected from Small Lake (white bars) and Amituk Lake (black bars). The concentrations of each element in the sub-cellular fractions are represented in upper panels (A, C, E, and G) and the element contribution (%) in lower panels (B, D, F, and H). Significant differences between Small Lake and Amituk fractions are denoted by * (Wilcoxon signed-rank test, $p<0.05$). M = mitochondria, M+L = microsomes and lysosomes, HSP = heat-stable proteins, HDP = heat denatured proteins, DE = cellular debris and nuclei, GR = granule-like structures.

Liver histology. Arctic char livers ranged from those that appeared healthy to livers that exhibited abnormal morphology. Healthy livers had homogenous parenchyma composed of chords of hepatocytes bordered by typical sinusoids (Figure 2). The most notable pathology in abnormal livers was fibrosis in the perisinusoidal area (Figure 3). A significantly greater number of individuals from Amituk Lake (83%) exhibited this abnormality than individuals from the other three lakes ($p<0.008$). Evidence of fibrosis was also found in individuals from 9-Mile (29%) and North Lake (27%). None of the livers from Small Lake char presented this abnormality. We also noted necrotic foci in three fish from Amituk Lake, two fish from North Lake, and one fish from Small Lake, but no significant differences could be determined based on the low number of fish with this pathology (Figure 4).

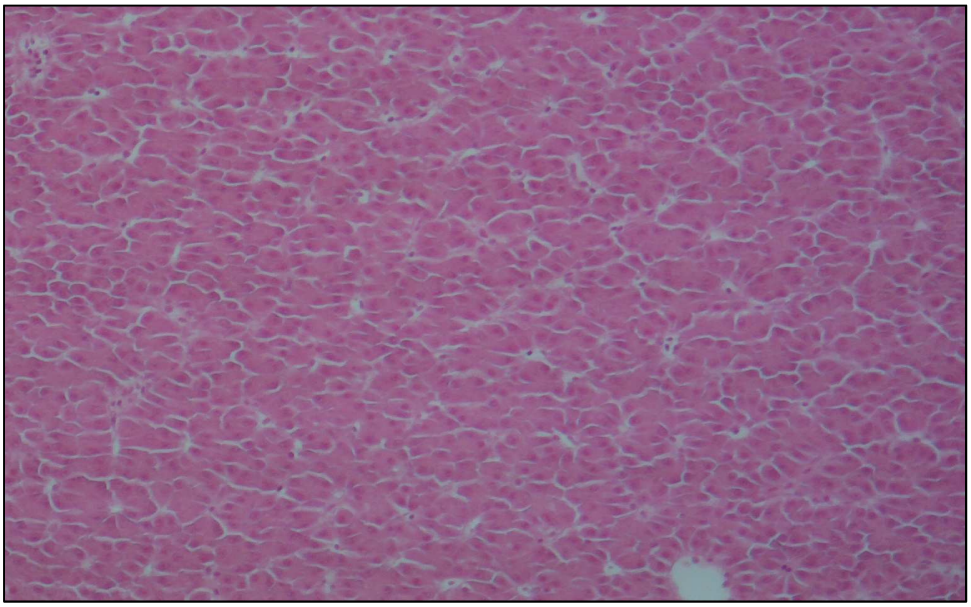

Figure 2. Micrograph of a liver section from a 13 year old female landlocked Arctic char (*Salvelinus alpinus*) from Small Lake showing normal liver morphology (H and E stained, 200X).

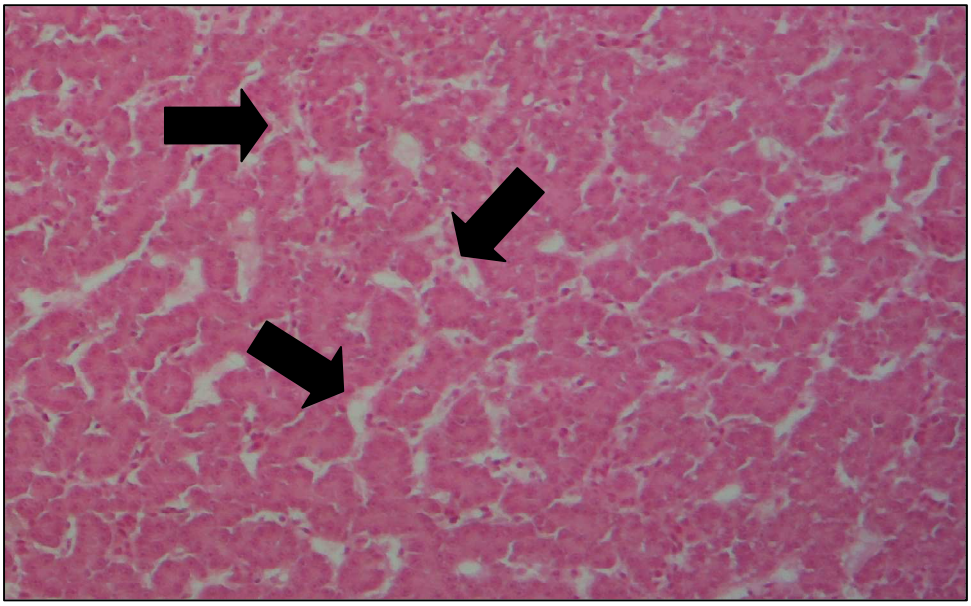

Figure 3. Micrograph of a liver section from a 13 year old female landlocked Arctic char (*Salvelinus alpinus*) from Amituk Lake showing abnormal perisinusoidal regions (black arrows) (H and E stained, 200X).

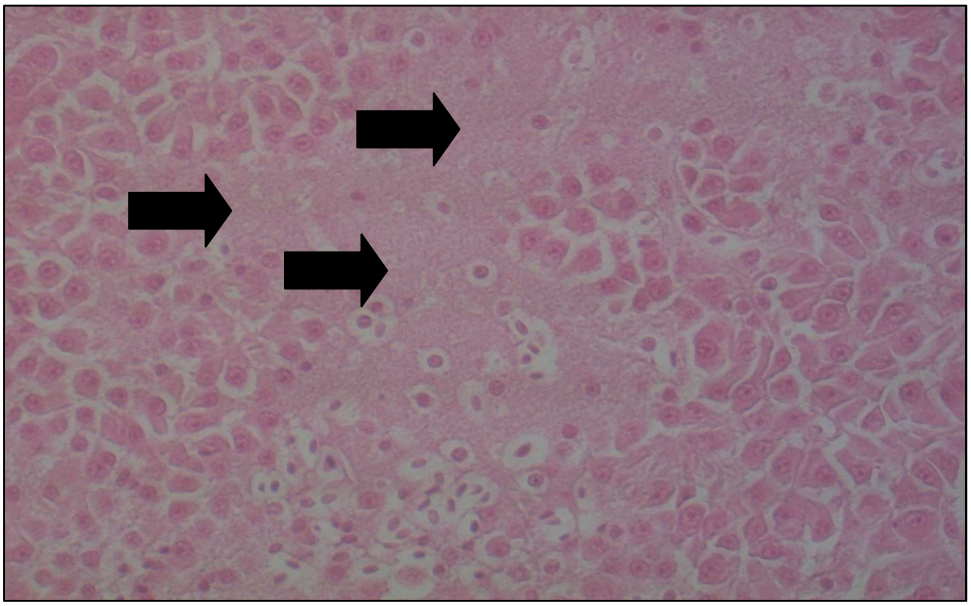

Figure 4. Micrograph of a liver section from a 14 year old male landlocked Arctic char (*Salvelinus alpinus*) from Amituk Lake showing severe necrosis (black arrows). (H and E stained, 400X)

In many of the char liver sections we identified MA as discrete pathologies containing pigments consistent with melanin, hemosiderin, and lipofuscin (Figure 5). Numbers of MA were positively correlated with age in char from 9-Mile, North, and Small Lake. There was no significant relationship between MA and age in Amituk char. Nevertheless, when combining data for char from all lakes, counts of hepatic MA were positively correlated with age (r^2=0.25, $p<0.0001$). During histological investigation of Arctic char livers, we expected to find higher numbers of hepatic MA in individuals from Amituk Lake, as char from this lake were known to have elevated Hg concentrations and several studies have reported MA in Hg-exposed fish.[12, 15] Surprisingly, we found very few MA in the livers of Amituk char (mean = 0.52) compared to char from 9-Mile (mean = 5.2) and Small Lake (mean = 5.4; $p<0.0001$).

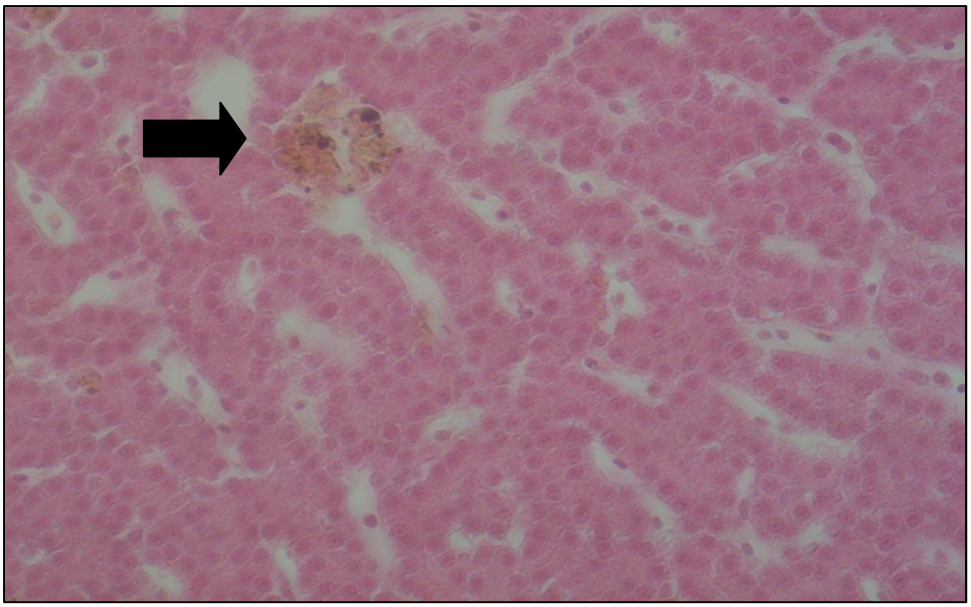

Figure 5. Micrograph of a liver section from a 17 year old female landlocked Arctic char (*Salvelinus alpinus*) from Small Lake showing a melano-macrophage aggregate (black arrow) (H and E stained, 400X).

We found positive correlations between Hg and MA in the livers of char from 9-Mile, North, and Small Lake, but there was no relationship between numbers of MA and Hg in Amituk char. We hypothesized that low numbers of these immune cells in Amituk livers could be due to an inhibitory effect of Hg, i.e. MA increase with increasing concentration

of Hg to a threshold, above which MA no longer form. We tested this by combining MA data for all fish and regressing it with Hg concentrations, however there was no significant relationship between the two variables.

None of the other metals were significantly correlated with numbers of MA, when regressions were performed by lake. This may have been an effect of low sample size as we only analyzed <10 individual livers from each lake for metals other than Hg. When combining data from all lakes, numbers of MA were positively correlated with hepatic Fe concentrations (r^2=0.21, p=0.016).

DISCUSSION

Metal concentrations in Arctic char. Gantner and colleagues[25] examined Hg concentrations in 27 Arctic char populations across the Canadian Arctic, including the four populations in the present study. Their results showed that char from Small Lake had the lowest muscle Hg concentrations and char from Amituk Lake had the highest. Therefore our study, though based on fewer populations, includes landlocked Arctic char with Hg concentrations that range from the lowest to the highest that are likely to be found in the Canadian Arctic. Differences in Arctic char Hg concentrations may be due to physical and chemical characteristics of lakes[26], as well as a result of char feeding ecology. Our finding that Hg correlates with δ^{15}N, reinforces results presented in previous studies[23, 26, 32] , which indicate that food web structure plays an important role in the level of Hg contamination in landlocked Arctic char. Similar to the results of Gantner et al.[25] as well as our own, Muir et al.[23] reported elevated concentrations of Hg in Arctic char from Amituk Lake, which were attributed to greater numbers of piscivorous char. Positive correlations between Tl and δ^{15}N have been previously reported for char from Amituk Lake[23] and char from Lake Hazen[33] (Nunavut, Canada), suggesting that Tl also biomagnifies in these food webs. Elevated Se concentrations in Amituk fish have previously been reported, as have positive correlations between Se and Hg in Arctic char muscle tissue.[23] The negative relationships between δ^{15}N and Fe suggest that char feeding at higher trophic positions, i.e. those feeding on other char, are less exposed to these metals. In the study lakes, the main food source for non-cannibalistic char is

chironomids,[23-24] which have high levels of Fe-rich hemoglobin in their tissues giving them the ability to live in areas of low oxygen.[34] A diet based predominately on larval chironomids could account for the elevated hepatic Fe concentrations in Small and 9-Mile char and the low Fe in Amituk char.

Health indices. We explored relationships between concentrations of metals in Arctic char and general measures of fish health. The body CF is the relation between a fish's length and weight, or "plumpness", whereas HSI gives the relative weight of the liver. Higher HSI and CF are generally interpreted as elevated fat or glycogen stores in the liver and body. How these indices relate to essential and non-essential metals varies throughout the literature as studies have documented positive, negative, and insignificant relationships as a result of metal exposure.[17] The positive correlation between $\delta^{15}N$ values and HSI may be interpreted as positive effects of feeding at higher trophic positions, i.e. char have greater fat reserves as a result of feeding on other char. This conclusion is supported by the positive correlation between C:N and $\delta^{15}N$ and C:N and HSI. The positive correlations of Hg and HSI, as well as Tl with HSI and body condition, are likely a reflection of trophic position rather than a positive effect of these metals on the health of char, though hormesis has been previously reported for Hg in laboratory studies[35] as has liver hyperplasia.[36] Higher hepatic concentrations of Fe were associated with relatively low HSI and CF, which may be an effect of these metals or a result of feeding on chironomids which are rich in Fe, but provide less energy to the consumer than smaller char.

Sub-cellular metal partitioning. Protocols used to determine the sub-cellular partitioning of metals, based on differential centrifugation, are operationally defined and may suffer from an overlap of sub-cellular fractions, organelle aggregation, and other issues.[20, 31] Note too that the lumping of fractions into sensitive (HDP, mitochondria, microsomes and lysosomes) and detoxified (HSP and granule-like) compartments is probably an oversimplification.[37] Nevertheless, partitioning procedures for the determination of metals at the sub-cellular level do provide useful information about how aquatic organisms deal with both essential and non-essential metals.

Elevated proportions of Hg bound to sensitive sites and similar Hg sub-cellular distributions suggest that Hg is not well detoxified in the livers of Arctic char from either lake. Mercury has strong binding affinities for Se and sulfur, which within organisms are mostly present as selenols and thiols. These biomolecules are well distributed throughout cells and thus there are numerous potential binding sites for Hg.[38] In the HSP fraction, Se is most likely incorporated as seleno-cysteine[39] in thermostable metal-binding proteins such as metallothionein, whereas in the HDP fraction it is likely incorporated into the active sites of Se-dependent enzymes (HDP) such as GSH peroxidase (GPx). Thiols are found in the amino acid cysteine, cysteine residues of proteins, and the tri-peptide GSH. Though Hg forms complexes with thiols in biological systems, it has a greater affinity for selenols.[38, 40] This high binding affinity makes Se a molecular target of Hg[41] and may explain the similar sub-cellular distributions of these two elements in Arctic char livers.

The accumulation of Hg in the mitochondria and HDP fractions may have negative consequences for cellular respiration and enzymes of the redox defense system. Evidence for this comes from laboratory studies which have demonstrated that dietary MeHg exposure leads to structural abnormalities of mitochondria and inhibition of respiration[42], as well as decreased activity of GPx.[43] Our findings that relatively little Hg accumulated in the fraction containing thermostable proteins (HSP), and presumably metallothionein, are similar to the results of a recent study which also found a low percentage of Hg in the HSP fraction of wild mullet (*Liza aurata*) livers. The low percentage of Hg in the HSP fraction could have been a result of Hg concentrations which did not exceed the physiological threshold for metallothionein induction.[44] However, given the extreme Hg concentrations in Amituk char livers, it seems unlikely that the low proportion of Hg in the HSP fraction is a result of metallothionein not being induced. One possible explanation for the low percentage of Hg in this fraction may be related to Hg speciation. We did not measure Hg speciation in individual liver fractions and therefore do not know how MeHg and Hg(II) were distributed at the sub-cellular level. However, MeHg was the predominant form of total Hg in Arctic char liver homogenates and MeHg's relative contribution increased with increasing total Hg. Inorganic Hg has been shown to induce and bind with metallothionein.[45] Conversely,

MeHg is not thought to induce metallothionein synthesis[45] and likely binds less strongly with its cysteine residues.[38] However, the assumption that a higher proportion of Hg(II) in the liver would lead to a greater proportion of Hg in the HSP fraction has not been tested in fish. Note that aquatic invertebrates exposed to Hg(II) also accumulated very little Hg in the HSP fraction.[46]

There is a scarcity of information about how Tl is distributed at the sub-cellular level in aquatic organisms, but a recent study found that Tl primarily accumulated in the HSP fraction in the livers of European (*Anguilla anguilla*) and American (*Anguilla rostrata*) eels. In eel livers, Tl also accumulated in granule-like structures and sensitive sub-cellular pools.[20] This is similar to our findings, which show that Tl accumulated in both sensitive and detoxified compartments with increasing total concentrations in whole liver. Interestingly, Amituk char maintained a smaller proportion of Tl in the sensitive compartment, suggesting that these fish may be better at detoxifying this metal.

Iron readily cycles between oxidized and reduced forms, and in its reduced form it has the ability to bind with oxygen. Thus, organisms have evolved to use Fe during biochemical processes such as respiration. Iron's redox activity also gives it the potential to damage cells, which is why organisms have also evolved mechanisms to tightly control Fe transport and storage.[47] In Small Lake char livers, Fe was predominately associated with the organelle fraction containing microsomes and lysosomes. Though we cannot be certain whether Fe mainly accumulated in microsomes or lysosomes within this fraction, accumulation in the latter is more likely given that lysosomes within macrophages are known to play an important role in the metabolism and storage of Fe.[47-48]

Liver Histology. Sinusoids serve as the microvasculature of the liver, which transport blood and dissolved substances throughout the organ. The basal surfaces of hepatocytes face these vessels and, thus come into close contact with both desirable and toxic substances.[17] Over time, the latter may cause chronic damage in the form of fibrosis.[49] Toxins are known to activate, directly or indirectly[50], fat-storing cells (stellate cells) which are located in the perisinusoidal space separating endothelial cells and hepatocytes. These cells secrete collagen,[51] which may be responsible for the

inflammation noted in Amituk char livers. In a lab study with Arctic char, Oliveira et al.[52] found exposure to dietary MeHg caused proliferation of connective tissue, of which collagen is a main component. Similar to our findings, Mela et al.[15] found evidence of tissue damage in the perisinusoidal space during ultrastructural examination of the livers of a neotropical fish exposed to dietary MeHg. Char from Amituk and Small were of similar age, therefore, the presence of fibrosis in the livers of Amituk char cannot be a result of the aging process. Arctic char are commonly hosts to cestode parasites, which have been shown to increase in number in piscivorous char.[53] These parasites may traverse the intestinal wall, enter the liver and other organs, resulting in fibrosis and visceral adhesions.[53-54] Parasite loads of Arctic char in the present study are significant and appear to increase with age.

Age may have played a role in the generation of hepatic MA, however Amituk and Small lake char had significantly different numbers of hepatic MA but similar ages, suggesting that age cannot fully explain their presence. The higher numbers of MA in Small Lake and 9-Mile char are most likely a result of elevated hepatic Fe concentrations. In higher vertebrates Fe is stored as ferritin, but above a certain threshold Fe is stored as hemosiderin, which is a common granular pigment found in the MA of fish and is consistent with what we observed in the hepatic MA of 9-Mile and Small Lake char. This was further supported by the visualization of Fe(III) in MA and the sub-cellular results, as lysosomes and macrophages are intimately linked in Fe metabolism.[47] Melano-macrophage aggregates have been shown to vary in morphology and tissue distribution across phylogenetic groups of fish and tend to be found in organs associated with blood formation (hematopoiesis), where one of their roles is to scavenge effete erythrocytes and sequester redox active Fe.[55] In salmonids, hematopoiesis mainly occurs in kidney and spleen and therefore, hepatic MA are less common,[56] however it may be that a substantial dietary source of Fe, e.g. larval chironomids, can stimulate the formation of hepatic MA in these fish as a means of handling the elevated Fe burden. The lack of correlation between Hg and MA across lakes was surprising given that correlations between Hg and hepatic MA have been previously reported for fish.[12, 57] Given that MA tissue distribution differs with fish phylogeny, it may be that MA respond to Hg in the

livers of fish which are predisposed to having hepatic MA. More research is needed to understand the relationship between MA and Hg in fish.

Conclusion

There appear to be both costs and benefits to cannibalism in landlocked Arctic char from the four study lakes. The benefit of better body condition comes with greater concentrations of non-essential metals like Tl and Hg, which based on the results of sub-cellular partitioning, are not being effectively detoxified in Arctic char livers. Furthermore, Arctic char feeding at higher elevated trophic positions may also be exposed to more parasites. Future work should focus on better understanding the interaction of metals and parasites in Arctic char. Additionally, the elevated Hg in Arctic char from Amituk Lake is of particular concern as concentrations are above those reported to negatively impact reproduction in laboratory fish. More research should focus on how char reproduction may be impacted by non-essential metals, especially Hg.

ASSOCIATED CONTENT

Supporting Information.

Methods used for sub-cellular partitioning of Arctic char livers

AUTHOR INFORMATION

Corresponding Author. *Phone: +1 734 763 6280; email:drevnick@umich.edu

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of R. Rodrigue, J. Perreault, S. Premont, and L. Rancourt. We also thank K. Hudelson, D. Iqaluk, and G. Lescord for their help in the field. We thank A. P. Roberts for help with histology and for reading an

earlier version of the manuscript. Funding for this project was provided by the Northern Contaminants Program. P.G.C.C. is supported by the Canada Research Chair program.

REFERENCES

1. Muir, D.; Wang, X.; Yang, F.; Nguyen, N.; Jackson, T.; Evans, M.; Douglas, M.; Kock, G.; Lamoureux, S.; Pienitz, R., Spatial trends and historical deposition of mercury in eastern and northern Canada inferred from lake sediment cores. *Environ. Sci. Technol.* **2009,** *43* (13), 4802-4809.

2. Selin, N. E., Global biogeochemical cycling of mercury: a review. *Annual Review of Environment and Resources* **2009,** *34*, 43-63.

3. Lockhart, W.; Stern, G.; Low, G.; Hendzel, M.; Boila, G.; Roach, P.; Evans, M.; Billeck, B.; DeLaronde, J.; Friesen, S., A history of total mercury in edible muscle of fish from lakes in northern Canada. *Sci. Total Environ.* **2005,** *351*, 427-463.

4. Beckvar, N.; Dillon, T. M.; Read, L. B., Approaches for linking whole-body fish tissue residues of mercury or DDT to biological effects thresholds. *Environ. Toxicol. Chem.* **2005,** *24* (8), 2094-2105.

5. Dillon, T.; Beckvar, N.; Kern, J., Residue based mercury dose response in fish: An analysis using lethality equivalent test endpoints. *Environ. Toxicol. Chem.* **2010**.

6. Sandheinrich, M. B.; Bhavsar, S. P.; Bodaly, R.; Drevnick, P. E.; Paul, E. A., Ecological risk of methylmercury to piscivorous fish of the Great Lakes region. *Ecotoxicology* **2011**, 1-11.

7. Depew, D. C.; Basu, N.; Burgess, N. M.; Campbell, L. M.; Devlin, E. W.; Drevnick, P. E.; Hammerschmidt, C. R.; Murphy, C. A.; Sandheinrich, M. B.; Wiener, J. G., Toxicity of dietary methylmercury to fish: Derivation of ecologically meaningful threshold concentrations. *Environ. Toxicol. Chem.* **2012,** *31* (7), 1536-1547.

8. AMAP *Mercury in the Arctic*; 2011.

9. Drevnick, P. *Investigation of mercury toxicity in landlocked char in high Arctic lakes. Synopsis of Research Conducted under the 2012–2013 Northern Contaminants*

Program; Aboriginal Affairs and Northern Development Canada: Ottawa, 2013; pp 305-315.

10. Scheuhammer, A.; Braune, B.; Chan, H. M.; Frouin, H.; Krey, A.; Letcher, R.; Loseto, L.; Noël, M.; Ostertag, S.; Ross, P., Recent progress on our understanding of the biological effects of mercury in fish and wildlife in the Canadian Arctic. *Sci. Total Environ.* **2014**.

11. Drevnick, P.; Roberts, A.; Otter, R.; Hammerschmidt, C.; Klaper, R.; Oris, J., Mercury toxicity in livers of northern pike (Esox lucius) from Isle Royale, USA. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **2008,** *147* (3), 331-338.

12. Barst, B. D.; Gevertz, A. K.; Chumchal, M. M.; Smith, J. D.; Rainwater, T.; Drevnick, P.; Hudelson, K. E.; Hart, A.; Verbeck, G. F.; Roberts, A. P., Laser Ablation ICP-MS Co-localization of Mercury and Immune Response in Fish. *Environ. Sci. Technol.* **2011**.

13. Raldúa, D.; Bayona, J.; Barceló, D., Mercury levels and liver pathology in feral fish living in the vicinity of a mercury cell chlor-alkali factory. *Chemosphere* **2007,** *66* (7), 1217-1225.

14. Schwindt, A. R.; Fournie, J. W.; Landers, D. H.; Schreck, C. B.; Kent, M. L., Mercury concentrations in salmonids from western US national parks and relationships with age and macrophage aggregates. *Environ. Sci. Technol.* **2008,** *42* (4), 1365-1370.

15. Mela, M.; Randi, M.; Ventura, D.; Carvalho, C.; Pelletier, E.; Oliveira Ribeiro, C., Effects of dietary methylmercury on liver and kidney histology in the neotropical fish Hoplias malabaricus. *Ecotoxicol. Environ. Saf.* **2007,** *68* (3), 426-435.

16. Larose, C.; Canuel, R.; Lucotte, M.; Di Giulio, R. T., Toxicological effects of methylmercury on walleye (Sander vitreus) and perch (Perca flavescens) from lakes of the boreal forest. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **2008,** *147* (2), 139-149.

17. Di Giulio, R. T.; Hinton, D. E., *The toxicology of fishes*. CRC: 2008.

18. Sigel, A.; Sigel, H.; Sigel, R. K., *Metallothioneins and related chelators*. Royal Society of Chemistry: 2009; Vol. 5.

19. Rosabal, M.; Hare, L.; Campbell, P. G., Subcellular metal partitioning in larvae of the insect Chaoborus collected along an environmental metal exposure gradient (Cd, Cu, Ni and Zn). *Aquat. Toxicol.* **2012,** *120*, 67-78.

20. Rosabal, M.; Pierron, F.; Couture, P.; Baudrimont, M.; Hare, L.; Campbell, P. G., Subcellular partitioning of non-essential trace metals (Ag, As, Cd, Ni, Pb, Tl) in livers of American (Anguilla rostrata) and European (Anguilla anguilla) yellow eels. *Aquat. Toxicol.* **2015**.

21. Rosabal, M.; Ponton, D. E.; Campbell, P. G.; Hare, L., Uptake and Subcellular Distributions of Cadmium and Selenium in Transplanted Aquatic Insect Larvae. *Environ. Sci. Technol.* **2014,** *48* (21), 12654-12661.

22. Wallace, W. G.; Lee, B.-G.; Luoma, S. N., Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar. Ecol. Prog. Ser.* **2003,** *249*, 183-197.

23. Muir, D.; Wang, X.; Bright, D.; Lockhart, L.; Kock, G., Spatial and temporal trends of mercury and other metals in landlocked char from lakes in the Canadian Arctic archipelago. *Sci. Total Environ.* **2005,** *351*, 464-478.

24. Chételat, J.; Amyot, M.; Cloutier, L.; Poulain, A., Metamorphosis in chironomids, more than mercury supply, controls methylmercury transfer to fish in High Arctic lakes. *Environ. Sci. Technol.* **2008,** *42* (24), 9110-9115.

25. Gantner, N.; Muir, D. C.; Power, M.; Iqaluk, D.; Reist, J. D.; Babaluk, J. A.; Meili, M.; Borg, H.; Hammar, J.; Michaud, W., Mercury concentrations in landlocked Arctic char (Salvelinus alpinus) from the Canadian Arctic. Part II: Influence of lake biotic and abiotic characteristics on geographic trends in 27 populations. *Environ. Toxicol. Chem.* **2010,** *29* (3), 633-643.

26. Lescord, G. L.; Kidd, K. A.; Kirk, J. L.; O'Driscoll, N. J.; Wang, X.; Muir, D. C., Factors affecting biotic mercury concentrations and biomagnification through lake food webs in the Canadian high Arctic. *Sci. Total Environ.* **2014**.

27. Swanson, H. K.; Kidd, K. A.; Babaluk, J. A.; Wastle, R. J.; Yang, P. P.; Halden, N. M.; Reist, J. D., Anadromy in Arctic populations of lake trout (Salvelinus namaycush): otolith microchemistry, stable isotopes, and comparisons with Arctic char (Salvelinus alpinus). *Can. J. Fish. Aquat. Sci.* **2010,** *67* (5), 842-853.

28. Blackwell, B. G.; Brown, M. L.; Willis, D. W., Relative weight (Wr) status and current use in fisheries assessment and management. *Rev. Fish. Sci.* **2000,** *8* (1), 1-44.

29. Swanson, H. K.; Kidd, K. A., Mercury concentrations in Arctic food fishes reflect the presence of anadromous Arctic charr (Salvelinus alpinus), species, and life history. *Environ. Sci. Technol.* **2010,** *44* (9), 3286-3292.

30. Barst, B. D.; Hammerschmidt, C. R.; Chumchal, M. M.; Muir, D. C.; Smith, J. D.; Roberts, A. P.; Rainwater, T. R.; Drevnick, P. E., Determination of mercury speciation in fish tissue with a direct mercury analyzer. *Environ. Toxicol. Chem.* **2013,** *32* (6), 1237-1241.

31. Rosabal, M.; Hare, L.; Campbell, P. G., Assessment of a subcellular metal partitioning protocol for aquatic invertebrates: preservation, homogenization, and subcellular fractionation. *Limnol. Oceanogr. Methods* **2014,** *12* (7), 507-518.

32. Gantner, N.; Power, M.; Iqaluk, D.; Meili, M.; Borg, H.; Sundbom, M.; Solomon, K. R.; Lawson, G.; Muir, D. C., Mercury concentrations in landlocked Arctic char (Salvelinus alpinus) from the Canadian Arctic. Part I: Insights from trophic relationships in 18 lakes. *Environ. Toxicol. Chem.* **2010,** *29* (3), 621-632.

33. Gantner, N.; Power, M.; Babaluk, J. A.; Reist, J. D.; Köck, G.; Lockhart, L. W.; Solomon, K. R.; Muir, D. C., Temporal trends of mercury, cesium, potassium, selenium, and thallium in arctic char (Salvelinus alpinus) from Lake Hazen, Nunavut, Canada: effects of trophic position, size, and age. *Environ. Toxicol. Chem.* **2009,** *28* (2), 254-263.

34. Armitage, P. D.; Cranston, P.; Pinder, L., *The Chironomidae: biology and ecology of non-biting midges*. Springer Science & Business Media: 1995.

35. Hammerschmidt, C.; Sandheinrich, M.; Wiener, J.; Rada, R., Effects of dietary methylmercury on reproduction of fathead minnows. *Environ. Sci. Technol* **2002,** *36* (5), 877-883.

36. Adams, S.; Shugart, L.; Southworth, G.; Hinton, D., Application of bioindicators in assessing the health of fish populations experiencing contaminant stress. *IN: Biomarkers of Environmental Contamination. Lewis Publishers, Chelsea, Michigan. 1990. p 333-353, 6 fig, 2 tab, 52 ref. DOE Contract DE-AC 05-84 OR 21400.* **1990**.

37. Campbell, P. G.; Hare, L., Metal detoxification in freshwater animals. Roles of metallothioneins. *Metallothioneins and related chelators* **2009**, 239-277.

38. Wang, F.; Lemes, M.; Khan, M., Metallomics of mercury: Role of thiol-and selenol-containing biomolecules. *Environmental Chemistry and Toxicology of Mercury* **2012**, 517.

39. Gladyshev, V. N., Selenoproteins and selenoproteomes. In *Selenium*, Springer: 2012; pp 109-123.

40. Khan, M. A. K.; Wang, F., Mercury selenium compounds and their toxicological significance: Toward a molecular understanding of the mercury selenium antagonism. *Environ. Toxicol. Chem.* **2009,** *28* (8), 1567-1577.

41. Ralston, N. V.; Raymond, L. J., Dietary selenium's protective effects against methylmercury toxicity. *Toxicology* **2010,** *278* (1), 112-123.

42. Cambier, S.; Benard, G.; Mesmer-Dudons, N.; Gonzalez, P.; Rossignol, R.; Brethes, D.; Bourdineaud, J.-P., At environmental doses, dietary methylmercury inhibits mitochondrial energy metabolism in skeletal muscles of the zebra fish (Danio rerio). *The international journal of biochemistry & cell biology* **2009,** *41* (4), 791-799.

43. Berntssen, M.; Aatland, A.; Handy, R., Chronic dietary mercury exposure causes oxidative stress, brain lesions, and altered behaviour in Atlantic salmon (Salmo salar) parr. *Aquat. Toxicol.* **2003,** *65* (1), 55-72.

44. Araújo, O.; Pereira, P.; Cesário, R.; Pacheco, M.; Raimundo, J., The sub-cellular fate of mercury in the liver of wild mullets (Liza aurata)–Contribution to the understanding of metal-induced cellular toxicity. *Mar. Pollut. Bull.* **2015**.

45. Kidd, K.; Batchelar, K., 5 - Mercury. In *Fish Physiology*, Chris M. Wood, A. P. F.; Colin, J. B., Eds. Academic Press: 2011; Vol. Volume 31, Part B, pp 237-295.

46. Xie, L.; Flippin, J. L.; Deighton, N.; Funk, D. H.; Dickey, D. A.; Buchwalter, D. B., Mercury (II) bioaccumulation and antioxidant physiology in four aquatic insects. *Environ. Sci. Technol.* **2008,** *43* (3), 934-940.

47. Kurz, T.; Eaton, J. W.; Brunk, U. T., The role of lysosomes in iron metabolism and recycling. *The international journal of biochemistry & cell biology* **2011,** *43* (12), 1686-1697.

48. Wolf, J. C.; Wolfe, M. J., A brief overview of nonneoplastic hepatic toxicity in fish. *Toxicol. Pathol.* **2005,** *33* (1), 75.

49. Zachary, J. F.; McGavin, M. D., *Pathologic basis of veterinary disease*. Elsevier Health Sciences: 2013.

50. Klaassen, C. D., *Casarett and Doull's Toxicology: the basic science of poisons*. McGraw-Hill New York (NY): 2013; Vol. 1236.

51. Blomhoff, R.; Wake, K., Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. *The FASEB journal* **1991,** *5* (3), 271-277.

52. de Oliveira Ribeiro, C.; Belger, L.; Pelletier, E.; Rouleau, C., Histopathological evidence of inorganic mercury and methyl mercury toxicity in the arctic charr (Salvelinus alpinus). *Environ. Res.* **2002,** *90* (3), 217-225.

53. Frandsen, F.; Malmquist, H. J.; Snorrason, S. S., Ecological parasitology of polymorphic Arctic charr, Salvelinus alpinus (L.), in Thingvallavatn, Iceland. *J. Fish Biol.* **1989,** *34* (2), 281-297.

54. Hammar, J., Cannibals and parasites: conflicting regulators of bimodality in high latitude Arctic char, Salvelinus alpinus. *Oikos* **2000,** *88* (1), 33-47.

55. Agius, C.; Roberts, R., Melano macrophage centres and their role in fish pathology. *J. Fish Dis.* **2003,** *26* (9), 499-509.

56. Agius, C., Phylogenetic development of melano–macrophage centres in fish. *J. Zool.* **1980,** *191* (1), 11-31.

57. Batchelar, K. L.; Kidd, K. A.; Drevnick, P. E.; Munkittrick, K. R.; Burgess, N. M.; Roberts, A. P.; Smith, J. D., Evidence of impaired health in yellow perch (Perca

flavescens) from a biological mercury hotspot in northeastern North America. *Environ. Toxicol. Chem.* **2013,** *32* (3), 627-637.

SUPORTING INFORMATION

Methods used for sub-cellular partitioning of Arctic char livers

Composite samples of Arctic char liver were allowed to thaw on ice in 1.5 mL of Tris buffer adjusted to pH 7.4 with HCl (25 mmol L-1, OmniPur, EM Science, affiliate of MERCK KGaA, Darmstadt, Hesse, Germany). Each sample was homogenized using a Pellet Pestle (Kontes, Vineland, NJ, USA) for 5 s at 30 s intervals for a total of 5 times. A 100 µL aliquot was removed from the homogenate for mass-balance quality-control measurements and the remainder was centrifuged at 800 x g for 15 min at 4 ℃. The resulting supernatant was removed and placed on ice until further separation.

The pellet was suspended in 500 µL of ultrapure water, heated for 2 min at 100 ℃ and digested with an additional 500 µL of 1N NaOH (99.998%, Sigma-Aldrich) at 65 ℃ for 1 hour. Centrifugation of the digestate at 10,000 x g for 10 min at ambient temperature was performed to separate the NaOH-resistant fraction (granule-like fraction) from the solubilized fraction containing cellular

debris and nuclei. The supernatant, taken after the original centrifugation step (800 x g for 15 min at 4 ℃), was centrifuged at 10,000 g for 30 min at 4 ℃ to yield the mitochondrial fraction. The supernatant was kept at 80 ℃ for 10 min, then placed on ice for 1 hour, before centrifugation at 50,000 x g for 10 min at 4 ℃ to isolate the pellet containing heat-denatured proteins (HDP) and a pellet containing heat-stable proteins (HSP). High-speed centrifugations (≥ 50,000 x g) were performed using a Beckman TLA-100 centrifuge equipped with a TLA-100.3 rotor (Beckman Counter). Lower-speed centrifugations were performed using an IEC Micromax centrifuge (Thermo IEC, Arlington, MA, USA). Each supernatant was acidified with nitric acid (final [HNO_3] 10%; v/v; Optima grade, Fisher Scientific) and kept at 4 ℃ until analysis. The pellets were frozen at −80 ℃ before lyophilisation and trace element analysis as described in the text.