UNIVERSITÉ DU QUÉBEC À MONTRÉAL

TOXICOCINÉTIQUE DU MERCURE CHEZ LE DORÉ ET LA PERCHAUDE DANS LES LACS DE LA FORÊT BORÉALE

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Für meine Großeltern

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RÉSUMÉ

Ce mémoire présente les résultats d'une étude portant sur les effets sanitaires du mercure (Hg) et de son principal dérivé organique, le méthylmercure (MeHg), chez le doré jaune (*Sander vitreus*) et la perchaude (*Perca flavescens*) capturés dans quatre lacs de la région de l'Abitibi-Témiscamingue (Québec, Canada). Effectué en août 2005, ce projet s'insère dans le cadre des travaux du Réseau collaboratif de recherche sur le mercure (COMERN) subventionné par le CRSNG. Les principaux objectifs de l'étude consistaient à **i)** examiner la toxicocinétique du Hg total/MeHg dans les tissus musculaire et hépatique du doré jaune, ainsi qu'à **ii)** évaluer les effets physiologiques et biochimiques de l'accumulation du Hg total/MeHg chez le doré jaune et la perchaude à l'aide d'une série de bioindicateurs. Il s'agissait plus spécifiquement de déterminer l'impact du Hg total/MeHg sur la condition générale et le système de détoxification du glutathion (GSH) de spécimens collectés à partir de sites peu impactés par ces xénobiotiques (0,3-0,79 ppm Hg total dans la chair).

À la lumière des analyses de Hg total/MeHg dans les muscles et les foies de dorés jaunes, le volet toxicocinétique révèle des différences significatives dans le taux d'accumulation du Hg total/MeHg entre les spécimens des différents lacs échantillonnés. Des différences ont également été observées au niveau de la cinétique du Hg total/MeHg entre les tissus musculaire et hépatique. Nos résultats suggèrent en outre la présence d'un lent processus de déméthylation hépatique chez les spécimens des trois lacs où les teneurs en Hg total dans les chairs étaient les plus faibles.

Le volet physiologique et biochimique indique que les dorés du lac Malartic, soit les poissons de notre échantillonnage avec les teneurs en MeHg dans les foies les plus élevées possèdent un indice hépatosomatique (HSI) et une activité enzymatique de la glutathion *S*-tranférase (GST) significativement plus faibles. Le HSI est négativement corrélé à la concentration hépatique en Hg total. L'activité des enzymes GST et de la glutathion réductase (GR) est reliée au HSI. Dans le lac Desjardins-Est, les perchaudes présentent les plus fortes concentrations hépatiques de MeHg. Chez ces spécimens, l'activité des enzymes GST et de la glutathion peroxydase dépendant du sélénium (GSH-Px SD) est négativement corrélée à la concentration hépatique de MeHg. Cette étude démontre que le Hg et le MeHg peuvent induire des effets physiologiques et cellulaires adverses chez le doré jaune et la perchaude et ce, à des concentrations environnementales significatives, mais faibles.

Mots-clés : toxicocinétique, mercure/méthylmercure, poissons, glutathion, lacs

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LISTE DES ABBRÉVIATIONS

COMERN	Collaborative Mercury Research Network
FQRNT	Fonds québécois de la recherche sur la nature et les technologies
NSERC	Natural Sciences and Engineering Research Council of Canada
C_L	LeCren condition factor
HSI	Hepatosomatic index
GCL	Glutamate-cysteine ligase
GR	Glutathione reductase
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSH-Px SD	Glutathione peroxidase selenium dependent
GSH-Px SI	Glutathione peroxidase selenium independent
GSSG	Glutathione disulfide
GST	Glutathione-*S*-transferase
Hg	Mercury
MeHg	Methylmercury
ppb	Parts per billion
ROS	Radical oxygen species
Se	Selenium

GENERAL INTRODUCTION

Research context

Mercury (Hg), a toxic element, is found both naturally and as introduced compound in the environment (Nriagu and Pacyna, 1988, Nriagu, 1989, Fitzgerald *et al.* 1998). Historically, Hg has been used as a pigment, as well as in the extraction of gold and silver through mercury-amalgamation. Hg was also used in a variety of industries, for example in certain types of chlor-alkali plants as well as pulp and paper mills. Industrial use of Hg, and its subsequent release to the environment, has contributed to augmenting Hg levels worldwide. In the last century alone, it has been estimated that anthropogenic emissions to the atmosphere have tripled the atmospheric and oceanic Hg concentrations (Mason *et al.* 1994). About 5000-6000 t of Hg is released each year of which about 50% can be attributed to anthropogenic activity (Mason *et al.* 1994, Lamborg *et al.*, 2002). The widespread use of Hg has lead to an increase in concentrations in soil, sediments and aquatic ecosystems worldwide (Perry et al., 2005, Gray and Hines, 2006). These circumstances are problematic to address because the physicochemical properties of Hg make this contaminant difficult to contain and to recover (Wiener *et al.*, 2003).

Physicochemical properties (particle size, lipophilicity, hydrophilicity, etc.) play an important role in determining xenobiotic toxicity (Malins and Ostrander, 1994). Hg causes diverse effects that are dependent on its chemical form (Boudou *et al.*, 1991, Ullrich *et al.*, 2001). Mercury exists in several forms in the environment: as a vapor (Hg^0), in an inorganic form (Hg^{2+}) and in an organic form of which methylmercury (MeHg) is the most important. Of the three, the organic form, MeHg, is considered the most toxic even at very low exposure doses

(Ullrich *et al.*, 2001). Highly neurotoxic (Yee and Choi, 1994), MeHg can cause damage to the visual cortex and to the sensory system in humans. Signs of intoxication include the constriction of the visual field, sensory impairment of extremities, hearing loss, muscle weakness, tremors, cardiovascular problems and mental deterioration (Harada, 1995, Castoldi *et al.*, 2001, Yokoo *et al.*, 2003). The main source of MeHg in humans is the consumption of contaminated fish (Castoldi *et al.*, 2001, Daré *et al.*, 2001). In light of all these adverse effects, many countries now have consumer advisories which encourage people to limit their intake of fish.

MeHg is a major environmental contaminant which affects many species. Although the main vector for MeHg contamination in humans is through the consumption of fish, fish have a beneficial nutritious value. Fish muscles are rich in proteins, antioxidants (vitamin E) as well as omega-3 fatty acids. Including fish in the diet may even lead to a reduction in cardiovascular diseases (Kris-Etherton *et al.*, 2001). Fishing is also an important cultural and economic activity (Scott and Crossman, 1973). It is therefore important to understand the mechanisms by which MeHg induces toxic effects in fish.

MeHg is formed through the methylation of inorganic Hg. It can be produced through abiotic and biotic pathways, although methylation by sulfate-reducing bacteria in the sediments of fresh and ocean water is the dominant pathway (Ullrich *et al.*, 2001). The exact biochemical processes which lead to the production of MeHg are still unknown.

Unlike inorganic mercury, MeHg easily accumulates along the food web (Berntssen *et al.*, 2003). Mercury levels, as well as the relative proportions of its organic and inorganic form, vary largely according to the trophic level, tissue studied and zoological group (Ancora *et al*, 2002, Thompson, 1990). There also seems to be a species-dependent variation in the distribution of organic mercury (Nigro and Leonzio, 1996). The concentrations of MeHg tend to increase with the

trophic level in a food web, with top predators being the most contaminated (Schultz and Newman, 1997). This phenomenon, known as MeHg biomagnification, is observed in most ecosystems, regardless of the Hg source. Within a fish species, MeHg concentrations tend to increase with length, age and weight (Wiener *et al.*, 2003).

Hg concentrations in fish are influenced by a series of biological, physicochemical and environmental factors (Simoneau *et al.*, 2005). These factors interact in complex ways which lead to highly variable Hg levels. Important inter-lake variability, even within the same geographic region, has been reported by several researchers (Schetagne and Verdon, 1999, Rose *et al.*, 1999). In order to explain this variability, a large body of research has focused on physicochemical characteristics of lakes including lake size, size and nature of the catchment, ratio of the watershed to the surface of the lake, water temperature, dissolved organic carbon and pH among others (Roué Le Gall *et al.*, 2005, Greenfield *et al.*, 2001, Balogh *et al.*, 1998). Other factors, including geographical location and exposure to either airborne or direct contaminants, have also been studied (Strom and Graves, 2001, Rose et al., 1999). These factors alone are unable however, to fully account for the Hg variability observed in fish populations in the natural environment.

In order to effectively manage the Hg issue in ecosystems, it is important to have a better understanding of the behavior of Hg compounds in the natural environment. However, gaining this knowledge is difficult when one remains within the framework of conventional monodisciplinary research, given that the chemical and biological processes that control Hg bioaccumulation are still not well understood, that the chemistry and speciation of Hg is complex (Ullrich *et al.*, 2001) and that ecosystems are dynamic. In order to address this complexity, the pan-Canadian Collaborative Mercury Research Network (COMERN) was established in 2001 through a five year grant awarded by the Natural Sciences and Engineering Research Council of Canada (NSERC). The general aim of

COMERN, based at the Université du Québec à Montréal (UQÀM), was to identify the causes of increasing Hg levels and its impacts on the health of communities using a novel integrated ecosystem approach based on interdisciplinary and the integration of knowledge. Working within this framework has led to scientific breakthroughs in Hg research such as, for example: the development of new low exposure-level Hg toxicity biomarkers (Stamler *et al.*, 2006), the importance of metabolism in human Hg bioaccumulation (Canuel *et al.*, 2006), the identification of fish growth rates as an important factor in MeHg bioaccumulation (Simoneau *et al.*, 2005) as well as to the development of modeling tools that assess the sensitivity of ecosystems to Hg loadings (Roué Le Gall *et al.*, 2005).

Although the understanding of Hg in the environment has improved tremendously, in part through innovative interdisciplinary approaches, knowledge gaps still remain. Relatively few studies have addressed the specific health effects of Hg on feral fish communities. Most studies on the toxicological effects of Hg in fish consist of controlled laboratory experiments conducted over a relatively short period of time and at unrealistically high concentrations of MeHg where the major focus has generally been either A) toxicokinetics (e.g. Schultz and Newman, 1997, Leaner and Mason, 2004) or B) neurotoxicity (e.g. Berntssen *et al.*, 2003, Tsai *et al.*, 1995). Rarer still, are field studies dealing specifically with the physiological and biochemical effects of Hg on fish populations at environmentally realistic exposure levels. The general object of the present research, which is part of a larger COMERN-developed regional case study on the Abitibi-Témiscamingue area in the Province of Québec, is to study the effects of MeHg on the health of two species of feral fish; walleye (*Sander vitreus*) and perch (*Perca flavescens*). This study was conducted *in vivo*, in lakes with realistic Hg exposure levels that were identified as frequently fished by local anglers. More specifically, the object was to look at the effects of a long-term, chronic exposure to low levels of Hg at the cellular, organism and population levels of

fish in order to determine A) the adverse effects of Hg exposure to fish and B) which mechanisms (if any) are involved in the protection against Hg.

MeHg toxicity in fish

In fish, MeHg can cause incoordination, loss of appetite, diminished swimming activity and mortality (Berntssen *et al.*, 2003). In addition, it may reduce reproductive success by affecting gonadal development or spawning success of adults or by reducing the hatching of eggs and the health and survival of embryolarval stages (Wiener *et al.*, 2003).

Most of the mercury in fish is in the methylated form. A study by Bloom (1992) has concluded that over 95% of the Hg found in fish tissues is MeHg. The body burden of a xenobiotic acquired by an individual animal depends on several factors such as the physicochemical properties of the chemical, the routes of exposure and the physiological and biochemical make-up of the animal (Malins and Ostrander, 1994). In wild fish, dietary uptake accounts for more than 90% of the total uptake of MeHg (Leaner and Mason, 2002). Fish probably assimilate 65-80% or more of the MeHg present in the food they eat (Wiener *et al.*, 2003).

Food exists as many different complex mixtures, dependent on trophic status, phylogeny of the food items, health status and body condition. The availability of toxicants to fish from these foods is dependent on the associated matrix, the nature of the association of toxicant with the matrix, the form of the toxicant and the favorability of the gastrointestinal milieu for absorption (Malins and Ostrander, 1994). Digestability of the foodstuff plays an important role in liberating toxicants for absorption and associating toxicants with media that facilitate absorption. The bioavailability of MeHg to fish may also be dependant on the digestive processes of the organism (Leaner and Mason, 2002).

One of the determinant factors in contaminant accumulation in any organism is the ability of the xenobiotic to cross the intestinal membranes and enter the systemic circulation (Malins and Ostrander, 1994). In fish, MeHg can enter the circulation by two processes, which depend on the speciation of the contaminant. If MeHg is uncomplexed, it can diffuse directly across intestinal membranes due to its lipid solubility. However, in the presence of complexing agents such as amino acids, absorption may occur through active processes (Aschner and Clarkson, 1988). In a study on intestinal MeHg absorption in catfish by Leaner and Mason (2002), uptake occurred via passive and active processes and/or via the energy dependant $_{L}$-neutral amino acid carrier depending on the form of the MeHg complex.

After being absorbed through the intestinal wall, MeHg binds to red blood cells and is distributed to all tissues and organs via the circulatory system (Wiener *et al.*, 2003). MeHg is found in virtually all fish tissues (Leaner and Mason, 2004), but much of the MeHg in the body is eventually relocated to the skeletal muscle where it is stored in a water-soluble protein-bound form (Wiener *et al.*, 2003). The storage of methylmercury in skeletal muscles may serve as a protective mechanism in fish, given that the sequestration in muscles may reduce the exposure of the central nervous system to MeHg (Wiener *et al.*, 2003).

Cellular Effects of MeHg

The primary target for the cytotoxic action of MeHg is the central nervous system (Bragadin *et al*, 2002). Although the molecular mechanisms for toxicity have yet to be completely clarified, certain studies have found that MeHg impairs mitochondria, which appear to be the prime cellular target (Bragadin *et al.*, 2002, Castoldi *et al.*, 2001). Mitochondria are the sites of adenosine triphosphate (ATP) production, molecules which provide the energy necessary for various cellular functions such as protein synthesis and the replication of DNA (Klaassen, 2001, Garrett and Grisham, 2000). Some ATP is generated directly during the Kreb's

cycle, but most of the ATP produced in tissue respiration is generated by the electron transfer chain which takes place across the membranes of mitochondria (Garrett and Grisham, 2000). During the Kreb's cycle, hydrogen ions (or electrons) are donated to two carrier molecules, nicotinamide adenine dinucleotide (NAD) or flavin adenine dinucleotide (FAD). These molecules are oxidized to reduced NAD (NADH) and reduced FAD (FADH) and carry the electrons to the respiratory or electron transport chain found in the mitochondrial cristae (Cooper, 1999). The NADH and FADH molecules diffuse from one complex to the next. At each site is a proton pump which transfers hydrogen from one side of the membrane to the other. This creates a gradient across the inner membrane with a higher concentration of hydrogen ions in the space between the inner and outer membranes of the mitochondrion. The ATP synthase enzyme uses the energy of the proton gradient to form ATP from adenosine diphosphate (ADP) and phosphate. It also produces water from the hydrogen and the oxygen (Garrett and Grisham, 2000).

Generally, the inner membrane of mitochondria is impermeable to chemical compounds (Garrett and Grisham, 2000). However, a study by Bragadin *et al.* (2002) has shown that MeHg induces the opening of a mitochondria permeability pore (MTP), which allows for the passage of large molecules with molecular masses in excess of 1500 Da. Other effects of MeHg include the uncoupling of the oxidative phosphorylation pathway as well as electron transport in mitochondria (Yee and Choi, 1994, Daré *et al*, 2001), the increase of inner membrane permeability to potassium, the collapse of the mitochondrial membrane potential, Ca^{2+} release and finally calpain and cytochrome c release (Castoldi *et al.*, 2001, Bragadin *et al.*, 2002). Depending on the concentration, MeHg can trigger either necrosis or apoptosis which leads to cell death. At high concentrations, MeHg has been reported to induce necrosis in vitro, while at low concentrations, apoptosis (Chandra *et al.*, 2000, Castoldi *et al.*, 2001).

Necrosis is the result of acute cellular dysfunction in response to severe stress conditions or after exposure to toxic agents (Chandra *et al.*, 2000). It is associated with a rapid depletion of cellular ATP. Necrosis is characterized by an increase in cellular volume and a rupture of the plasma membrane which causes the contents of the cell to spill into the intracellular milieu (Chandra *et al.*, 2000). This release can cause subsequent tissue damage by affecting neighboring cells or by initiating an inflammatory response (Daré *et al.*, 2001, Janeway *et al.*, 2001).

Apoptosis is a form of programmed cell death that occurs during a variety of pathological situations (Chandra *et al.*, 2000). Apoptosis is a normal cellular process which constitutes a mechanism of cell replacement, tissue remodeling and removal of damaged cells (Janeway *et al.*, 2001). It is a process characterized by alterations in cell morphology, cellular shrinkage, chromatin condensation and DNA fragmentation (Chandra *et al.*, 2000, Janeway *et al.*, 2001). A number of protease families are implicated in apoptosis, namely the caspases, which are a family of cysteine proteases that cleave proteins (Janeway *et al.*, 2001). Apoptosis can be triggered via a variety of pathways. The one most likely involved in MeHg induced apoptosis focuses on mitochondrial dysfunction, which can cause the release of cytochrome c and can lead to the initiation of the caspase cascade (Chandra *et al.*, 2000, Castoldi *et al.*, 2001, Gatti *et al.*, 2004). Recent reports have also suggested a role for glutathione (GSH) and oxidative stress in the triggering of apoptosis (Hall, 1999, Chandra *et al.*, 2000).

Glutathione

Like many other heavy metal organic compounds, MeHg has a high affinity for sulfhydryl groups, including those of glutathione (GSH) as well as those of proteins (Canesi *et al.*, 1999).[1] MeHg binds with such avidity to sulfhydryl groups (SG) that its association with other ligands in biological systems is

[1] Heavy metal cations are characterized as having a high affinity for sulfhydryl groups, the rank of affinity being : Hg(II) > Cu(I) > Cd(II) > Cu(II) > Zn(II).

inconsequential (Hughes, 1957). The high affinity of MeHg for thiols makes proteins and peptides bearing cysteines susceptible to structural and functional modifications in all subcellular compartments (Castoldi *et al.*, 2001). MeHg-SG complexes have been identified in several mammalian and piscine tissues. According to a recent report by Harris *et al.* (2003), the dominant chemical form of MeHg in fish tissue is that of a MeHg-cysteinyl conjugate, where the cysteine moiety is part of a larger peptide such as GSH or protein.

Thiols are organic molecules containing a sulfhydryl (-SH) functional group found on proteins as well as in non-protein compounds such as GSH (Klaassen, 2001). Thiols are integral to many proteins; they contribute to the stability of tertiary structures by forming disulfide bonds, and in some cases, constitute the active site of enzymes (e.g. GSH-Px) (Dickson and Forman, 2002, Nagai *et al.*, 2002). Non-protein thiols, or low molecular weight thiols, play essential roles in many biochemical reactions because they are easily oxidized and regenerated (Dickinson and Forman, 2002). GSH (L-γ-glutamyl-cysteinyl-glycine) is a major non-protein thiol that is involved in a variety of cellular functions such as transport and metabolic processes, cellular signaling, the maintenance of protein thiols critical to enzyme activity, as well as cellular defense against xenobiotics and oxidative stress (e.g. Peña *et al.*, 2000, Canesi *et al.*, 1999, Hasspieler *et al.*, 1994). GSH exists in two forms, the active, or reduced, form (GSH) and the oxidized form as a disulfide (GSSG) (Klaassen, 2001). The main producer of GSH is the liver, which exports the molecule via the blood (Viña, 1990). However, it can also be synthesized in the cytosol and then transported into organelles such as the mitochondrion and nucleus (Chandra *et al.*, 2000). The cellular response to stress often involves a change in thiol content, where GSH is consumed in reactions that protect the cell through the removal of the xenobiotic compound, and is subsequently replaced through enzymatic reduction of disulfide or by *de novo* synthesis (Dickinson and Forman, 2002). Binding of MeHg to GSH may cause a depletion of the intra-cellular pool of antioxidants, which can

upset the redox balance in cells leading to oxidative stress (Halliwell and Gutteridge, 1989). [2]

Free Radicals and Oxidative Stress

Cells have antioxidant systems which protect them against oxidative metabolites including free radicals and radical oxygen species (ROS) (Viña, 1990). A free radical is any species capable of independent existence that contains one or more unpaired electrons. They can be formed by a gain or a loss of a single electron in non-reactive species or by homolytic fission (break of a covalent bond) (Halliwell and Gutteridge, 1989). These radicals involve oxygen and are referred to as reactive oxygen species (ROS) (e.g. hydrogen peroxide and singlet oxygen) (Kelly *et al.*, 1998). These ROS are generated in organisms via several different cellular processes that involve either endogenous or xenobiotic compounds (Winston and Di Giulio, 1991). These free radicals then randomly attack all cell components, including proteins, lipids and nucleic acids, potentially causing extensive cellular damage (Klaassen, 2001). When there is an imbalance between the generation and removal of radical species within an organism, the resultant is oxidative stress (Kelly et al, 1998). The generation of oxidative stress may be a pre-cursor to apoptosis or necrosis (Chandra *et al.*, 2000).

MeHg deposits in mitochondria where it disrupts the electron transport chain, inducing the formation of free radicals and lipid peroxidation (Berntssen *et al.*, 2003, Daré *et al.*, 2001, Yee and Choi, 1994) which lead to the disruption of membranes (Daré *et al.*, 2001). As well, the homolytic breakdown of MeHg can produce alkyl and free radicals (Daré *et al.*, 2001, Ganther, 1978). By virtue of the high concentrations of polyunsaturated fatty acids in fish tissue, fish may be

[2] Antioxidants are defined as any substrate that, when present in low concentrations compared with those of the substrate, significantly delay or prevent the oxidation of that substrate. Oxidation is defined as a loss of one or more electrons.

more susceptible to contaminant induced lipid peroxidative damage (Winston and Di Giulio, 1991).

Not only does MeHg induce lipid peroxidation, it also binds the molecules that protect the organism against oxidative stress. MeHg binds GSH, thereby leading to a reduction in the intracellular pool of the GSH molecules. As a protective response, the organism may increase GSH regeneration and biosynthesis (Winston and Di Giulio, 1991).

Antioxidant Defense Systems

Fish have several different types of cellular antioxidant defense mechanisms, one of the most important being the glutathione system. Reduced glutathione protects cellular components from toxicity, both as a substrate for conjugation and as an antioxidant defense. Many compounds conjugate with GSH, either spontaneously (non-enzymatically) when the electrophile is very reactive, or enzymatically via glutathione S-transferase (GST) (Dickinson and Forman, 2002, Hasspieler *et al.*, 1994).[3] Conjugation is a protective measure, as it facilitates xenobiotic excretion and protects other cellular targets such as protein thiols (Hasspieler *et al.*, 1994). GSH can either quench ROS directly, or enzymatically, in a reaction catalyzed by glutathione peroxidase (GSH-Px).[4] GSH-Px catalyses the reduction of fatty acid hydroperoxides and/or H_2O_2 to stable products through the oxidation of two molecules of GSH, which leads to the formation of glutathione disulfide (GSSG) (Berntssen *et al.*, 2003, Kelly *et al.*, 1998, Hasspieler *et al.*, 1994). The exposure to large doses of hydrogen peroxide can lead to ATP depletion by inhibition of glycolysis. GSH likely reduces Se and the reduced form of the enzyme then reacts with hydrogen peroxide (Halliwell and Gutteridge, 1989). Conjugation can

[3] In mammals, GSTs are a multigene family of enzymes (isoforms) named alpha, mu, pi, theta, sigma, kappa, zeta and omega. They were grouped into classes based upon sequence homology and ability to catalyze the conjugation of glutathione to a broad range of electrophilic substrates in animal organisms (Hoarau *et al.*, 2002).

[4] GSH-Px is an enzyme that is made up of four subunits, each containing one atom of selenium (Se) at its active site (Halliwell and Gutteridge, 1989).

result in the depletion of GSH (Dickinson and Forman, 2002). The cell must then regenerate the oxidized molecules, either through reduction of GSSG to GSH by glutathione reductase (GR) which uses NADPH as the reducing agent or by *de novo* synthesis (Dickinson and Forman, 2002, Peña-Llopis *et al.*, 2003). GR is an enzyme which contains two protein subunits, each with flavin (FAD) at its active site. NADPH probably reduces the FAD, which then passes its electrons onto a disulfide bridge (-S-S-) between two cysteine residues in the protein. The two –SH groups then interact with GSSG and reduce it to two molecules of GSH (Halliwell and Gutteridge, 1989). The NADPH required is provided by the oxidative pentose phosphate pathway. As GR operates and lowers the $NADPH/NADP^+$ ratio, the pentose phosphate pathway speeds up to replace the NADPH (Halliwell and Gutteridge, 1989).

De novo synthesis involves two separate ATP dependent enzyme systems: glutamate-cysteine ligase (GCL) and glutathione synthetase (GS) (Peña-Llopis *et al.*, 2003). GCL, a heterodimer which is composed of two subunits, a light and a heavy one, catalyses the step in which $_L$-cysteine is linked to $_L$-glutamate, which is rate-limiting (Dickinson and Forman, 2002).

The capacity of the glutathione system to cope depends on the activity of GSH-Px, GR and the pentose phosphate pathway enzymes among others (Halliwell and Gutteridge, 1989). Compounds that are able to generate oxidative stress can also, in some cases, lead to the induction of antioxidant enzymes, which is considered an adaptive protective response (Winston and Di Giulio, 1991). Many compounds have been shown to induce GSH synthesis through increased transcription of GCL (Dickinson and Forman, 2002). Results from studies in rats indicate that short and long-term exposure to MeHg in drinking water resulted in a two- to three-fold up-regulation of messenger ribonucleic acid (mRNA) encoding for GCL. Concomitantly there was a similar magnitude of increase in the levels of GSH, and the activities of GR and GSH-Px (Sarafin *et al.*, 1996, Woods *et al.*,

1995). Therefore, it is possible that fish chronically exposed to MeHg may also exhibit a comparable upregulation of these enzymes.

The two other important antioxidant enzymes are superoxide dismutase (SOD) which converts oxygen into H_2O_2 and catalase (CA) which detoxifies H_2O_2 into H_2O and O_2 (Halliwell and Gutteridge, 1989). In a series of experiments on basal enzyme activities in fish, consistently high activities of peroxidase and low activties of catalase were observed, whereas SOD activity was highly variable. Carnivorous fish showed higher GSH-Px and CA activities and much lower SOD activities than herbivorous fish (Winston and Di Giulio, 1991).

Modulators of Antioxidant Systems in Fish

GSH concentrations can be modulated by a number of factors including hormones, such as adrenaline and glucagon, fasting (which reduces the quantity of cysteine, the precursor to GSH synthesis), species, sex, age and metabolism. Another factor is temperature, which can alter the metabolism in fish (Viña, 1990). The photoperiod is also an important factor (Craig, 2000).

Test Species

All of the following information was taken from Scott and Crossman's *Freshwater Fishes of Canada* (1973). The test species are walleye (*Sander vitreus*) and perch (*Perca flavescens*). Both of these species are prized by commercial and sports fishers alike.

Perch exhibit a circumpolar distribution. They are generally found in freshwater and spawn in the spring when the water temperature is between 8.9°C and 12.2°C. Their growth rate is highly variable and is dependent on lake productivity, lake size and population density. Perch adapt easily to various habitats, though they

prefer open areas with clear water, moderate vegetation and a gravelly bottom. Perch are generally more active during the day and hunt between sunrise and sunset. Their prey varies according to size and season, but is mainly composed of immature insects, large invertebrates and small fish. Perch are preyed upon by most predatory fish, including pike, walleye and trout.

Walleye are an almost exclusively freshwater species. Spawning occurs between spring and summertime depending on latitude and temperature, the ideal temperature varying between 6.7°C and 8.9°C. They exhibit a relatively rapid growth rate, with females growing faster than males. Walleye prefer large, shallow and turbid lakes. Walleye are sensitive to light; therefore when the water is too transparent, most of the activity occurs at night. Their diet is mainly composed of fish and invertebrates. Their main predator is the pike, which is also a direct competitor for food.

Specific research objectives

The general objective of this investigation is to assess the toxicological effects of Hg in feral fish at environmentally relevant exposure levels. The results of this Master's thesis are presented in the following two chapters as scientific articles to be submitted for publication in refereed journals. The first article explores the toxicokinetics of different forms of Hg in the liver and muscle of walleye. The liver is a focal point of this paper because of its role in xenobiotic detoxification. The hypotheses are that Hg concentrations increase relative to age and that MeHg kinetics vary according to the type of tissue studied.

The second article looks at the physiological and biochemical effects of Hg in the livers of walleye and perch. The main objective of this paper is to gauge the effects of MeHg on the physiological condition of fish as well as on the GSH system. The hypothesis is that a long-term, low-level exposure to MeHg will lead to an altered response in the glutathione system and adversely affect the health of

fish. In addition, it is hypothesized that species-differences in response will be observed.

In summary of these objectives, it will be shown that fish rely on active endogenous antioxidant systems which participate in detoxification reactions when exposed to MeHg. Furthermore, this may have consequences on the general condition of the animal. These findings will lead to an improvement in the understanding of the mechanisms of action of MeHg, as well as provide insights into how organisms cope with xenobiotics.

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CHAPTER I*

MERCURY TOXICOKINETICS: A COMPARISON IN THE LIVER AND MUSCLE OF WALLEYE (*Sander vitreus*) FROM LAKES OF THE BOREAL FOREST

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Abstract

The toxicokinetics of two forms of mercury (Hg) were examined in walleye (*Sander vitreus*) from four lakes of the Abitibi-Témiscamingue region in Québec. Methylmercury (MeHg) and total Hg concentrations were determined in the livers of walleye, while total Hg concentrations were determined in the muscle. Hg accumulation rates were compared in both organs and growth rates were established. Differences in muscle Hg accumulation rates were not attributable to growth alone and Hg kinetics differed between livers and muscles. In three of the sampled lakes, liver total Hg and muscle total Hg concentrations increased with age while liver MeHg concentrations remained stable. However, in walleye livers, the percentage of MeHg decreased with age, possibly indicating the presence of a slow demethylation process. These results are among the first to suggest demethylation in wild-caught freshwater fish populations.

1.1 Introduction

The environmental and human health consequences of mercury (Hg) have been the focus of numerous studies in Canada and the World (e.g. Bjorklund *et al.*, 1984, Mason, 1987, Jackson, 1991, Bahnick and Sauer, 1994, Rose et al., 1999). In fish flesh, Hg is primarily found in an organic form as methylmercury (MeHg) (Bloom, 1992). Unlike inorganic Hg, which is not very efficiently absorbed and rapidly eliminated (Boudou *et al.*, 1991), MeHg easily accumulates and biomagnifies along the aquatic food web with top predators being the most contaminated (e.g.: Cabana and Rassmussen, 1994, Watras *et al.*, 1998, Clarkson, 2002, Berntssen *et al.*, 2003). MeHg is known to cause adverse effects in most living organisms by targeting the nervous system (Yee and Choi, 1994). Reported effects include anorexia, lethargy, muscle ataxia, visual impairment and death, depending on the dose (e.g.: Wolfe *et al.*, 1998, Castoldi *et al.*, 2001, Berntssen *et al.*, 2003, Wiener *et al.*, 2003). The main route of MeHg exposure in humans occurs through the consumption of fish (Castoldi *et al.*, 2001, Daré *et al.*, 2001) and in light of the associated adverse effects, many countries now have consumer advisories that encourage people to limit their intake of fish (Canada, 1985, WHO, 1990, USEPA, 1998).

Since fish are the main vector of Hg exposure in humans, a large body of research has focused on quantifying Hg concentrations in fish muscle (Goldstein *et al.*, 1996, Cizdiel *et al.*, 2002, Kamman *et al.*, 2005). However, less is known about Hg kinetics in other tissues (Cizdziel *et al.*, 2003), which is an important issue to address when attempting to understand Hg bioaccumulation and health effects in fish. Laboratory studies have demonstrated that MeHg accumulation is a dynamic process, with initial distribution to the blood, followed by accumulation in the kidney, spleen, liver and brain (e.g. Goldstein *et al.*, 1996, Schultz and Neuman, 1997). In a study on the uptake and distribution kinetics of MeHg in sheepshead minnows, Leaner and Mason (2004) found that the liver was the preferential storage organ for MeHg during the first few days following exposure, but that the

long-term sink was the muscle, where MeHg is sequestered in a water-soluble protein-bound form (Harris *et al.*, 2003, Wiener *et al.*, 2003). The storage of MeHg in skeletal muscles may serve as a protective mechanism in fish, given that the sequestration in muscles may reduce Hg exposure to the central nervous system (Wiener *et al.*, 2003).

The object of this investigation was to compare Hg bioaccumulation in the livers and muscles of wild-caught walleye (*Sander vitreus*) from Canadian Boreal Shield lakes in order to gain insights on freshwater fish Hg toxicokinetics. We also focused on Hg kinetics in the liver because of its capacity to detoxify xenobiotics (Cizdziel *et al.*, 2003) as well as its fundamental role in MeHg redistribution within organisms (Leaner and Mason, 2004).

1.2 Methodology

This study was conducted as part of a larger investigation on the ecosystem effects of Hg in the Abitibi-Témiscamingue region of Québec. Four lakes representing a gradient of fish Hg concentrations were selected on the basis of a previous study (Simoneau *et al.*, 2005); Lake Malartic (78°05′59″N, 46°38′35″W), Lake Preissac (78° 21′ 57″ N, 48° 22′ 33″ W), Lake Desjardins-East (78° 15′ 30″ N, 46° 38′ 35″ W) and Lake Desjardins-West (78° 15′ 30″ N, 46° 38′ 40″ W). Sampling occurred during the first two weeks of August, 2005. A minimum of 30 walleye per lake were collected using experimental gill nets (61 m x 2.4 m) with mesh sizes ranging from 2.5 cm to 15.2 cm. Fish were sacrificed by a sharp blow to the head followed by cervical dislocation. Walleye were measured and weighed before being bled and samples of liver and dorsal muscle tissues were harvested for mercury analysis.

Total Hg concentrations were measured in liver and muscle tissues using cold vapor atomic fluorescence spectrometry (CV-AFS). One hundred grams of muscle tissue were freeze dried and digested in a 10:1 mixture of 1 N HNO_3 and 6

N HCl as described in Pichet *et al.* (1999). Samples were analyzed in duplicate or triplicate. MeHg concentrations were measured in liver tissues according to the protocol outlined in Pichet *et al.* (1999). Briefly, 5 mg of liver were digested in a KOH/MeOH solution and analyzed in duplicate or triplicate. Quality control included procedural blanks and National Research Council of Canada certified calibration standards (lobster hepatopancreas or Tort-2 and dogfish muscle or Dorm-1). The percentage of liver MeHg (% liver MeHg) was calculated as follows:

(Liver MeHg concentration/Liver total Hg concentration) x 100 (equation 1)

Walleye ages were determined using the otolith method described in Pépin and Lévesque (1985). Structures were submerged in alcohol and read using a binocular. Each structure was read by two independent readers.

Statistical data analysis was performed using JMP 5.1 software. In order to facilitate among-lake comparisons, we decided to group fish into age classes ranging from 1 to 6 because only one of the four lakes had walleye that were over the age of 6. Simple linear regression analysis was carried out to detect associations between 1) muscle total Hg concentrations and age; 2) walleye length and age; and 3) liver total Hg concentrations and muscle total Hg concentrations for each of the lakes. The relation between length and age for lake Desjardins-East was best described by a polynomial relationship; however, in order to compare the walleye from this lake to those of the others, we used a linear fit. Before using the linear relation, we tested both models in order to ensure that the slopes and the model outputs did not differ significantly. Analysis of covariance (ANCOVA) was then carried out in order to determine whether slopes were different among lakes for relations established between muscle total Hg concentrations and age and length and age. Mean values of liver total Hg concentrations, liver MeHg concentrations, muscle total and liver total Hg concentrations and % MeHg were calculated for walleye from each age group in

all four lakes. Analysis of variance (ANOVA) and Tukey-Kramer HSD multiple comparison tests were then used to determine significant differences in Hg forms in the different compartments of walleye among lakes. Lakes Preissac, Desjardins-East and Desjardins-West were not significantly different and were grouped for further analysis. Finally, linear regression analysis was used in order to explore the relation between Hg forms and age for the pooled data set (pooled mean values per age for lakes Preissac, Desjardins-East and Desjardins-West) and for the entire data set in Lake Malartic. Statistical significance was set at a probability level $\alpha < 0.05$.

1.3 Results

Muscle total Hg concentrations ranged anywhere from 155 ppb in Lake Desjardins-East, to 837 ppb in Lake Malartic, which is similar to the overall range of liver total concentrations (140 ppb in Lake Desjardins-East to 859 ppb in Lake Malartic). Walleye muscle total Hg concentrations were positively related to age in each of the lakes sampled (Figure 1.1). The slopes describing the relation between muscle total Hg concentrations and age, or in other words, the Hg muscle accumulation rates, were not statistically different for lakes Malartic, Desjardins-East and Desjardins-West as determined using ANCOVA analysis (Figure 1.1), although the origins differed between Lake Malartic and lakes Desjardins-East and Desjardins-West. Walleye from Lake Preissac had lower Hg accumulation rates than walleye from the other lakes. Liver total Hg concentrations increased proportionally with muscle total Hg concentrations (Table 1.1). Growth patterns were determined using linear regression analyses, since the growth of fish in the 1 to 6 year old cohorts in these lakes was generally best described using this model (Figure 1.2). Fish from Lake Desjardins-East had a significantly higher growth rate than fish from lakes Malartic, Desjardins-West and Preissac. Growth rates for walleye from Desjardins-West, Malartic and Preissac did not differ significantly according to ANCOVA analyses on the slopes (Figure 1.2).

Walleye from Lake Malartic had significantly higher mean muscle total Hg, liver total Hg and liver MeHg concentrations than those of the other lakes. The mean values for these Hg forms did not differ significantly among lakes Desjardins-East, Desjardins-West and Preissac (Table 1.2). The % MeHg did not vary significantly among walleye from lakes Preissac, Desjardins-East and Desjardins-West, while significant differences existed between Lake Malartic and lakes Desjardins-West and Preissac.

Based on the data in Table 1.2, lakes Preissac, Desjardins-West and Desjardins-East were grouped together for subsequent analysis. Muscle total Hg concentrations were positively related to age (Figure 1.3, R^2=0.82, P<0.0001, n=17) as were liver total Hg concentrations (Figure 1.3, R^2=0.66, P<0.0001, n=17). Liver MeHg concentrations also tended to increase proportionally with age, however, this relation was only marginally significant (Figure 1.3, R^2=0.22, P=0.0891, n=17) and mean values never exceed 200 ppb. The % liver MeHg is negatively related to age (Figure 1.4A, R^2=0.48, P=0.0022, n=17) and this relation is stronger when the outlier is removed (Figure 1.4 B, R^2=0.64, P=0.0002, n=16).

In Lake Malartic, the relations for the organic and inorganic forms of Hg in the liver increase linearly with age (Figure 1.5, R^2=0.51, P=0.0137, n=11 and R^2=0.46, P=0.0021, n=11 for liver MeHg concentrations and liver total Hg concentrations, respectively), but most of the Hg in the liver appears to be in the methylated form. An ANCOVA analysis determined that there were no significant differences between the origins and slopes of these two linear regressions (P=0.6015 and P=0.5839, respectively). There was no significant relation between the % liver MeHg values and age (P=0.45, n=11).

1.4 Discussion

A study by Bloom (1992) has concluded that over 95% of the Hg found in fish muscle is in the methylated form. This has been corroborated by several other

studies (e.g. Goldstein *et al.*, 1996, Oliveira Ribeiro *et al.*, 1999, Leaner and Mason, 2004). Within a fish species, muscle total Hg concentrations tend to increase with length, age and/or weight (Wiener *et al.*, 2003, Simoneau *et al.*, 2005). The results from the present study corroborate this finding, as walleye muscle total Hg concentrations increase linearly with age in all the lakes under study (Figure 1.1). Walleye liver total Hg concentrations are also related to muscle total Hg concentrations (Table 1.1), which is similar to the results reported by Cizdziel *et al.* (2003) and others (Goldstein *et al.*, 1996). However, according to our results, the Hg accumulation rate in fish muscles varies from one lake to the next with walleye from lakes Malartic, Desjardins-East and Desjardins-West accumulating Hg as a function of age more rapidly than walleye from Lake Preissac. While differences in accumulation rates have been linked to differences in growth rates in the past (Simoneau *et al.*, 2005), this is not reflected by our data (Figure 1.2). If growth were to modulate Hg concentrations in the fish from our study, walleye from Desjardins-East would have the lowest Hg concentrations while walleye from lakes Malartic, Desjardins-West and Preissac would have higher and statistically similar muscle Hg concentrations. This is not the case, as the walleye sampled from Lake Malartic have significantly higher mean total muscle Hg concentrations than those of Desjardins-East, Desjardins-West and Preissac, which did not differ significantly (Table 1.2). However, the fish from this study, which are grouped into a cohort ranging from ages 1 to 6, are relatively young and are not entirely representative of the entire age spectrum in feral fish populations. It is possible that growth may play a larger role when the full spectrum of ages is considered, but our data do not allow for such inferences. It is also possible that these differences in accumulation rates may be attributable to other factors such as genetic polymorphisms which can affect the delivery of Hg to target organs and affect organ response or to the intake of nutrients that might influence absorption, uptake, distribution and metabolism of Hg (NRC 2000).

In order to understand Hg accumulation in fish, it is important to consider both uptake and absorption (Fournier *et al.*, 2002). In freshwater ecosystems, Hg can

enter the organism via two routes: the direct route, resulting from uptake by the gills of the metal in the water, and the indirect or trophic route, resulting from the consumption of prey (Boudou *et al.*, 1991). In piscivorous fish such as walleye, the dominant pathway for MeHg bioaccumulation is through the indirect route (Leaner and Mason, 2002). Trudel *et al.* (2000) recently reported that less than 0.1 % of the Hg accumulated in fish resulted from the direct uptake of Hg from water. In wild fish, it has been suggested that dietary uptake accounts for more than 90 % of the total uptake of MeHg (Wiener *et al.*, 2003). While both organic and inorganic forms of Hg exist in prey items, almost no inorganic Hg is absorbed by the organism. Inorganic Hg was shown to accumulate in the intestine, but since the intestinal walls are relatively impermeable to this form of Hg, most is excreted in the feces (Boudou *et al.*, 1991, Oliveira Ribeiro *et al.*, 1999). In a study on Hg distribution kinetics in arctic charr, Oliveira Ribeiro *et al.* (1999) reported that less than 4% of inorganic Hg was distributed to the blood and visceral organs following feeding and that the concentrations in organs remained constant once the fecal elimination of the non retained metal occurred. MeHg on the other hand, is very efficiently absorbed with estimated transfer rates between 76 and 86 % (Boudou *et al.*, 1991).

It should therefore be expected that the majority of Hg that accumulates in fish is in the methylated form. However, according to the results presented in Figure 1.3, a portion of the MeHg appears to be transformed in the livers of walleye that have relatively low mean liver total Hg concentrations (under 300 ppb). As stated above, muscle total Hg concentrations increase steadily with age (pooled mean data for lakes Preissac, Desjardins-East and Desjardins-West (Figure 1.3, R^2=0.82, P<0.0001, n=17), as commonly reported (Trudel and Rassmussen, 2006), and liver total Hg concentrations increase as well (Figure 1.3, R^2=0.66, P<0.0001, n=17), which is in agreement with the findings reported by Cizdziel *et al.* (2003). However, in these three lakes, liver MeHg concentrations do not increase significantly with age, never exceed 200 ppb (Figure 1.3, R^2=0.22, P=0.0891, n=16) and the % liver MeHg decreases with age (Figure 1.4, R^2=0.64,

P=0.0002, n=16). These data suggest that there may be a demethylation process in walleye livers and that this process is relatively slow. To our knowledge, no other researchers have reported similar findings in freshwater fish. Demethylation has, however, been demonstrated in different species. In guinea pig livers, 30% of total Hg concentrations were in the inorganic form three weeks after the administration of MeHg (Komsta-Szumska *et al.*, 1983) and research with monkeys has shown the existence of a slow demethylation process in the brain (Lind *et al.*, 1988, Vahter *et al.*, 1995). Indirect evidence in avian species also indicates the presence of a similar process (Scheuhammer *et al.*, 1998).

The physiological process governing MeHg demethylation is unclear - it may be enzymatic, bacterial or chemical (Storelli *et al.*, 1998), but several authors suggest that selenium (Se) may play a role (Koeman *et al.*, 1975, Yoneda and Suzuki, 1997, Scheuhammer *et al.*, 1998). The demethylation of MeHg is believed to be a two step process involving the initial storage of MeHg in the liver, followed by the formation of an equimolar inorganic Hg-Se complex when MeHg concentrations increase above a certain threshold value (Koeman *et al.*, 1975, Scheuhammer *et al.*, 1998, Storelli *et al.*, 1998). The formation of this Hg-Se complex may reduce the toxicity of MeHg to the organism (Yoneda and Suzuki, 1997). It would be interesting to measure the Se concentrations in future studies in order to verify the existence of an equimolar relation between Hg and Se.

Several authors have also reported the presence of a MeHg threshold value at which the concentration of liver MeHg is maintained. Threshold values for MeHg appear to vary according to class; Storelli *et al.* (1998) reported threshold values of 1 ppm and 100 ppm for turtles and dolphins, respectively, Wagemann *et al.* (1998) indicate a possible value of 2 ppm for marine mammals, while Scheuhammer *et al.* (1998) found that liver MeHg concentrations remained below 10 ppm in two piscivorous birds. These values are much higher than that observed in our study, which can be estimated at 200 ppb for walleye between

ages 1 and 6 in the three pooled lakes. Additional research is needed in order to verify this value.

According to our data, a possible liver demethylation pathway may exist in walleye with relatively low mean liver total Hg concentrations as discussed above. However, in Lake Malartic, where walleye had the highest mean liver total Hg concentration (343.50 ± 52.43 ppb), the Hg and MeHg distribution patterns observed were different than those for fish from the other lakes. As shown in Figure 1.5, the relations for the different liver Hg species increase linearly with age, unlike in the other three lakes, where liver MeHg concentrations remained relatively stable. Both regressions (i.e. slopes and origins) were not statistically different which indicates that all the Hg in walleye livers from Lake Malartic is in the methylated form. In addition, there was no significant relation between the % liver MeHg and age (P=0.45, n=11). These results are surprising and suggest that there is no demethylation taking place in the livers of walleye in the age range sampled from Lake Malartic. One possible explanation may be that the demethylation process is saturated, which would result in the accumulation of MeHg in the liver. The process might also be inhibited by other xenobiotics. It would be interesting to repeat this study in lakes representing a wider range of fish Hg concentrations in order to fully verify these hypotheses.

1.5 Conclusion

This study has shown that the distribution of Hg varies according to the tissue sampled and that Hg toxicokinetics differ in livers relative to muscles. In three of the lakes, the percentage of MeHg in walleye livers was shown to decrease with age, which may constitute indirect evidence of a possible demethylation process. This is, to our knowledge, this first study to suggest such a phenomenon in freshwater, feral fish species.

1.6 Acknowledgements

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Figure 1.1: Relation between total muscle Hg and age in walleye among the four lakes. The equations are as follows: Desjardins-East: muscle Hg = 107.16 + 85.47 age (R^2=0.83, n=35, P<0.0001); Desjardins-West: muscle Hg = 147.37 + 72.05 age (R^2=0.79, n=38, P<0.0001); Malartic: muscle Hg = 308.33 + 72.52 age (R^2=0.77, n=15, P<0.0001); Preissac: muscle Hg =231.27 + 31.54 age (R^2=0.41, n=21, P=0.0019). Linear regressions with the same letter represent relations with slopes that are not significantly different (statistical significance was set at a probability level of α 0.05).

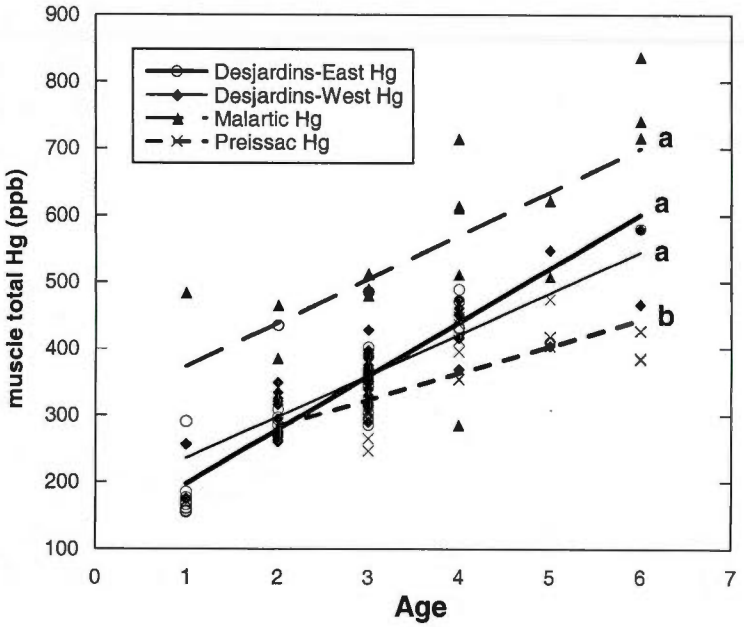


Table 1.1: Linear regression equations, coefficients and statistical parameters for the relation between liver total Hg concentrations and muscle total Hg concentrations among lakes.

Lake	Regression coefficients and P values
Desjardins-East	Liver total Hg=150.16 + 0.26 muscle Hg R^2=0.41 n=31 P<0.0001
Desjardins-West	Liver total Hg=69.32 + 0.46 muscle Hg R^2=0.71 n=34 P<0.0001
Malartic	Liver total Hg=29.87+ 0.65 muscle Hg R^2=0.76 n=15 P<0.0001
Preissac	Liver total Hg=-15.61 + 0.71 muscle Hg R^2=0.60 n=20 P<0.0001

Figure 1.2: Relation between length and age in walleye among the four lakes. The equations are as follows: Desjardins-East: length = 156.86 + 50.87 age (R^2=0.83, n=39, P<0.0001); Desjardins-West: length = 210.23 + 27.07 age (R^2=0.52, n=38, P<0.0001); Malartic: length = 210.67 + 23.98 age (R^2=0.73, n=15, P<0.0001); Preissac: length =207.84 + 23.41 age (R^2=0.63, n=21, P<0.0001). Linear regressions with the same letter represent relations with slopes that are not significantly different (statistical significance was set at a probability level of α 0.05).

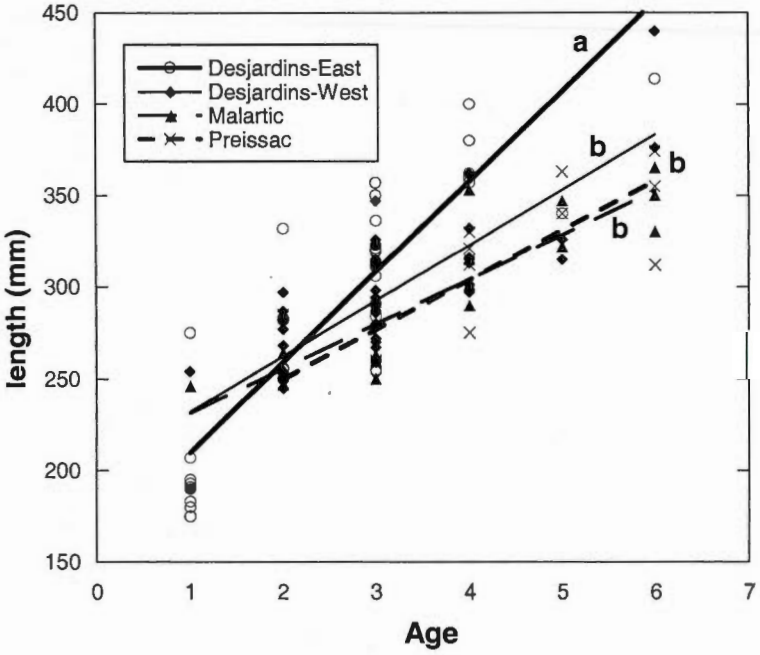


Table 1.2: Mean and standard error of different Hg forms in liver and muscle of walleye among the different lakes sampled. Hg concentrations are expressed in ppb. Values with the same letter are not significantly different (Tukey-Kramer HSD α 0.05).

	Lake			
Hg species	Desjardins-East	Desjardins-West	Malartic	Preissac
Muscle total Hg	331.11 ± 18.02^{b} n=36	358.97 ± 16.88^{b} n=41	554.25 ± 27.03^{a} n=16	371.13 ± 22.54^{b} n=23
Liver total Hg	237.24 ± 14.48^{b} n=33	239.49 ± 13.86^{b} n=36	395.27 ± 20.17^{a} n=17	257.44 ± 18.59^{b} n=20
Liver MeHg	187.06 ± 5.73^{b} n=33	182.92 ± 6.57^{b} n=36	343.50 ± 52.43^{a} n=11	176.99 ± 9.37^{b} n=20
% MeHg	$77.80 \pm 2.11^{a,b}$ n=33	73.26 ± 1.99^{b} n=36	88.45 ± 3.42^{a} n=11	69.83 ± 2.76^{b} n=20

Figure 1.3: Relation between the different forms of Hg and age in liver and muscle of walleye. Pooled data set of mean Hg concentrations for each age class for lakes Preissac, Desjardins-East and Desjardins-West are presented. The equations for each relation are as follows: muscle Hg = 195.25 + 51.56age (R^2=0.82, n=17, P<0.0001); liver Hg =185.79+19.59age (R^2=0.66, n=17, P<0.0001); liver MeHg=160.43+5.19 age (R^2=0.22, n=16, P=0.0891).

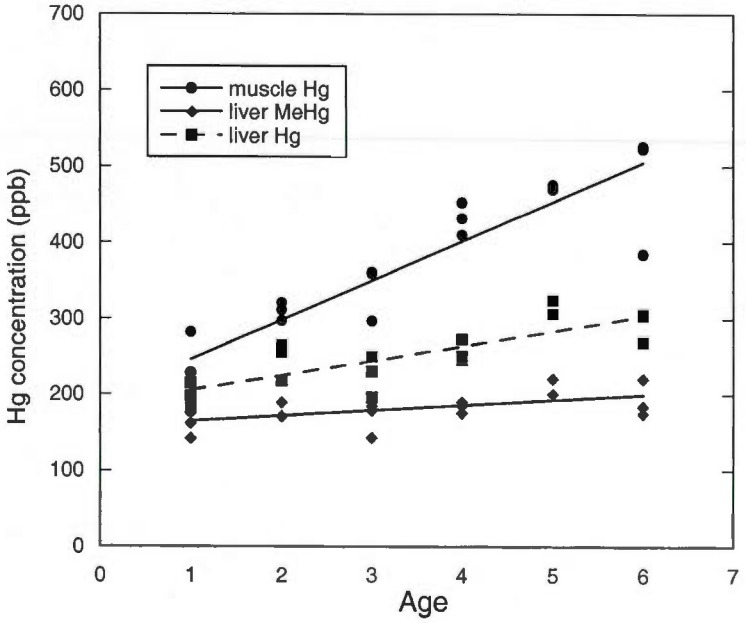


Figure 1.4: The % MeHg in walleye liver as a function of age for the pooled data set of mean Hg concentrations for each age class for lakes Preissac, Desjardins-East and Desjardins-West A) with outlier and B) without.

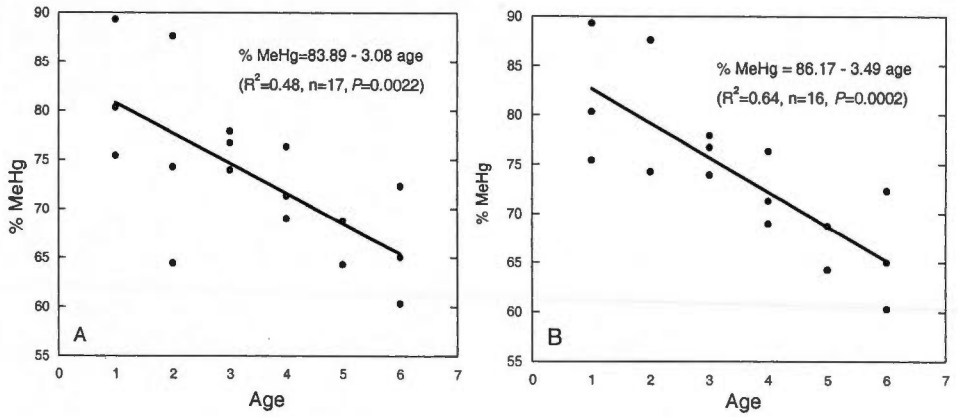
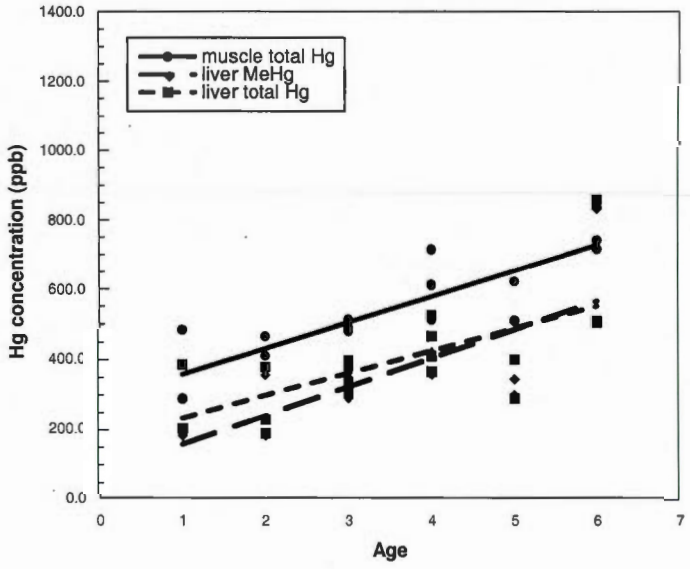


Figure 1.5: Relation between the different forms of Hg and age in liver and muscle of walleye from Lake Malartic. The equations for each relation are as follows: muscle Hg = 280.44 + 74.38 age (R^2=0.70, n=17, P<0.0001); liver Hg =188.99+65.39age (R^2=0.46, n=18, P=0.0021); liver MeHg = 71.84 + 82.27 age (R^2=0.51, n=11, P=0.0137).



CHAPTER II*

TOXICOLOGICAL EFFECTS OF METHYLMERCURY ON WALLEYE (*Sander vitreus*) AND PERCH (*Perca flavescens*) FROM LAKES OF THE BOREAL FOREST

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Abstract

Biochemical and physiological responses of walleye (*Sander vitreus*) and perch (*Perca flavescens*) were studied in four Canadian boreal forest lakes representing a mercury (Hg) Hg exposure gradient. The aim of this study was to assess the effects of Hg and methylmercury (MeHg) on the general physiological condition of fish as well as to gauge the relationship between MeHg and the glutathione (GSH) system in metal-contaminated and reference sites using a series of biomarkers. Walleye from Lake Malartic had the highest liver MeHg concentrations, exhibited lower hepatosomatic indices (HSI) and lower glutathione *S*-transferase (GST) activity. HSI was negatively related to liver total Hg concentrations in walleye (R^2 = 0.33, n=108, P< 0.0001). Glutathione reductase (GR) and GST activity for walleye from Lake Malartic were related to HSI (R^2 = 0.38, n=25, P= 0.0010; R^2 = 0.46, n=27, P< 0.0001, respectively). In Lake Desjardins-East, where perch had the highest liver MeHg concentrations, glutathione peroxidase selenium dependent activity (GSH-Px SD) and GST activity were negatively related to liver MeHg concentrations (R^2 = 0.39, n=21, P= 0.0026; R^2 = 0.22, n=21, P= 0.0298, respectively). This study suggests that Hg may induce adverse effects on the physiology and cellular metabolism of walleye and perch at environmentally relevant concentrations.

2.1 Introduction

The presence of Mercury (Hg) in ecosystems is ubiquitous and has continued to rise steadily since the beginning of the industrial period (Sweet and Zelikoff, 2001). In terms of Hg burdens in fish, aquatic ecosystems exhibit important inter-lake variability, even within the same geographic region (Rose *et al.*, 1999, Schetagne and Verdon, 1999). In a study of 775 water bodies across the province of Québec, walleye (*Sander Vitreus*) and perch (*Perca fluvescens*) Hg muscle levels were reported to range from 0.09 to 4.90 mg/kg and from 0.05 to 1.90 mg/kg (ppm), respectively (Laliberté, 2004). Many lakes are reported to have fish with Hg concentrations that exceed commercialization guidelines set at 0.5 mg/kg by the government of Canada. While the adverse human health effects of Hg and methylmercury (MeHg), the more toxic organic form of Hg, are well documented (Castoldi *et al.*, 2001, Daré *et al.*, 2001, Chan *et al.*, 2003), little is known on the effect of these contaminants on fish health or condition. Of the few studies published on the physiological and toxicological aspects of Hg contamination in fish, Berntssen *et al.* (2003) found that MeHg can cause incoordination, loss of appetite, diminished swimming activity and mortality. Wiener *et al.* (2003) suggest that MeHg may reduce reproductive success by affecting gonadal development or spawning success of adults or by reducing the hatching of eggs and the health and survival of embryolarval stages. Hammerschmidt *et al.* (2002) also reported Hg-related reproductive effects such as delayed spawning in adult fathead minnows. However, these studies consist of controlled laboratory experiments in which fish are exposed to different concentrations of MeHg over a relatively short period of time. MeHg also has similar effects in birds (Wiener *et al.*, 2003). In humans, MeHg can cause damage to the visual cortex and to the sensory system. Signs of intoxication include the constriction of the visual field, sensory impairment of extremities, hearing loss, muscle weakness, tremors and mental deterioration (Castoldi *et al.*, 2001).

Although the molecular mechanisms for mercury toxicity have yet to be completely clarified, recent reports suggest that its biochemical mode of action involves the generation of radical oxygen species (ROS) and lipid peroxidation through mitochondrial impairment (Yee and Choi, 1994, Daré *et al*, 2001) leading to cell death via necrosis or apoptosis (Castoldi *et al.*, 2001, Bragadin *et al.*, 2002). These ROS, generated in organisms via several different cellular processes that involve either endogenous or xenobiotic compounds (Winston and Di Giulio, 1991), then randomly attack all cell components, including proteins, lipids and nucleic acids, potentially causing extensive cellular damage (Leonard *et al.*, 2004). When there is an imbalance between the generation and removal of radical species within an organism, the resultant is oxidative stress (Kelly *et al.*, 1998).

Fish have several different types of cellular antioxidant defense mechanisms, one of the most important being the glutathione (GSH) system. Glutathione (L-γ-glutamyl-cysteinyl-glycine) is a major non-protein thiol that is involved in a variety of cellular functions such as transport and metabolic processes, cellular signaling, the maintenance of protein thiols critical to enzyme activity, as well as cellular defense against xenobiotics and oxidative stress (e.g. Hasspieler *et al.*, 1994, Canesi *et al.*, 1999, Peña *et al.*, 2000). MeHg has a high affinity for sulfhydryl groups, including those of GSH as well as those of proteins (Canesi *et al.*, 1999). MeHg binds with such avidity to sulfhydryl groups that its association with other ligands in biological systems is inconsequential (Hughes, 1957). In binding to GSH, MeHg reduces the intracellular pool of available GSH molecules. As a protective response, the organism may increase GSH regeneration and biosynthesis via a variety of enzyme systems such as glutathione reductase (GR), which catalyzes the regeneration of GSH by reduction of glutathione disulfide (GSSG), and by *de novo* synthesis, involving two separate ATP dependent enzyme systems: glutamate-cysteine ligase (GCL) (rate-limiting step) and glutathione synthetase (GS) (Winston and Di Giulio, 1991, Llopis-Peña *et al.*, 2003).

The aim of this study was to determine whether an association exists between fish Hg/MeHg concentrations and biochemical and physiological effects in field-collected fish. We measured a series of physiological (LeCren condition factor and hepatosomatic index) and biochemical biomarkers (GSH and associated enzymes) in walleye and perch collected from four Canadian boreal forest lakes. Our hypothesis is that MeHg induces adverse effects on the physiological condition of fish at environmentally relevant concentrations.

2.2 Methodology

2.2.1 Lake selection and description

Study lakes were selected on the basis of a previous survey of mercury in lakes of the boreal forest. High, intermediate, and low mercury sites were chosen based on reported mean muscle mercury concentrations in walleye that were size-adjusted to a standard length of 350 mm using polynomial regressions (Simoneau *et al.*, 2005). Two lakes from the Abitibi region were selected; Lake Malartic (78° 05′ 59″ N, 46° 38′ 35″ W) which has walleye with a reported length-standardized mean Hg muscle concentration of 0.79 ppm, and Lake Preissac (78° 21′ 57″ N, 48° 22′ 33″ W), which had walleye with a reported mean Hg length-standardized muscle concentration of 0.32 ppm. In the Témiscamingue region, Lake Desjardins was selected. Lake Desjardins is separated into two arms that are connected by a weir. Previous research has shown that the mean Hg length-standardized muscle concentration in walleye from the upstream arm, or Desjardins-East (78° 15′ 30″ N, 46° 38′ 35″ W), is 0.5 ppm, which is nearly double those of walleye from the downstream arm, or Desjardins-West (78° 15′ 30″ N, 46° 38′ 40″ W) with a mean Hg length-standardized muscle concentration of 0.3 ppm (Simoneau *et al.*, 2005). Therefore, these two water bodies were considered separately (i.e. as two lakes). The length-standardized walleye muscle Hg concentrations from these four lakes fall into the lower range of reported Hg concentrations for the province of Québec (0.09 to 4.9 ppm) and we consider that

these four lakes represent a low, but environmentally relevant Hg exposure gradient.

2.2.2 Sample collection and preparation

From each of the chosen lakes, a minimum of 30 walleye were collected using experimental gill nets (61 m x 2.4 m) with mesh sizes ranging from 2.5 cm to 15.2 cm, while a minimum of 30 perch were caught using a seine. The sampling campaign was held during the first two weeks of August, 2005. Sampling occurred between 18h00 and 22h00 for every lake and sampling sites were chosen randomly. The nets were lifted every hour and the fish were collected alive and placed in a floating fish trap. The sample schedule was strictly followed so as to ensure that the hormonal and diurnal cycles are similar for all the fish sampled (Sepúlveda *et al.*, 2004).

Walleye and perch were sacrificed by a sharp blow to the head followed by cervical dislocation within two hours of capture. They were measured and weighed before being bled (Sepúlveda *et al.*, 2004). Livers from both species were excised and blotted within 10 minutes of sacrificing the animal (Anulacion *et al.*, 1997). Walleye livers were weighed in order to establish the hepatosomatic index (HSI). The HSI is specific for the liver, but can be used as an indicator of the overall health of the animal. Perch livers were too small to be weighed accurately; therefore the HSI was not determined for this species and the LeCren condition index, which describes the general health of fish, was used instead. Livers were rinsed in a phosphate buffered saline solution (PBS) to remove excess blood before being sliced transversally and sectioned into pieces, placed in 2ml cryovials and snap frozen in liquid nitrogen. Liver samples were stored at -80°C until analysis in the laboratory (Anulacion *et al.*, 1997, Krüner and Westernhagen, 1999). Finally, age structures (otholiths and opercula) were collected and stored at room temperature (Pépin and Lévesque, 1985).

2.2.3 Condition factors

For walleye, the hepatosomatic index (HSI) was calculated using the following equation (Sepúlveda *et al.*, 2004):

$$\text{(weight of organ/ weight of fish) x 100} \qquad \text{(equation 1)}$$

For perch, the LeCren (C_L) condition factor was calculated according to the following equation:

$$C_L = W/aL^b \qquad \text{(equation 2)}$$

where W represents the weight of the individual and L represents length. The values for a and b were derived empirically using the following function which describes the relationship between weight and length (Eastwood and Couture, 2002):

$$\log W = \log a + b \log L \qquad \text{(equation 3)}$$

2.2.4 Mercury Analysis

Total mercury (Hg) was measured in liver tissues using cold vapor atomic fluorescence spectrometry (CV-AFS). Five mg of liver were freeze dried and digested in a 10:1 mixture of 1 N HNO_3 and 6 N HCl as described in Pichet *et al.* (1999). Samples were analyzed in duplicate or triplicate. Methlymercury (MeHg) was analyzed according to the protocol outlined in Pichet *et al.* (1999). Five mg of liver were digested in KOH/MeOH solution and analyzed in duplicate or triplicate. Quality control included procedural blanks and National Research Council of Canada certified calibration standards (lobster hepatopancreas or Tort-2 and dogfish muscle or Dorm-1).

2.2.5 Age determination

The ages of walleye were estimated using the otholith method, while the ages of perch were estimated using the opercula method, which are both described in Pépin and Lévesque (1985). Ages were estimated by two independent readers.

2.2.6 Cytosolic enzyme sample preparation

Samples for glutathione associated enzyme activities were processed according to Meyer *et al.* (2003). Liver tissues were homogenized for 30 seconds in 4 volumes of an ice-cold Tris-HCl homogenizing buffer (0.1 M Tris-HCl, 0.25 M sucrose, 1mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethylsulfonyl fluoride (PMSF) at a pH of 7.4, using a Brinkman Polytron homogenizer. The homogenates were then centrifuged at 10,000 g for 20 minutes at 4°C. The resulting supernatants were centrifuged at 105,000 g for 1 hour at 4°C. The 105,000 g supernatants were then transferred and snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis (Meyer *et al.*, 2003).

2.2.7 Enzyme assays

Glutathione reductase: Glutathione reductase (GR) activity was measured by a plate reader on cytosolic supernatants according to the method described by Smith *et al.* (1989) as modified for a plate reader by Cribb *et al.* (1989) in a final reaction mixture of 0.1 M sodium phosphate buffer (NaH_2PO_4), 1mM EDTA, 6 mM nicotinamide adenine dinucleotide phosphate (NADPH), 2 mM GSSG, and 1.625 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

Glutathione peroxidase: Glutathione peroxidase (GSH-Px) activity was measured by a plate reader as described by Gallagher and Di Giulio (1992) and modified by Meyer *et al.* (2003) in a final reaction mixture of 50 mM potassium phosphate

(KPO_4) at pH 8.3, 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.2 mM NADPH, 1 U/ml GR, 1 mM GSH and either 1.5 mM cumene hydroperoxide (to measure total GSH-Px activity) or 0.25 mM hydrogen peroxide (to measure glutathione peroxidase selenium dependent (GSH-Px SD) activity). Glutathione peroxidase selenium independent (GSH-Px SI) activity was determined by subtracting GSH-Px SD activity from total GSH-Px activity (Gallagher and Di Giulio, 1992).

Glutathione S-transferase: Glutathione *S*-transferase (GST) analysis was based on the methods described by Habig and Jackoby (1981) as modified for a plate reader by Gallagher *et al.* (2000) in a final reaction mixture of 1mM GSH, 0.01 M KPO_4 and 1 mM 1-chloro-2,4-nitrobenzene (CDNB).

Glutamate cysteine ligase and glutathione concentrations: Glutamate cysteine ligase (GCL) activity and baseline GSH concentrations were measured by a dual beam spectrophotometer in accordance with the protocol outlined by White *et al.* (2003) except that samples were incubated at room temperature instead of 37°C.

Protein dosing: All enzyme activities were expressed as nmol/min/mg protein (Hasspieler *et al.*, 1994). Therefore, protein content in the different fractions of liver samples was measured using the Biorad assay (Meyer *et al.*, 2003).

2.2.8 Statistical analysis

Data were analyzed using JMP 5.1 software (SAS Institute, 2003). Data were not normally distributed in all cases and the variance was unequal for some parameters, therefore non-parametric tests were used. Correlations between variables based on Spearman's index (data not shown) were examined to explore possible associations among all variables measured (liver MeHg and total Hg concentrations, age, length, condition indices, GSH concentrations, GST, GSH-Px SD, GSH-Px SI and GR activities). Non-parametric Wilcoxon/Kruscal-Wallis tests were used to determine significant differences between the four fish

populations for liver MeHg concentrations, enzymatic activity and condition indices. When the test was significant, a Noether test for multiple comparisons of unbalanced groups was used to determine which lakes were significantly different (Scherrer, 1984). In perch, liver Hg and liver MeHg concentrations were unrelated to age and/or length in all lakes sampled. In walleye, liver MeHg concentrations were unrelated to age in three of the four lakes, with the exception of Lake Malartic (Chapter 1). However, in Lake Malartic, this relation was rendered significant by only one sample. In order to account for this factor, we compared walleye from the same age range (pooled ages from 1 to 6, since only Lake Malartic had older walleye) among all the lakes for liver MeHg concentrations. All other parameters, including condition indices and biochemical biomarkers, were unrelated to age or length in both species. A Michaelis-Menten non-linear model was used to describe the relation between HSI and liver Hg concentrations in walleye. Then a regression of HSI, using the fitted estimates from the Michaelis-Menten non-linear model, on Hg values was carried out in order to determine the probability and the correlation coefficient value of the relationship. Finally, simple linear regression analysis was carried out to detect associations between variables in the two lakes with the highest MeHg concentrations. Statistical significance was set at a probability level $\alpha < 0.05$.

2.3 Results

2.3.1 MeHg concentrations

The distribution of liver MeHg concentrations was significantly higher in walleye from Lake Malartic (roughly 40%) than that of the other three walleye populations, independent of age. There were no significant differences among the other populations (Figure 2.1). For perch, the distribution of liver MeHg concentrations was highest in Lake Desjardins-East, followed by Lake Desjardins-West, Lake Malartic and Lake Preissac. There were no significant differences in

perch liver MeHg concentrations between lakes Malartic and Preissac (Figure 2.2).

2.3.2 Condition indices

There were significant differences in the distributions of HSI values among the four walleye populations as determined by a Wilcoxon/Kruskal-Wallis test ($P<$ 0.0001). The distribution of walleye HSI was highest in lakes Desjardins-East and Desjardins West, followed by Lake Preissac and was lowest in Lake Malartic (Figure 2.3). The distribution of walleye HSI values for lakes Malartic and Preissac were significantly different than those of the other two lakes. A Noether test determined that there was no significant difference in walleye HSI values from lakes Desjardins-East and Desjardins-West (Figure 2.3). HSI values were negatively related to liver total Hg concentrations and the fitted values of the Michaelis-Menten model explained 33% of the variability observed (n=108, $P<$ 0.0001, Figure 2.4). HSI values were not correlated to age or length (P=0.0950 and P=0.1813, respectively).

Significant differences in the distribution of the C_L were observed among the four perch populations studied (Figure 2.5, Wilcoxon/Kruskal Wallis, $P<$ 0.0001). Perch from Lake Desjardins-West had a significantly lower C_L distribution than populations from the other lakes. There were no significant differences among lakes Malartic, Desjardins-East and Preissac (Figure 2.5). The C_L was not correlated to liver MeHg or Hg concentrations (P=0.0605 and P=0.1122, respectively) or to length or age (P=0.5729 and P=0.5885, respectively).

2.3.3 Biochemical biomarkers

In walleye populations, pairwise correlations determined that GST GR, GSH-Px SD and GSH-Px SI activity were all positively correlated to one another (Table

2.1, B). In perch populations, a similar situation was observed, with the exception of GSH-Px SI activity, which was only correlated to GST activity (Table 2.1, B).

For both species, biochemical biomarkers were unrelated to age and length (Table 2.1 A, B). In walleye populations, there were no significant differences in GSH concentrations, GR, GSH-Px SD or GSH-Px SI activities among the different lakes. Walleye from Lake Malartic had a significantly lower GST activity (roughly 30%) than those from Lake Desjardins-West (Table 2.2, A). Fish from Lake Preissac had significantly lower GCL activity than lakes Desjardins-East and Desjardins-West, while GCL activity in Lake Malartic did not differ significantly from those of the other lakes (Table 2.2, A).

In perch populations, there were no significant differences in GR, GSH-Px SI and GCL activities among the lakes sampled. Perch from Lake Desjardins-West had significantly higher GST activity than those of Malartic and Preissac. GSH-Px SD activity was significantly higher in perch from Lake Desjardins-East than in lakes Desjardins-West, Malartic and Preissac (Table 2.2, B). GSH concentrations were significantly different in perch from lakes Desjardins-West and Preissac (Table 2.2, B).

In Lake Malartic, walleye liver GR and GST activities increased with HSI (Figure 2.6: R^2 = 0.38, n=25, P= 0.0010; R^2 = 0.46, n=27, P< 0.0001, respectively). Enzyme activity was unrelated to MeHg or Hg concentrations. In Lake Desjardins-East, perch GST and GSH-Px SD activities decreased as a function of MeHg liver concentrations (Figure 2.7: R^2 = 0.39, n=21, P= 0.0026; R^2 = 0.22, n=21, P= 0.0298, respectively).

2.4 Discussion

Field studies are useful for ascertaining the potential effects of pollutants on organisms at environmentally relevant concentrations, but unlike an experimental

design where all the variables are controlled, the results generated through a field study are often more ambiguous, making causality difficult to establish (Scherrer, 1984).

It is possible that the fish sampled in this study were subjected to stressors like other types of heavy metals (e.g. cadmium or copper) or pesticides which may have influenced biomarker responses (e.g. Peña-Llopis *et al.*, 2003, Canesi *et al.*, 1999). However, the fish from these lakes were previously sampled for other contaminants, such as polychlorinated biphenyls (PCB) and metals and the concentrations were found to be marginal ($\leq$ 20 µg/kg)(D Laliberté, personal communication). We therefore consider Hg to be the major xenobiotic stressor in these environments.

2.4.1 Methylmercury concentrations

When studying Hg in fish, a large body of research has focused on deriving muscle Hg concentrations (Goldstein *et al.*, 1996, Cizdiel *et al.*, 2002, Kamman *et al.*, 2005). However, less is known about what occurs in other tissues, such as the liver, which plays an important role in xenobiotic detoxification. Moreover, Hg kinetics in this organ differs from that of muscles (Chapter 1). In this study, we focused on the liver and measured both MeHg and total Hg concentrations in order to determine whether mercuric compounds adversely affect this organ.

MeHg concentrations were significantly higher (40%) in walleye livers from Lake Malartic as compared to the other walleye populations (Figure 2.1). Perch from Lake Desjardins-East had the highest liver MeHg concentrations followed by Lake Desjardins-West and lakes Preissac and Malartic (Figure 2.2). This trend differs from that observed in walleye. One possible explanation may be that these two species do not belong to the same food chain in these lakes. Hg is known to bioaccumulate along the food web, therefore species connected to one another in a food web should exhibit similar patterns in Hg concentrations, which is not the

case in this study. Cabana *et al.* (1994) observed that when littoral trophic chains are unconnected to pelagic trophic chains, Hg distribution patterns differ more than when these two food chains are connected. In addition, Mathers and Johansen (1985) found that in certain lakes, walleye feed almost exclusively on the pelagic food chain, which may be the case in the lakes under study and could account for the differences observed in species Hg bioaccumulation patterns.

2.4.2 Condition indices

The HSI is a physiological biomarker that reflects responses following chemical and cellular interactions which are generally indicative of irreversible damage (Hinton *et al.*, 1992). Albeit not specific, it can be a useful indicator of toxicant exposure and provide information on energy reserves and the general health of the organism (van der Oost et al., 2003). Pollutants can induce varying responses in the liver. A number of studies on fish, both field and laboratory based, have reported increases in HSI upon exposure to organic contaminants such as polychlorinated biphenyls and organophosphates (see review by van der Oost *et al.*, 2003). A reduction in HSI, or liver atrophy, is caused by a reduction in the size of liver cells, by a loss of lipids (lipid peroxidation or depletion), or via nuclear and cytoplasmic inclusions, and may be used as a biomarker for heavy metal exposure (Hinton et Laurén, 1990). According to the results of the present study, walleye HSI varied according to population and was related to liver total mercury concentrations. Lake Malartic, which had walleye with the highest liver MeHg concentrations (Figure 2.1), also had the lowest distribution of HSI values, followed by Lake Preissac (Figure 2.3). Walleye HSI was negatively related to liver total Hg concentrations, which explained 33% of the variability observed ($P< 0.0001$, Figure 2.4). The shape of the curve seems to indicate the presence of a threshold value situated at a liver total Hg concentration of about 200 ppb, over which the HSI declines rapidly. Although the negative relationship between HSI and liver Hg concentrations appears to be attributed solely to Lake Malartic, the relationship remains similar when this lake is removed from analysis (data not

shown). These results are consistent with other studies that have also reported reductions in HSI when fish were exposed to heavy metals (Goede and Barton, 1990, Ricard *et al.*, 1998, Norris *et al.*, 2000). Hg is a known pro-oxidant which induces oxidative stress through peroxide production and causes lipid peroxidation (Elia *et al.* 2003), which could lead to reduced HSI (Goede and Barton, 1990). Oliveira Ribeiro *et al.* (2002) found that a single oral dose of MeHg had a severe effect on the liver and caused alterations such as a reduction in the lipid reserves of hepatocytes as well as massive necrosis. A study by Friedmann *et al.* (2002) on the health effects of Hg on largemouth bass (*Micropterus salmoides*) also reported a reduction in HSI in fish from Hg-contaminated sites, but partially attributed the observed decrease to limited forage availability. In a study on the regulation of endogenous energy stores in golden perch (*Macquaria ambigua*), Collins and Anderson (1995) found that food deprivation and re-feeding had an affect on the relative size of fish liver. During deprivation, liver size reduced as energy reserves were mobilized and upon re-feeding, liver reserves were renewed (Collins and Anderson, 1995). It is possible that food deprivation may have also had an effect on HSI values in this study, which is perhaps reflected by the significant effect of population on HSI data (Figure 2.3).

The C_L did not differ significantly for perch among lakes Malartic, Desjardins-East and Preissac and the median value was above 1 (Figure 2.5). For healthy fish populations, the condition factor is usually situated around 1; a superior value indicates a better condition, while an inferior value indicates a poorer one (Couture and Rajotte, 2003). Perch from lake Desjardins-West had a significantly lower C_L, indicating a poorer condition. However, the condition factor was not related to MeHg or Hg concentrations in this lake. Our result contrasts with that of Suns and Hitchin (1990), who found a significant negative relationship between mercury concentrations and the condition factor in a study on perch from 16 lakes in Ontario. It is possible that the poorer condition of perch from Lake Desjardins-West may be attributed to other factors such as temperature, dissolved oxygen

concentrations and food availability (Eastwood and Couture, 2002), which are all known to affect condition indices.

2.4.3 Biochemical biomarkers

Reduced glutathione (GSH) protects cellular components from toxicity, both as a substrate for conjugation and as an antioxidant defense. Many compounds conjugate with GSH, either spontaneously (non-enzymatically) when the toxicant is very reactive, or enzymatically via glutathione S-transferase (GST) (Hasspieler *et al.,* 1994, Dickinson and Forman, 2002). Conjugation is a protective measure, as it facilitates xenobiotic excretion and protects other cellular targets such as protein thiols (Hasspieler *et al.*, 1994). The capacity of the glutathione system to cope depends on the activity of glutathione peroxidase (GSH-Px), glutathione reductase (GR) and the pentose phosphate pathway enzymes among others (Halliwell and Gutteridge, 1989). Therefore, as GSH molecules are consumed by reducing agents by GSH-Px activity, GR activity increases in order to maintain intracellular concentrations of reduced glutathione (Dickson and Foreman, 2002). This is reflected by our data in both perch and walleye, where glutathione peroxidase selenium dependent (GSH-Px SD), GST and GR enzyme activities were positively correlated to one another (Table 2.1 A, B). There appear to be differences between these species; in walleye, glutathione peroxidase selenium independent (GSH-Px SI) activity was correlated to GST, GR and GSH-Px SD activity, whereas in perch, GSH-Px SI activity was only correlated to GST activity.

The cellular response to stress often involves a change in thiol content, where GSH is consumed in reactions that protect the cell through the removal of the xenobiotic compound, and is subsequently replaced through enzymatic reduction of disulfide or by *de novo* synthesis (Dickinson and Forman, 2002). There were no significant differences in GSH concentrations among the different walleye

populations (Table 2.2, A). However, perch from Lake Desjardins-West had higher GSH concentrations than those of Malartic and Preissac (Table 2.2, B). Overall, GSH concentrations tended to increase with liver MeHg concentrations, but this relationship was not significant (data not shown). GR, which regenerates GSH from GSSG by using NADPH as the reducing agent, did not vary among lakes for either species (Table 2.2, A, B). In addition, GR activity was not related to liver Hg concentrations (data not shown). To our knowledge, the only other study on the effects of mercury on GR activity in fish also reported similar results (Elia *et al.*, 2003).

Compounds that are able to generate oxidative stress can also, in some cases, lead to the induction of antioxidant enzymes, which is considered an adaptive protective response (Winston and Di Giulio, 1991). Many compounds have been shown to induce GSH synthesis through increased transcription of GCL (Dickinson and Forman, 2002). Results from studies in rats indicate that short and long-term exposure to MeHg in drinking water resulted in a two- to three-fold up-regulation of mRNA encoding for GCL. Concomitantly there was a similar magnitude of increase in the levels of GSH, and the activities of GR and GSH-Px (Woods *et al.*, 1995, Sarafin *et al.*, 1996). However, in this study, there were no significant differences in GCL activity in walleye populations among the different lakes sampled (Table 2.2, A). Perch from Lake Preissac exhibited slight but significantly lower GCL activities. All the other lakes had statistically comparable GCL activities (Table 2.2, B). It is possible that, considering the relatively low range of liver MeHg concentrations in the sampled fish, GR was effective enough at maintaining the intracellular concentrations of GSH such that GCL-related GSH synthesis did not occur.

Some of the most sensitive markers of toxicant effects are alterations in the activities of biotransformation enzymes such as GST (van der Oost *et al.*, 2003) and previous research has shown that Hg may induce GST activity (Canesi *et al.*, 1999). In our study, GST activity varied according to population for both species

of fish. Walleye from Lake Malartic had a significantly lower GST activity than walleye from Desjardins-West. There were no significant differences in GST activity for lakes Desjardins-West, Desjardins-East and Preissac or among lakes Desjardins-East, Preissac and Malartic (Table 2.2, A). In perch, GST activity was highest in Lake Desjardins-West and differed significantly from lakes Malartic and Preissac. Perch from Lake Desjardins-West were also in the poorest condition. Increased GST activity, coupled with poor condition and higher GSH concentrations, may indicate the presence of another stressor since none of these biomarkers were related to liver MeHg concentrations in fish from Lake Desjardins-West. These data also suggest the presence of a GST substrate, capable of inducing GST activity.

Overall, the differences in biochemical biomarkers observed among the different fish populations were slight. This may be due to the fact that the liver MeHg concentrations measured in this study were relatively low (ranging from 15.2 to 293.5 ppb in perch and from 107.8 to 1024.5 ppb in walleye, all lakes taken together). Sampling fish over a wider range of MeHg concentrations would provide a more in-depth analysis of the relationship between MeHg exposure and these biochemical endpoints. However, despite the low MeHg concentrations, we still observe adverse effects which are surprising considering that the threshold value for Hg toxicity in fish has been estimated at a body burden of 1000 to 5000 ppb (Niimi and Kissoon, 1994, Wolfe *et al.*, 1998, Sweet and Zelikoff, 2001).

In order to explore possible effects of Hg on biochemical biomarkers, we decided to focus on the lakes with the highest and the widest range of liver MeHg concentrations. In perch from Lake Desjardins-East, GST activity was negatively correlated to liver MeHg concentrations, which explained 39% of the variability (P= 0.0026), indicating a possible inhibition of this enzyme by MeHg (Figure 2.7 A). Although the properties of MeHg as a weak inhibitor of GST are well known (Reddy *et al.*, 1981, Fu *et al.*, 1991), conflicting results in terms of the effects of a chronic, sub-lethal exposure to Hg on GST activity are reported in the scientific

literature. In a recent study, Ferrat *et al.* (2002) observed that GST activity increased in seagrass treated with inorganic Hg, but did not vary significantly when treated with MeHg. Canesi *et al.* (1999) also observed an increase in GST activity related to Hg concentrations in mussel tissues (Canesi *et al.*, 1999). Other studies have reported GST inhibition by Hg and other heavy metals (Elia *et al.*, 2000, van der Oost *et al.*, 2003).

Our results also indicate an inhibitory effect of liver MeHg on GSH-Px SD activity in perch from Lake Desjardins-East. Although GSH-Px SD activity was significantly higher in Lake Desjardins-East, its activity was negatively correlated with MeHg concentrations (R^2=0.23, P=0.0298, Figure 2.7 B). While we did not come across other studies published on GSH-Px enzyme activities in fish in relation to mercury concentrations, our data are consistent with research on birds and mammals which report a decrease in GSH-Px activity (Hoffman et al., 1998). This drop in activity may have important consequences as even a slight alteration in biotransformation enzyme activity may be harmful to an organism (Kelly *et al.*, 1998).

The response of enzyme activities seems to depend on species, experimental exposure times (in laboratory studies) and metal concentrations (Elia *et al.*, 2003). It appears that perch may be more sensitive to enzymatic inhibition by MeHg than walleye. Our results suggest that, in walleye, antioxidant response is dependent on liver condition. In Lake Malartic, enzyme activity was correlated to liver condition rather than liver MeHg concentrations. GR and GST activities were all positively correlated to HSI. As the liver condition becomes poorer, enzyme activity decreases (figure 2.7 A, B). This may in turn adversely affect the organism's capacity to mount an adequate response to stressors.

2.5 Conclusion

The results of this study suggest that Hg induces adverse effects on the physiology of walleye at environmentally relevant concentrations. Lower hepatosomatic indices were found in fish from Lake Malartic, the lake with the highest liver Hg concentrations. The hepatosomatic index was also negatively correlated to total Hg concentrations. In Lake Malartic, GR and GST activity were related to liver condition. In perch from Lake Desjardins-East, MeHg was associated with reduced GST and GSH-Px SD activities, thereby possibly compromising the organism's capacity to mount an adequate response to other environmental challenges. These results, which are among the first to document adverse effects on the condition and biochemical processes in indigenous fish populations, are even more surprising considering the narrow range in Hg concentrations (total muscle Hg concentrations ranging from 0.3 ppm to 0.79 ppm). Finally, more research is needed to link biochemical changes and physiological alterations to organism and population effects such as altered growth and survival.

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Figure 2.1: Distribution of liver MeHg concentrations in walleye ranging from 1 to 6 years old among the different lakes. A Wilcoxon/Kruskal-Wallis test determined that the distribution of liver MeHg concentrations differed significantly according to location (P <0.0001). Values with the same letter are not significantly different (Noether α 0.05).

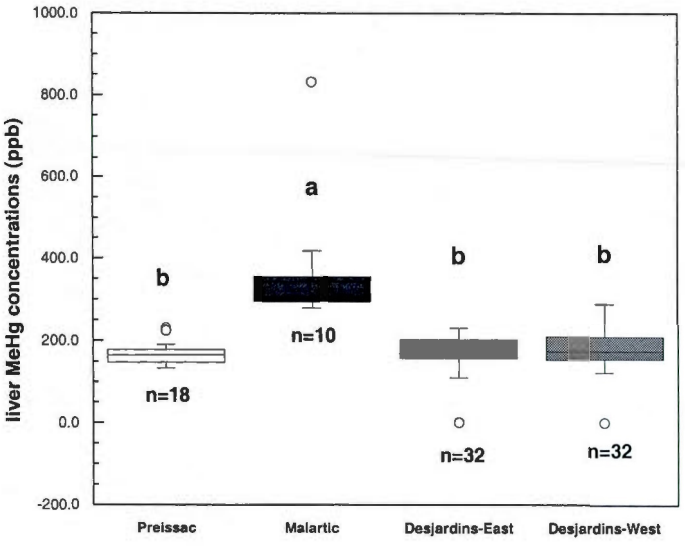


Figure 2.2: Distribution of liver MeHg concentrations in perch populations. A Wilcoxon/Kruskal-Wallis test determined that the distribution of liver MeHg concentrations differed significantly according to location (P <0.0001). Values with the same letter are not significantly different (Noether α 0.05).

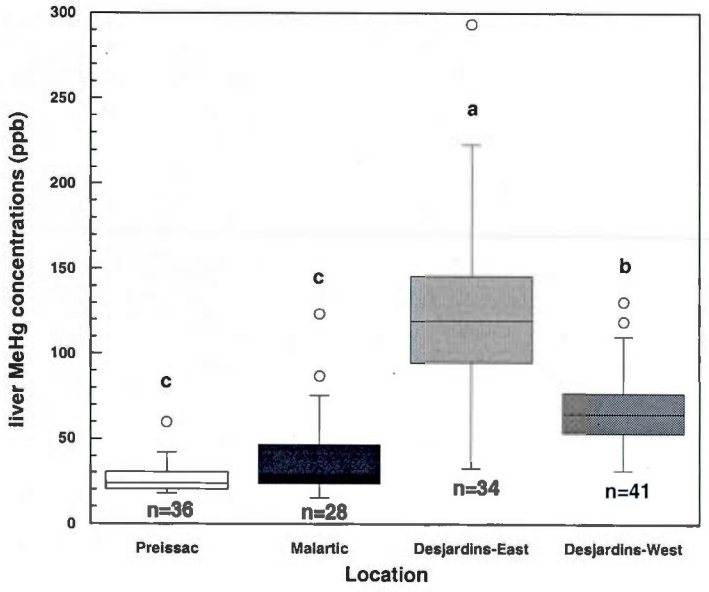


Figure 2.3: Distribution of hepatosomatic (HSI) values in walleye populations. A Wilcoxon/Kruskal-Wallis test determined that the distribution of HSI values differed significantly according to location (P <0.0001). Values with the same letter are not significantly different (Noether α 0.05).

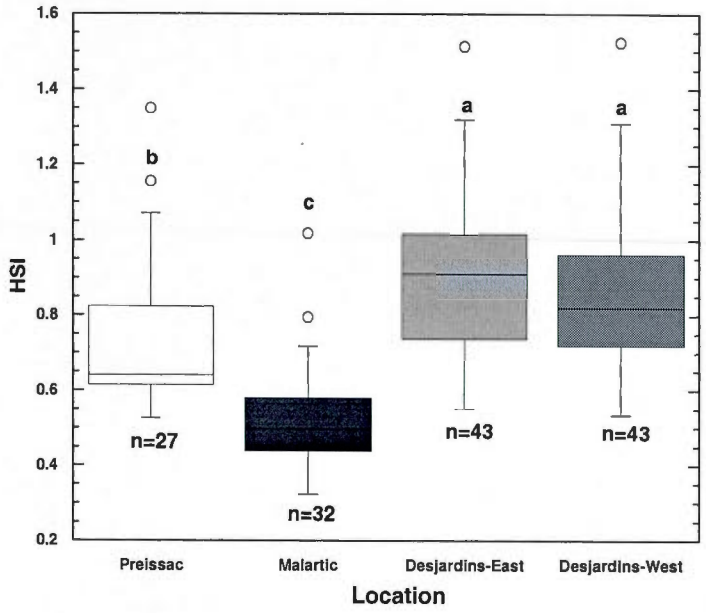


Figure 2.4: Hepatosomatic index as a function of liver mercury (Hg) concentrations. Michaelis-Menten fitted curve (R^2=0.33, P <0.0001, n=108).

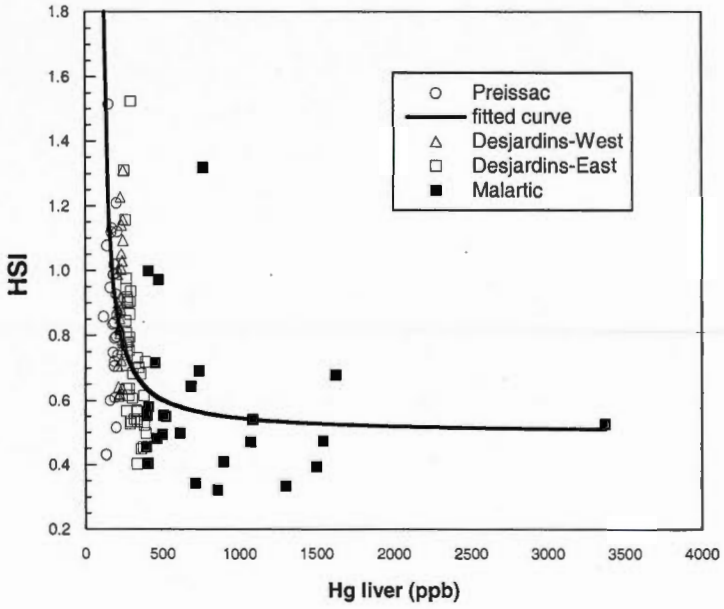


Figure 2.5: Distribution of LeCren condition factor (C_L) values in perch populations. A Wilcoxon/Kruskal-Wallis test determined that the distribution of C_L values differed significantly according to location (P <0.0001). Values with the same letter are not significantly different (Noether α 0.05).

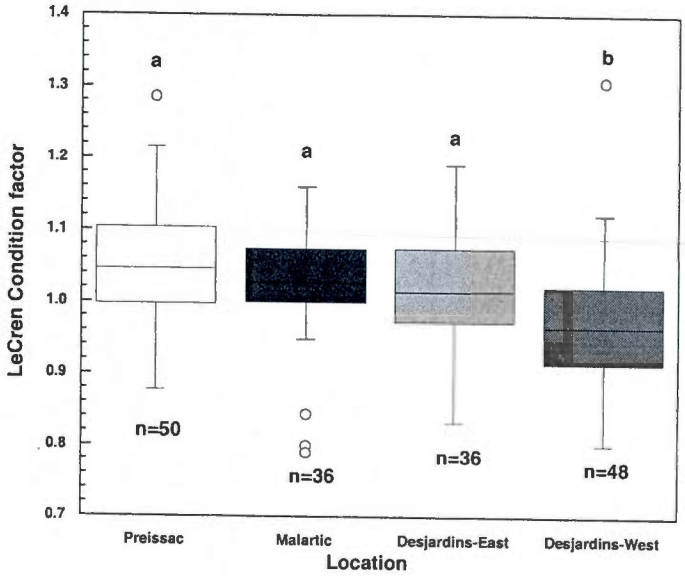


Table 2.1: Spearman pairwise correlations for biochemical biomarkers for walleye (A) and perch (B).The *P* value of the test is in parentheses.

A	GST activity	GR activity	GSH-Px SD activity	GSH-Px SI activity	length	age
GST activity	1					
GR activity	0.7454 (0.0000)	1				
GSH-Px SD activity	0.5921 (0.0000)	0.6105 (0.0000)	1			
GSH-Px SI activity	0.3035 (0.0024)	0.5831 (0.0000)	0.3890 (0.0001)	1		
length	0.1538 (0.0938)	-0.0417 (0.6500)	-0.0481 (0.5991)	0.1596 (0.0843)	1	
age	0.1551 (0.0907)	0.0247 (0.7884)	0.0091 (0.9208)	0.1645 (0.0751)	0.7937 (0.0001)	1

B	GST activity	GR activity	GSH-Px SD activity	GSH-Px SI activity	length	age
GST activity	1					
GR activity	0,4688 (0.0000)	1				
GSH-Px SD activity	0,6690 (0.0000)	0,6631 (0.0000)	1			
GSH-Px SI activity	0,3718 (0.0002)	0,1330 (0.2090)	0,0798 (0.4521)	1		
length	-0.2189 (0.1200)	-0.0234 (0.7899)	-0.0453 (0.6145)	-0.0618 (0.5538)	I	
age	-0.1777 (0.0623)	-0.0612 (0.4854)	-0.1034 (0.2491)	-0.0329 (0.7528)	0.6661 (0.0001)	1

Table 2.2: Median and range of hepatic biochemical biomarkers for walleye (A) and perch (B) among lakes. GSH concentrations expressed as μM/g tissue. Enzyme activity expressed as nmol/min/mg protein. Values with the same letter are not significantly different (Noether α 0.05).

A	Location			
Analyte	Desjardins-East	Desjardins-West	Malartic	Preissac
GSH	1.88 (0.55-3.23) n=40	1.67 (0.59-3.82) n=40	2.07 (0.48-4.16) n=32	1.45 (0.83-2.79) n=22
GR activity	6.89 (2.74-22.07) n=40	5.81 (2.81-21.64) n=41	6.12 (1.79-29.82) n=29	8.10 (1.36-14.20) n=20
GST activity	44.39 [a,b] (9.86-210.58) n=40	73.09 [a] (7.76-298.75) n=40	39.43 [b] (9.26-155.13) n=29	63.92 [a,b] (34.08-137.70) n=20
GCL activity	5.64 [a] (1.84-9.38) n=38	4.33 [a] (1.16-14.81) n=39	3.81 [a,b] (0.11-12.38) n=28	3.33 [b] (0.28-7.06) n=22
GSH-Px SD	15.78 (2.67-46.57) n=40	17.37 (6.52-51.66) n=40	14.35 (2.10-53.25) n=28	18.10 (8.58-66.65) n=21
GSH-Px SI	15.12 (1.74-87.86) n=33	14.87 (1.70-66.30) n=34	13.95 (4.06-71.72) n=25	18.95 (7.06-60.26) n=14

B	Location			
Analyte	Desjardins-East	Desjardins-West	Malartic	Preissac
GSH	0.23 [a,b] (0.08-0.62) n=39	0.24 [a] (0.05-0.72) n=44	0.19 [a,b] (0.05-0.59) n=30	0.17 [b] (0.04-0.73) n=37
GR activity	6.81 (1.21-15.6) n=30	7.37 (3.44-31.56) n=41	6.25 (0.99-25.82) n=33	6.70 (2.45-15.97) n=42
GST activity	58.09 [a,b] (22.15-145.01) n=31	73.02 [a] (23.16-134.08) n=40	45.09 [b] (13.29-103.21) n=33	44.24 [b] (23.82-90.21) n=42
GCL activity	5.03 (0.83-22.96) n=37	4.46 (0.24-15.17) n=44	4.85 (0.87-21.31) n=32	5.63 (0.54-17.09) n=36
GSH-Px SD	44.71 [a] (24.15-152.14) n=29	33.05 [b] (10.87-131.21) n=37	31.59 [b] (9.72-136.43) n=33	34.29 [b] (3.79-97.92) n=41
GSH-Px SI	13.93 (3.49-34.79) n=17	19.07 (1.56-99.36) n=30	14.17 (5.97-58.23) n=27	11.44 (0.77-47.15) n=32

Figure 2.6: Simple linear regression model of GR activity (A) and GST activity (B) as a function of HSI for walleye in Lake Malartic. Enzyme activity expressed as nmol/min/mg protein.

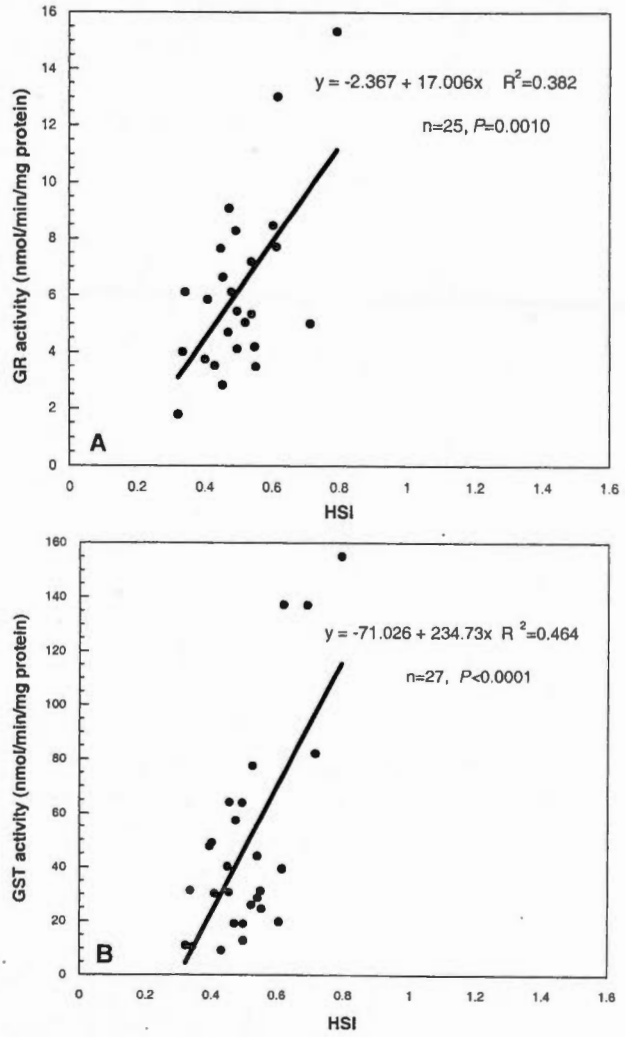
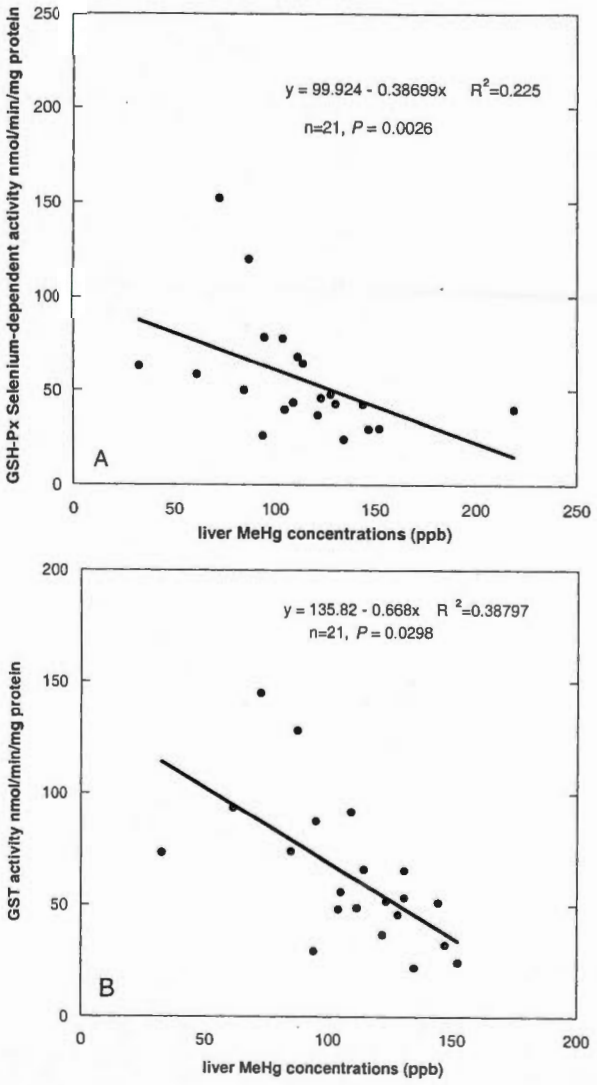


Figure 2.7: Simple linear regression model of GSH-Px SD activity (A) and GST activity (B) as a function of liver MeHg concentrations for perch in Lake Desjardins-East. Enzyme activity expressed as nmol/min/mg protein.



CONCLUSION GÉNÉRALE

Cette recherche s'insère dans le cadre d'un vaste programme de recherche entrepris par le Réseau collaboratif de la recherche sur le mercure (COMERN) et qui traite de la vulnérabilité des écosystèmes aux composés mercuriels, de même que de leurs effets sur la santé humaine. L'objectif général de ce projet de mémoire visait à évaluer l'effet du mercure (Hg) sur la santé des poissons. À la lumière de cette étude, nous apportons des éléments d'information sur le rôle du foie dans la cinétique du Hg d'une part et sur les réponses physiologiques des poissons exposés de façon chronique au Hg/MeHg d'autre part. Ces résultats inédits démontrent certains effets adverses du Hg/MeHg et ce à des concentrations relativement faibles. Cette recherche suggère de nouvelles pistes de recherche concernant en particulier les processus de déméthylation hépatiques et le rôle du système du glutathion dans l'homéostasie des organismes ichthyens.

Dans le premier chapitre, nous avons observé que la bioaccumulation du Hg et de son principal dérivé organique, le méthylmercure (MeHg), variait parmi les dorés jaunes des différents lacs échantillonnés. De plus, en étudiant la toxicocinétique de ces composés mercuriels de façon plus détaillée dans le foie des dorés jaunes, nous avons observé que, dans des lacs avec des poissons faiblement exposés au Hg, le pourcentage de MeHg diminuait avec l'âge, ce qui suggère la présence d'un lent processus de déméthylation hépatique. Ce résultat nous indique que le foie joue un rôle important dans le métabolisme de ce xénobiotique et que cet organe mérite plus d'attention lorsque des études toxicocinétiques sont effectuées. Les résultats du deuxième chapitre, qui portent sur les effets physiologiques et biochimiques des composés mercuriels, démontrent que, même à des

concentrations environnementales relativement faibles de Hg/MeHg, l'activité des enzymes du système du glutathion peut être altérée, ce qui peut potentiellement freiner la capacité des poissons de se protéger contre d'autres stress environnementaux.

Cette recherche suggère que les poissons d'eau douce sont plus sensibles aux composés mercuriels que préalablement considéré. Ceci peut avoir des conséquences sur les preneurs de décision qui doivent gérer les risques associés au Hg. Plus de recherches sont nécessaires pour réduire les incertitudes dans les modèles prédictifs et l'utilisation ainsi que la validation de biomarqueurs plus sensibles sont à préconiser.