Methyl Mercury Bioaccumulation: A Study of Factors Influencing Uptake and Elimination in Fish

by

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ABSTRACT

This research examined aspects of MeHg bioaccumulation in juvenile salmonids (Atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss*). The first aspect studied was the interaction of growth and MeHg bioaccumulation in short-term growth exposures. In two experiments using four different concentrations of dietary MeHg, it was found that rapidly growing fish actually accumulated Hg faster than slow growing fish. However, accumulation efficiency decreased inversely with ration; per unit of growth, slow growing fish accumulated MeHg with efficiencies approaching 100%, while for fish growing at 50 $mg \cdot g^{-1} \cdot d^{-1}$, accumulation efficiency dropped to less than 20%.

A second aspect of this research examined the depuration of MeHg from juvenile rainbow trout in relation to dietary selenium. After short depuration times (25 days), fish fed selenium had lower body burdens than control fish. Elimination half lives for Hg were reduced from ca. ∞ to 289 days. The use of muscle Hg analysis to calculate body burdens has several advantages for the study of Hg elimination in fish. Muscle comprises the largest fraction of body mass, it is the slowest-clearing pool of MeHg in fish, and in time the major distributive flux of MeHg in fish is to the muscle rather than to elimination pathways. The slow half-lives for MeHg in this experiment reflect the true nature of MeHg elimination in fish, namely that it is a slow process. Current literature estimates of rapid clearance of MeHg from fish are fundamentally flawed for their failure to accurately measure slow-clearing Hg pools in fish.

Simple and accurate methods for measuring growth rates are important to fisheries biology and ecotoxicology. An ancillary aspect of this research on growth rate interactions with MeHg bioaccumulation in fish entailed the development of new growth estimators utilizing erythrocytes. The approach is based on the theory that erythropoiesis (the synthesis of erythrocytes) will be stimulated in order to support

increased oxygen demand under conditions of rapid growth. In erythropoiesis, immature erythrocytes (reticulocytes) are released into circulation without hemoglobin, which is synthesized as the reticulocytes mature. Coincident with erythrocyte maturation, the hemoglobin synthetic apparatus will be initially active followed by senescence. Therefore, if erythropoiesis is stimulated, the net effect should be a rapid increase in the proportion of reticulocytes in the blood. This change should be measurable either by nucleic acid analysis (RNA concentrations or RNA/DNA ratios) or by reticulocyte enumeration on blood smears. In one experiment, RNA concentrations in whole blood, liver, and muscle, were analysed to compare the relative value of these parameters for estimating growth rates. A second experiment examined the use of microscopic enumeration of reticulocytes for estimating growth rates. In both experiments, fish were individually marked for determination of growth by increase in weight. In terms of RNA analysis, blood compared favourably with muscle and liver and was technically simpler to work with. Reticulocyte enumeration by microscope was technically simple and provided excellent results. Due to its low requirement for equipment, this technique for growth determination has great potential application for field situations.

ACKNOWLEDGEMENTS

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In addition to generic fishheads, specific thanks must go to (in reverse alphabetical order so I don't hurt Barry's feelings – he's so sensitive about the years wasted waiting for his name to be called!) Barry Zajdlik, Paul Welsh, Mike vandenHeuvel, Paul "I couldn't put a puck through your legs if my life depended on it" Sibley, Roman "crash" Lanno, Ulysses Klee, Bruce "chisels" Kilgour, Beth Goebel, Dean Fitzgerald, Cat "your hockey equipment makes our office smell like an abattoir" Evans, and Janine Clemons (*Neeeeennn!!!!).* These colleagues spent hours and hours listening to me *bitch, bitch, bitch!!!* for which I will be eternally grateful.

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1.0 General Introduction

The presence of high levels of mercury (Hg) in aquatic biota is a widespread phenomenon with both human and ecosystem health concerns (Lindqvist et al. 1991, Clarkson 1994). The factors responsible for this ongoing problem include: (1) the atmospheric transport of Hg and subsequent deposition on lakes and their watersheds; (2) the bacterial methylation of Hg once in place; and (3) the tendency toward accumulation of Hg in aquatic biota. It is the last of these three areas which is the focus of this thesis.

The field of bioaccumulation research is not without controversy. There are numerous areas which remain unresolved, including problems of growth and other temporal factors which interact with the accumulation process, clearance issues, and the utility and relevance of bioaccumulation models. These areas provided the basis for undertaking this research.

Methyl mercury (MeHg) is typically present at low levels in freshwater throughout the northern hemisphere (ca. 1 $ng \cdot L^{-1}$) (Lindqvist et al. 1991). MeHg bioaccumulates in fish from these waters, although the aqueous MeHg concentrations are too low to account for this in terms of MeHg uptake from water alone (Lindqvist et al. 1991). This behaviour of Hg in aquatic environments is due to biomagnification, in which Hg bioaccumulates to higher concentrations in organisms at higher trophic levels relative to lower trophic levels. The characteristic biomagnification of Hg is conferred on MeHg by virtue of the methyl group; inorganic Hg does not biomagnify. Coupled with the properties which allow for Hg to be released into the environment, the chemical behaviour of Hg, once in the environment, contributes to its importance as an environmental contaminant.

The processes of bioaccumulation and biomagnification are the defining features of fish-Hg interactions in aquatic environments. Although Hg bioaccumulation has been studied for decades, much remains unclear about this phenomenon. There are numerous possible reasons for this, not the least of which is semantic. For example,

bioaccumulation is defined in numerous ways, such as "the uptake and sequestration of contaminants by organisms from their ambient environment" (Phillips 1993) or as "the transfer of contaminants from the external environment to an organism" (Barron 1995). These definitions are really only those of uptake, and while ample for short-term toxicology, they do not convey a sense of the time frame, on a scale of years rather than hours, over which Hg bioaccumulation occurs in the natural environment. Routes of contaminant uptake have taken on specific usage, so that uptake from water is termed bioconcentration, while dietary uptake is just called bioaccumulation. In some cases, biomagnification (an ecotoxicological term referring to "the increase in tissue concentration of contaminants or substances through a series of predator-prey relationships, primarily by means of ingestive accumulation" (Schmitz, 1996)) is used to define bioaccumulation via the diet (Sijm et al. 1992, Gobas et al. 1993). However, the uptake of substances from commercial fish food in bioaccumulation experiments stretches the definition of biomagnification, as there is no actual transfer of contaminant between predators and prey. To clarify the issue, bioaccumulation can be defined simply as the phenomenon of time-dependent increase in the concentration of a substance in any organ or tissue of the body. By referring to the uptake vectors simply as waterborne or dietary, the term biomagnification can be reserved to convey its ecological sense.

Whether a substance bioaccumulates can be determined only in terms of concentration. Although body burden (the total amount of the substance in the body) also increases in bioaccumulation, it is not the defining feature. For example, as a fish grows, the composition of the body remains essentially constant for the bulk elements such as carbon (approximately 18% by weight) (Hoar 1983). Therefore, on a whole body basis, a 10 g fish contains 1.8 g of carbon, while a 100 g fish from the same population contains 18 g of carbon. When corrected to a whole body concentration (body burden of carbon ÷ body weight), it can be seen that the concentration is identical in both fish. The higher body burden in the larger fish is offset by the greater body weight; carbon is not bioaccumulating. Therefore, although body burden is correlated

with age, bioaccumulation can be said to be occurring only if the body burden of an older fish is greater than that of a younger fish when corrected to a unit mass basis (ie. a concentration). This is not to say that body burden cannot be used in bioaccumulation studies and calculations, once it is established that a substance bioaccumulates.

Growth (the increase in body size (Weatherly and Gill 1987)) and reproduction are defining biotic characteristics, with reproduction simply representing growth at the population level rather than at that of the individual. Time is implicit in growth; an organism of a given size could be young and rapidly growing or old and slow growing. Similarly, time is critical to the study of contaminant bioaccumulation, in that an organism with a given quantity of contaminant in its tissues could have received the contaminant quickly, at high doses, or slowly at low doses. The common occurrence of growth and contaminant accumulation over time gives the appearance of interactions between these two phenomena, making their study potentially difficult.

Potential growth interactions exist not only for uptake, but for the elimination of mercury as well. Niimi (1983, 1987) has noted that the study of contaminant elimination is not free from semantic issues either. For example, half lives for elimination have been defined in two different ways: (1) as "the time required for a body to eliminate half the amount of an administered substance through the normal channels of elimination" (Daniel 1978); or (2) as "the time required to reduce the concentration of a chemical by one-half in a designated medium or organism such as soil, water, and fish" (ASTM 1978). Definition (1) is preferred, as it does not have to deal with the effects of growth dilution on elimination estimates. This may seem contradictory, given that bioaccumulation is defined in terms of concentration, but in this case, the issue is one of 'book-keeping' (mass balancing) not semantics. With the exception of short depuration studies, fish growth can become significant. The terms of definition (2) can be met without actually eliminating any contaminant whatsoever. For example, Laarman et al. (1976) showed that Hg concentrations of white perch and rock bass dropped to one half and one third of the original levels after two years of depuration. During that time, however, body burdens were not significantly different from those of

the initial sampling, indicating that little if any Hg was actually eliminated. The differences in concentration can be attributed to growth dilution, in which the original amount of chemical becomes distributed in a larger amount of newly accrued body mass. The use of both elimination definitions probably accounts for the wide range of Hg turnover estimates in the literature (Headon et al. 1996). Definition (1) reflects true elimination, and can be derived without concern for growth.

The issues identified here as problematic are not the only ones of importance to understanding Hg in the environment. For example, the current view that the lipophilicity of MeHg is the predominant reason for the biomagnification of Hg has been challenged as simplistic and refuted experimentally (Mason et al. 1995, 1996). With this in mind, this work was originally intended to simplify Hg bioaccumulation according to the calls of Hamelink and Spacie (1977), who suggested twenty years ago that "What is needed now are more simple, universal approximations which can serve to guide future studies intended to resolve particular issues and unique situations, not more complex solutions to the particular problem posed by a few chlorinated hydrocarbons...these results will be dramatic for their simplicity and sobriety. As such, they will not be very newsworthy, but for those of us who have muddled through the myriad of environmental toxicology, they will be most welcome."

The approach in this study of Hg bioaccumulation pays attention to simplicity in an area of apparent complexity, attempting to clarify some of the issues in bioaccumulation research which remain incompletely understood. This dissertation is partitioned into five chapters, each dealing with particular aspects of Hg bioaccumulation research. Chapter 2 examines the interaction between growth and Hg bioaccumulation. Chapter 3 deals with the issues of Hg clearance, as the elimination of Hg has a significant bearing on bioaccumulation rates. Chapter 4 develops new techniques for measuring growth in fish. These methods have potential application for field bioaccumulation studies and fisheries biology in general. Chapter 5 attempts to unify the dissertation by providing a general discussion of the research.

The experimental research in this thesis consists of five experiments. For

simplicity, these experiments will be numbered in sequence of appearance in the thesis. Any reference to an experiment in subsequent chapters will use the same number. The five experiments are listed below:

Experiment Number	Subject Matter	Chapter
1	Dietary MeHg/growth interactions	2
2	Dietary MeHg/growth interactions	2
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4	Selenium/MeHg elimination interactions	3
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2.0 Interactions between Mercury Accumulation and Growth in Juvenile Salmonids

2.1 Introduction

Hg accumulation profiles for a cohort of fish normally reveal a characteristic pattern of increasing Hg concentrations with age. Typically, this elevation of Hg in older fish has focussed monitoring on adult fish, although Hg-age profiles for top predator fish species indicates that the early life stages are also potentially useful for revealing Hg accumulation trends. The accumulation of Hg often occurs most rapidly in young fish: for perch (*Perca flavescens* (Mitchell)) (Norstrom et al.1976), pike (*Esox lucius* L.) (Fagerström et al. 1974), walleye (*Stizostedion vitreum* (Mitchell)) (Munn and Short 1997), and lake trout (*Salvelinus namaycush* (Walbaum)) (Macrimmon et al. 1983), the steepest slope in the Hg-age profile occurs in the earliest (larval-juvenile) life phase. This is surprising, since even for piscivorous species, early life stages feed on small prey items with relatively low Hg concentrations. Since this early life period encompasses the highest feeding rate (up to 3000 $mg \cdot g^{-1} \cdot d^{-1}$), assimilation efficiency (up to 50% gross conversion efficiency), and growth rate (up to 800 $mg \cdot g^{-1} \cdot d^{-1}$) of any life stage (Kamler 1992), the combination of low dietary Hg and high growth rate should complicate the study of Hg bioaccumulation.

This chapter examines the interaction of growth and Hg bioaccumulation in juvenile salmonids in experiments that manipulated MeHg dose and growth rate directly, by feeding food of a given MeHg concentration at different ration sizes.

2.2 Materials and Methods

2.2.1 Experiment 1

This experiment was undertaken at the Huntsman Marine Sciences Centre, St.

Andrew's, NB. Atlantic salmon (*Salmo salar* L.) parr purchased from the Atlantic Salmon Federation (St. Andrew's, NB) were weighed and marked with fin clips and placed into one of 27 11L circular plastic tanks, five fish per tank. Loading densities were approximately 5 g (body weight)·L^{-1}.Tanks were randomized to one of three rations (5, 10, and 15 mg·g^{-1}·d^{-1}) and one of three MeHg concentrations (8.77±1.39, 14.26±0.44, and 19.43±1.87 µg·g^{-1} food; mean ±s.d. for three replicate food samples).

Freshwater for the experiment was drawn from the Town of St. Andrew's mains and had a temperature of 13°C for the duration of the experiment. Sodium thiosulphate was added to the inflow water at a concentration of 13 ± 7 µg·L^{-1} (n=7 samples) both to bind waterborne Hg or MeHg, minimizing the potential for uptake from water, and to protect against any possible spikes of chlorine in the water supply. Sodium thiosulphate is a common reagent used for chelation of MeHg in Hg analysis (Schintu et al. 1992). Flow rates to the tanks were approximately 170 mL·min^{-1}, providing 95% water turnover in approximately 3 h (Sprague 1969). This rapid water turnover time further minimized any possible waterborne exposure to Hg.

Fish were sampled (one per tank) after 4, 8, 12, 16, and 20 days, with the following exceptions: (1) 3 fish were lost from each of the 14 and 19 µg·g^{-1} treatments, and 2 fish were lost from the 9 µg·g^{-1} treatment. In all cases, this was due to jumping into the nylon mesh covering the tanks.

Relative growth rate for each fish (mg·g^{-1}·d^{-1}) was calculated as follows (Ricker 1979):

$$Relative\ Growth\ Rate = \frac{(WT_t - WT_0)}{(WT_0 * t)}$$

where WT_0 = initial weight (g)

WT_t = weight at sampling (g)

t = time (days)

2.2.2 Experiment 2

Juvenile rainbow trout (*Oncorhynchus mykiss*) were weighed and tagged by fin clips and placed in one of four 12 L glass aquaria, 8 fish per tank. Initial loading densities were approximately 7 g (body weight)·L^{-1}. Each tank was assigned a feeding rate at random. Feeding rates of 5, 15, 25, and 35 mg·g^{-1}·d^{-1} were chosen to provide a range of growth. The experiment used commercial fish food (Martin Feed Mills, Elmira ON) which was spiked with methyl mercuric chloride (MeHgCl) at a concentration of 23.94 ± 1.78 μg·g^{-1} (n=3 samples). Fish were fed for 20 days. Three days were allowed between final feeding and sampling to allow complete emptying of the digestive tract. Water temperature was 12°C for the entire experiment. Flow rates were 250 mL·min^{-1} to the tanks, providing 95% water turnover in approximately 2.5 h (Sprague 1969). Growth rates were calculated as per experiment 1.

2.2.3 Experiment 3

To compare differences in accumulation between inorganic Hg and MeHg, a final experiment was conducted. Mercuric chloride ($HgCl_2$) was added to Martin trout chow to a final concentration of 201.3 ± 46.5 μg·g^{-1}. Juvenile trout were weighed, marked by fin clip, and placed in one of four glass aquaria and fed at 5, 15, 25, and 35 mg·g^{-1}·d^{-1} for 11 days. Fish were sampled after 15 days. Water temperature and flow rates were the same as in experiment 2.

Experiments 2 and 3 were conducted in the biology wet lab facility at the University of Waterloo. Unchlorinated well water was used in these experiments (and experiments 4 and 5 (chapters 3 and 4)). The water is hard (approximately 392 mg·L^{-1} as $CaCO_3$) and has been characterized in detail by Lanno (1990).

2.2.4 Food Preparation

MeHg-spiked food was prepared by adding MeHg (dissolved in acetone) to pre-measured aliquots of food in aluminum pie plates. Acetone was removed by drying the food in a fume hood to evaporate the acetone. For experiment 1, a stock solution of

MeHg in acetone was serially diluted to provide three concentrations of MeHg in acetone. To prevent food from crumbling due to excess wetting, MeHg was added to all three food aliquots in quantities of acetone amounting to approximately 10% (v/w) of the food.

MeHg-spiked food for experiment 2 and inorganic Hg-spiked food for experiment 3 were prepared in a similar manner.

2.2.5 Mercury Analysis

Sample preparation involved the digestion of fish muscle (0.05-0.2 g) in 2 mL of concentrated nitric acid (Trace Metal Grade, Fisher Scientific) at 75°C in 16×150 mm disposable borosilicate culture tubes (VWR Scientific) with several boiling chips (BDH anti-bumping granules). After at least four hours (samples can be left up to 24 hours with no loss of analyte), 1 mL of 30% hydrogen peroxide was added to the samples. Initially, the samples were removed from the heating block for 1-2 minutes prior to peroxide addition and left off the block until the most vigorous boiling subsided. The boiling chips enhance the surface area for bubble formation and aid in the oxidation of the matrix. Depending on sample size, a second addition of hydrogen peroxide was sometimes required. Once the samples cleared, they were removed from the heat and made up to volume with water.

Mercury (total) was analyzed by cold vapour atomic absorption spectrophotometry (CVAAS). The measurements were carried out using a Perkin-Elmer Model 4000 flame atomic absorption spectrophotometer, with a 25×150 mm quartz-windowed cell affixed on top of the burner. Signals (peak heights) were recorded on a Perkin-Elmer R100 chart recorder. Mercury vapour was generated by adding 5 mL of 10% solution of stannous chloride (BDH) in 1% nitric acid to the test tubes containing digested sample. One mL of a 1% solution of silicone antifoam (G.E. AF-72) in water was added to prevent sample foaming into the tubing and absorption cell. Argon was sparged through the sample at 200 mL/min to carry the Hg vapour into the detection cell, and was introduced to the sample with a glass sparger (Ace Glass, Vineland NJ, ASTM 25-

50µm).

Hg concentrations are reported as $ng \cdot g^{-1}$ wet weight. Hg accumulation rates ($ng \cdot g^{-1} \cdot d^{-1}$) were calculated by dividing muscle mercury concentrations by the exposure time, assuming that no Hg was present in muscle initially. QA/QC issues were addressed by digesting and analyzing 3 samples of certified reference material (CRM) with each batch of samples. National Research Council of Canada DORM-1 (dogfish muscle) with a certified Hg concentration of 798 ± 74 $ng \cdot g^{-1}$ was used as the CRM throughout sample analysis. For 20 samples analyzed in conjunction with 6 batches of samples, Hg concentration was 817 ± 74 $ng \cdot g^{-1}$ (102% of the certified value).

2.2.6 Statistical Analysis

For each experiment, growth and Hg concentration was determined for individual fish. These values were converted to rates, and linear regression was used to ascertain relationships between these variables. Treating fish as the experimental units was essential, as growth of individual fish within tanks can vary widely. Because fish were weighed and marked, growth could be measured in individual fish, thereby avoiding this problem. The SYSTAT statistical package (version 6.0 for Windows, SPSS Inc., Chicago, IL) was used for data analysis.

Outliers were detected by SYSTAT (one per dietary MeHg concentration) in experiment 1. The outliers were removed for subsequent regression analysis.

2.2.7 Calculations of Growth and Hg Bioaccumulation

The growth and Hg accumulation data from experiment 2 were used to calculate several derived variables relating to Hg accumulation. The results of the experiment can be summarized by two equations: (1) the regression equation of growth rate as a function of ration; and (2) the Hg accumulation rate as a function of growth rate (the regression parameters for both these equations are presented in Table 2). Details of these calculations, the assumptions use, and sample calculations are presented in Appendix 1.

2.3 Results

2.3.1 Experiment 1

On a per tank basis, the fish in this experiment never accepted all of their ration. As a consequence of this poor consumption, growth on ration relationships were not reliable. Regardless of the mathematical significance of any analysis utilizing this data, it would underestimate growth per unit ration. Therefore, the factorial analysis of variance comparing growth and MeHg accumulation (for which this experiment was designed) was not conducted.

To avoid fouling of their tanks and incidental uptake of MeHg from the water, tanks were siphoned within 15 minutes of daily feeding. Even in the absence of thiosulphate, it is unlikely that MeHg desorbed from unconsumed food during this time. The strong binding of MeHg to sulphydryl groups of proteins ($k_{dissoc.} \approx 10^{-15}$) (Carty and Malone 1979), and low solubility of MeHg in water (Craig 1986), make it unlikely that significant quantities of MeHg were available for waterborne uptake by the fish, although Hg in water was not measured.

Dietary MeHg concentration was the major determinant of Hg accumulation rate, as indicated by steeper slope of Hg accumulation rate ($ng \cdot g^{-1} \cdot d^{-1}$) with increasing dietary Hg concentration (Figs. 2.1 - 2.3, Table 2.1). Regression equations for Hg accumulation rate ($ng \cdot g^{-1} \cdot d^{-1}$) as a function of growth rate ($mg \cdot g^{-1} \cdot d^{-1}$) are presented in Table 2.1. The 95% confidence bands for the slopes were 0.247-0.608 (9 $\mu g \cdot g^{-1}$), 0.329-1.086 (14 $\mu g \cdot g^{-1}$), and 1.074-2.264 (19 $\mu g \cdot g^{-1}$), indicating a significant difference between Hg accumulation profiles in the low and high (9 and 19 $\mu g \cdot g^{-1}$ diets). The Hg accumulation rate is in units of increase in Hg concentration and, as such, already accounts for growth by correcting the Hg burden to a unit mass basis. So, for example, in Fig. 2.3, at growth rates of 40-50 $mg \cdot g^{-1} \cdot d^{-1}$, fish were accumulating Hg roughly 2.5-fold faster per unit mass than fish that were not growing.

2.3.2 Experiment 2

To rule out any potential effect due to varying exposure time, all fish were fed for the same length of time (20 days). The relationship between Hg accumulation rate and growth rate was similar to those observed in experiment 1 (Fig. 2.4) although the linear fit was the best among the four MeHg diets used in these two experiments (Table 2.1). The growth data for experiment 2 (Fig. 2.5) indicate that a linear increase in growth occurred across the range of rations used (Table 2.1). The variability around the 15 and 25 $mg \cdot g^{-1} \cdot d^{-1}$ rations was considerable.

2.3.3 Experiment 3. In spite of the extremely high $HgCl_2$ concentration in this experiment (201 $\mu g \cdot g^{-1}$), accumulation was minimal and unrelated to growth (Fig. 2.6, Table 2.1).

2.3.4 Calculations of Growth and Hg Bioaccumulation The assignment of a growth rate was the starting point for these calculations. Based on the Hg accumulation-growth relationship (Fig. 2.4) and the growth-ration curve for experiment 2 (Fig. 2.5), it was possible to back calculate food consumption rates, and in turn, the dose of MeHg received from the diet. For these calculations, fish size was assumed to be 10 g. Details of these calculations are included in Appendix 1.

Supply of Hg (dose) is the product of ration and Hg concentration in the food; the body burden increases as a proportion of dose (Fig. 2.7). When expressed as an "uptake efficiency" (body burden ÷ dose), it is seen that the uptake efficiency decreases in proportion with dose, with the greatest uptake efficiency occurring under low growth conditions (Fig. 2.7).

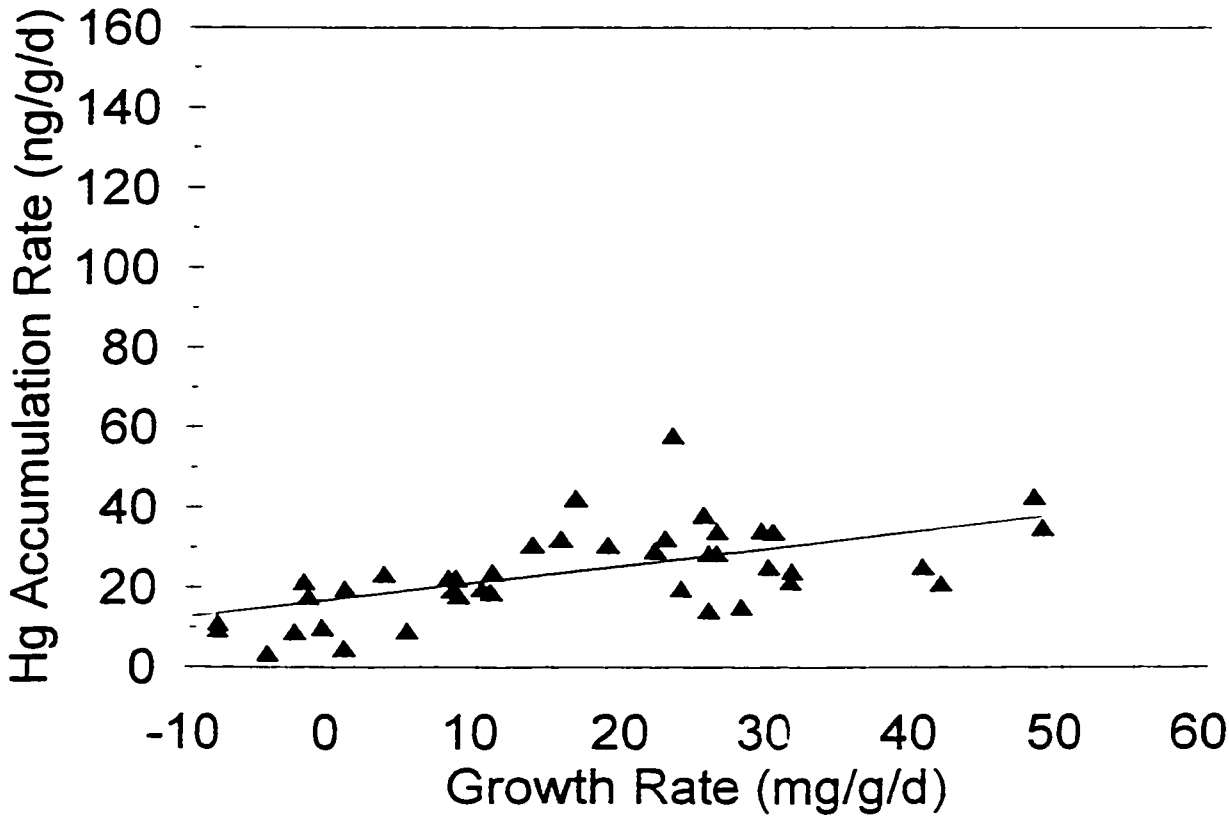


Figure 2.1. Hg accumulation in juvenile Atlantic salmon from experiment 1. Hg accumulation rate with respect to growth rate for dietary MeHg concentration of 9 $\mu g \cdot g^{-1}$. Regression line from Table 2.1 is plotted in addition to individual data points.

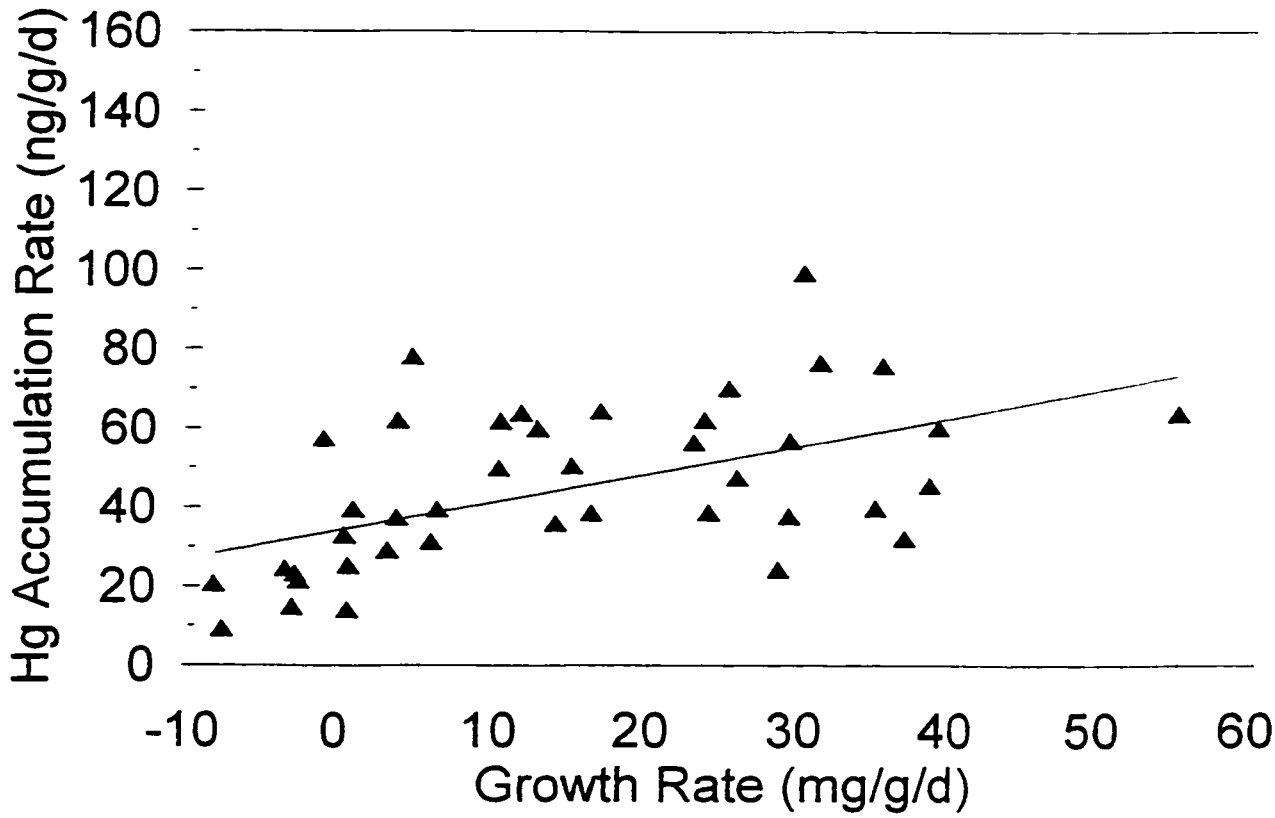


Figure 2.2. Hg accumulation in juvenile Atlantic salmon from experiment 1. Hg accumulation rate with respect to growth rate for dietary MeHg concentration of 14 $\mu g \cdot g^{-1}$. Regression line from Table 2.1 is plotted in addition to individual data points.

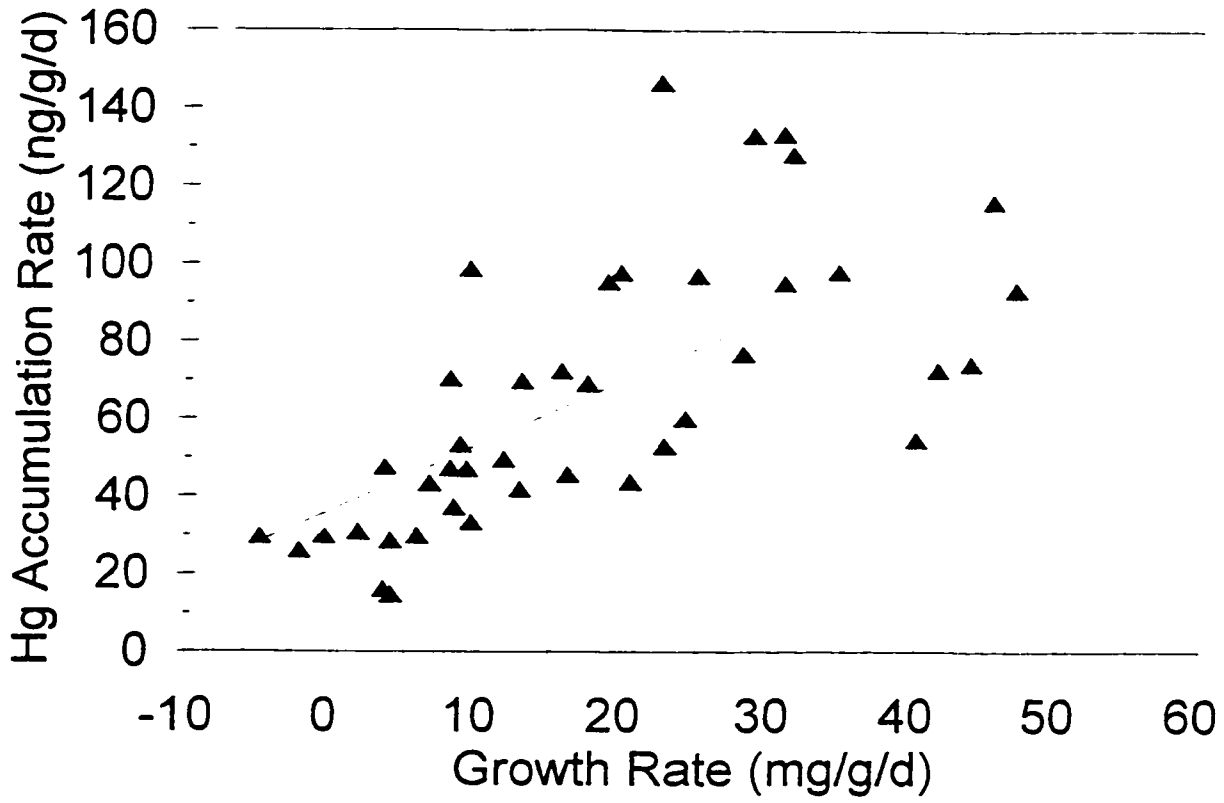


Figure 2.3. Hg accumulation in juvenile Atlantic salmon from experiment 1. Hg accumulation rate with respect to growth rate for dietary MeHg concentration of 19 $\mu g \cdot g^{-1}$. Regression line from Table 2.1 is plotted in addition to individual data points.

Table 2.1. Regression parameters for Hg accumulation with respect to growth rate. Regression equation is: Hg accumulation rate ($ng \cdot g^{-1} \cdot d^{-1}$) = Slope × Growth Rate ($mg \cdot g^{-1} \cdot d^{-1}$) + Y-intercept ($ng \cdot g^{-1} \cdot d^{-1}$).

Experiment	Dietary [Hg]	Slope	Y-intercept	r^2	F	p
1	9	0.427	17.122	0.364	22.867	<0.001
1	14	0.707	34.371	0.263	14.259	<0.001
1	19	1.669	36.623	0.452	32.139	<0.001
2	24	2.635	87.127	0.555	37.370	<0.001
3	200	0.020	2.883	0.117	1.725	0.212
2[a]	24	0.824	-4.000	0.630	51.498	<0.001

[a] This equation is that of growth yield ($mg \cdot g^{-1} \cdot d^{-1}$) per unit ration ($mg \cdot g^{-1} \cdot d^{-1}$) for experiment 2 , not Hg accumulation rate as in the previous 5 rows. The y-intercept for this equation has units of $mg \cdot g^{-1} \cdot d^{-1}$.

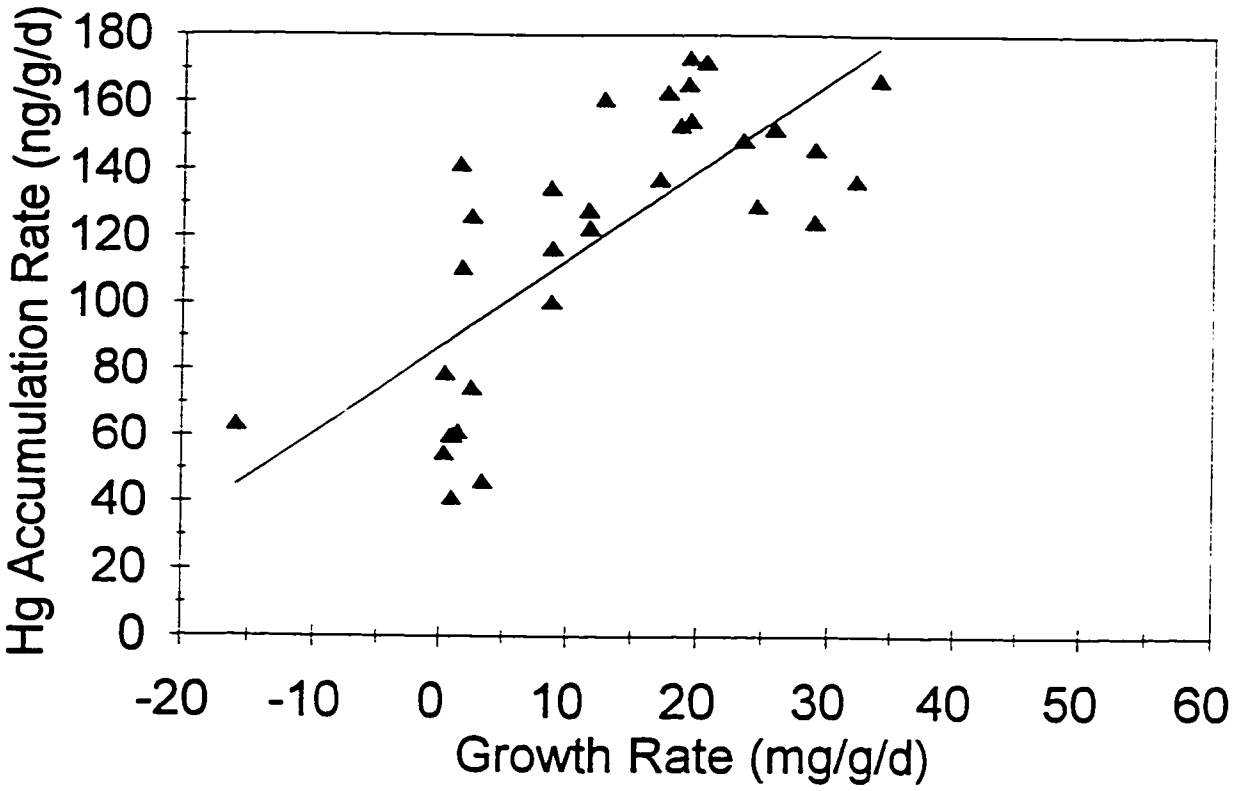


Figure 2.4. Hg accumulation in juvenile rainbow trout from experiment 2. Hg accumulation rate with respect to growth rate for dietary MeHg concentration of 24 $\mu g \cdot g^{-1}$. Regression line from Table 2.1 is plotted in addition to individual data points.

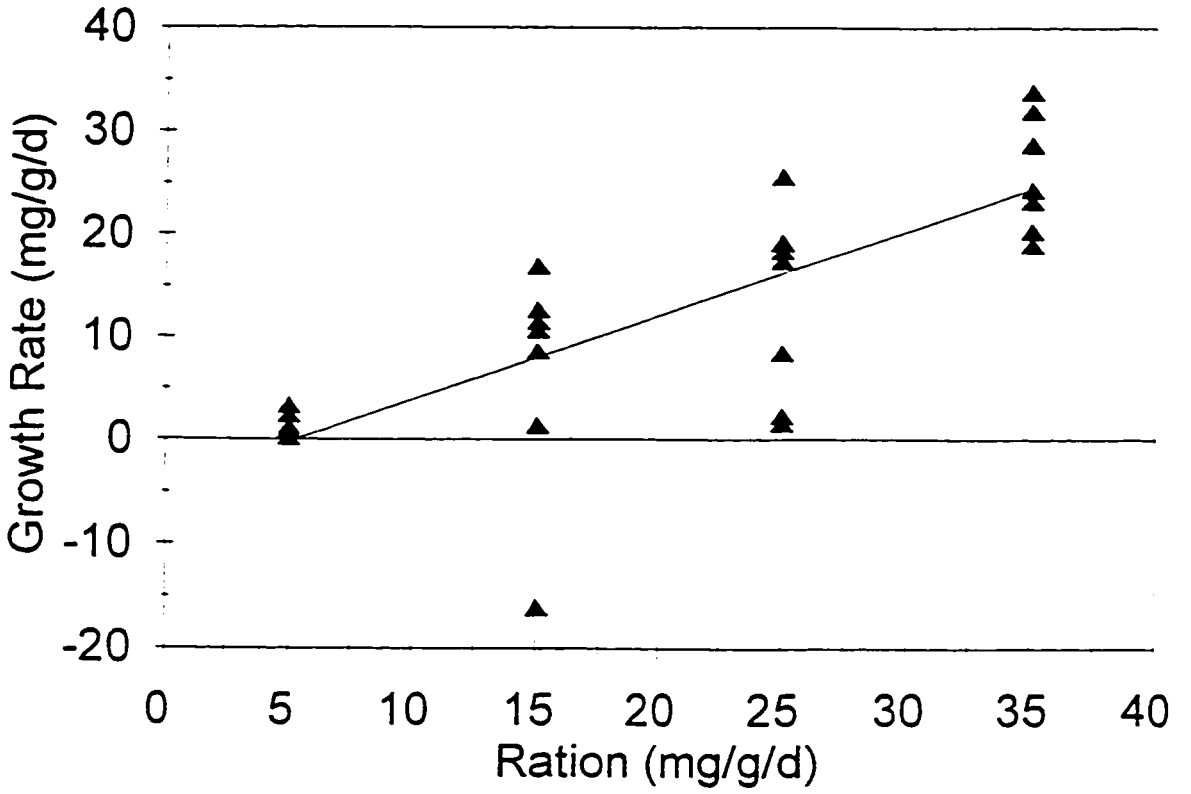


Figure 2.5. Growth increase in juvenile rainbow trout from experiment 2. Growth rate with respect to rations of 5, 15, 25, and 35 $mg \cdot g^{-1} \cdot d^{-1}$ for dietary MeHg concentration of 24 $\mu g \cdot g^{-1}$. Regression line from Table 2.1 is plotted in addition to individual data points.

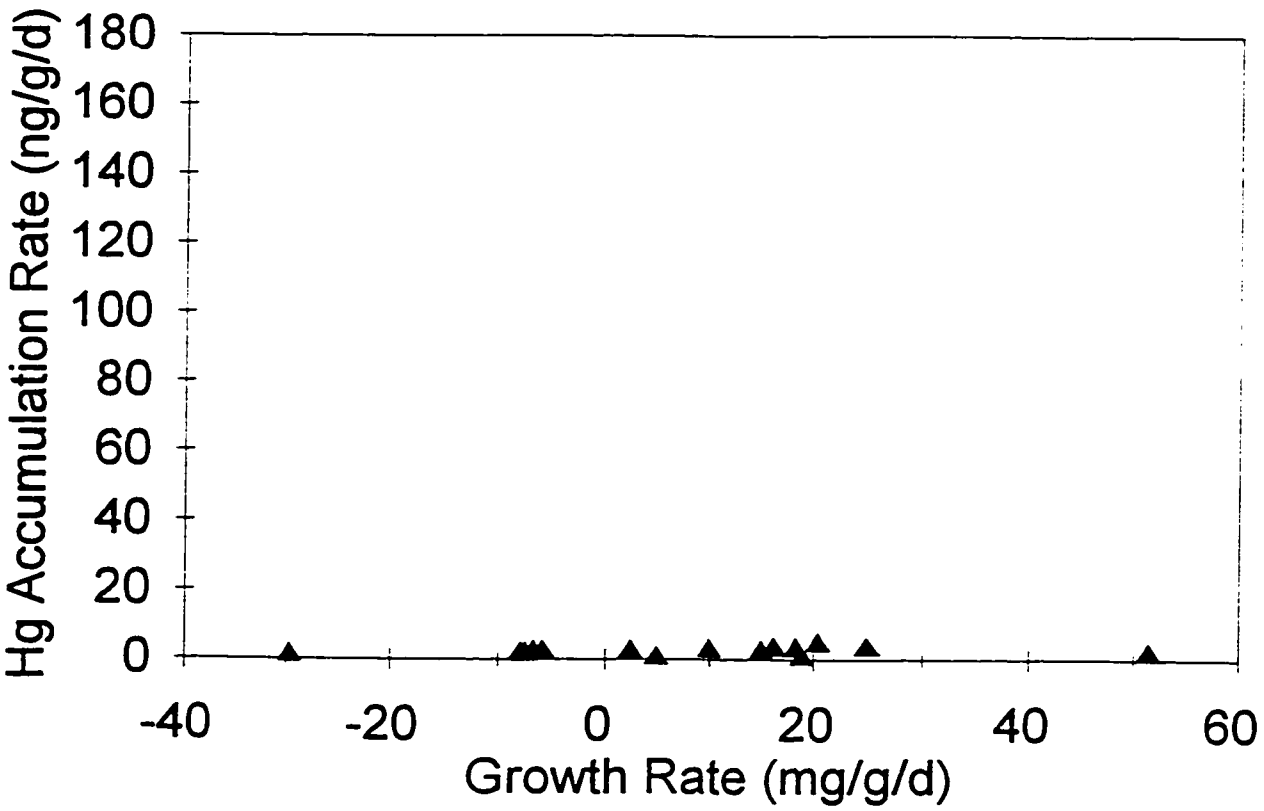


Figure 2.6. Hg accumulation in juvenile rainbow trout from experiment 3. Hg accumulation rate with respect to growth rate for dietary inorganic Hg ($HgCl_2$) concentration of 200 $\mu g \cdot g^{-1}$.

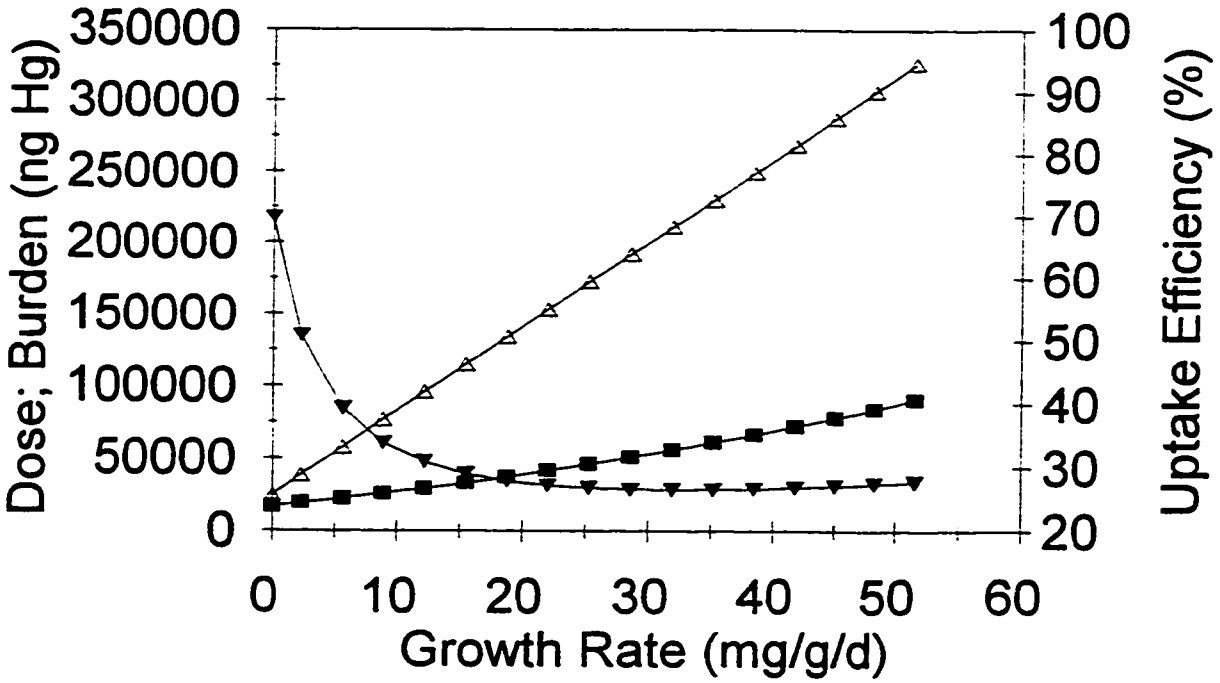


Figure 2.7. Plots of calculated total dose (open triangle), body burden (filled square), and Hg uptake efficiency (filled triangle) derived from growth-ration and Hg accumulation growth equations from experiment 2 (Table 2.1). Initial fish size was 10 g, and exposure was 20 days, as in experiment 2.

2.4 Discussion

The experiments presented in this chapter indicate that faster growing fish do accumulate MeHg faster than slower growing fish, at least in the short term. Rogers and Beamish (1982) also conducted a growth experiment with salmonids exposed to dietary MeHg, and, although they tagged individual fish, no examination of growth-accumulation interactions was attempted. In their 84 day experiment, fish fed higher rations at a given MeHg concentration did have higher muscle Hg concentrations than slower-growing fish. No estimate of error was provided by the authors, but the trends were similar to the findings presented here.

The occurrence of bioaccumulation in conjunction with growth in the time domain confounds its study. In addition to accounting for uptake, internal distribution, biotransformation, and excretion of MeHg, the bioenergetic considerations of organisms must be appreciated. Some researchers (Norstrom et al. 1976, Fagerström et al. 1974, Barber et al. 1991) attempt to account for both these aspects of bioaccumulation in a very detailed way, estimating bioenergetic factors (energy utilization partitioning, foraging, swimming, reproduction, growth) as well as MeHg bioavailability, clearance, and uptake from both water and food. The drawbacks to such an approach have been noted (Stow and Carpenter 1994, Hamelink and Spacie 1977), and there is a need for simpler approaches for the study of MeHg bioaccumulation (Hamelink and Spacie 1977).

Variable growth essentially represents different bioenergetic endpoints. The cumulative dose in a twenty day exposure increases dramatically with increasing ration (Fig. 2.7). The actual amount accumulated in muscle is a decreasing proportion of the dose as ration increases. For example, at a maintenance ration (zero growth), no change in weight occurs, and virtually all of the dose is accumulated in the fish, while large growth rates (ca. 50 $mg \cdot g^{-1} \cdot d^{-1}$) correspond to a decrease in accumulation to roughly 15% of the dose. This scenario demonstrates that although the Hg concentration increases more rapidly with increasing rate of growth, it is achieved with decreasing

efficiency. At low ration size, efficiency of absorption of dietary constituents is high (Brett and Groves 1979) as is MeHg absorption (Fig. 2.7, Clarkson et al. 1984). Presumably, absorbed MeHg is efficiently transported to and distributed among the body tissues while excretion rates are probably quite low (discussed in chapter 3). With increasing ration (and growth rate), the mass balancing of Hg accumulation is more difficult. The lower accumulation efficiency of MeHg at higher rations is likely due to some combination of higher excretion and lower uptake efficiency.

The fact that even 10-fold higher concentrations of inorganic Hg do not accumulate more rapidly at higher growth rates suggests that a mechanism unique to MeHg exists which might enhance dietary uptake and allow for this accumulation phenomenon. This same differential accumulation pattern for inorganic and methyl Hg accumulation was observed by Boudou and Ribyere (1983).

The most common justification for the relative ease of accumulation of MeHg relative to inorganic Hg is that the methyl group of MeHg confers lipophilicity to the molecule (Carty and Malone 1979). This view has been challenged as simplistic and refuted experimentally (Mason et al. 1995, Mason et al. 1996). Furthermore, Boudou and Ribyere (1983) concluded that "In the case of methylmercury, it appears that its liposolubility, contrary to what has been generally accepted, is not the entire reason for its toxicity and does not play a major role in its transport." An increasing body of evidence indicates that transmembrane fluxes of mercuric and methyl mercuric ions are virtually identical, so an alternate explanation may be more realistic.

According to Clarkson (1993), the most realistic explanation for MeHg accumulation involves uptake mechanisms across the intestine. MeHg is thought to undergo a significant enterohepatic circulation in vertebrates (Norseth 1973, Clarkson 1994). Clarkson (1994) contends that this is the major causative factor for the efficient bioaccumulation of MeHg observed in so many vertebrate species. Disruption of this enterohepatic circulation can be achieved by dietary sulphydryl compounds with a high affinity for Hg (Takahashi and Hirayama 1971, Clarkson et al. 1973). Mounting evidence points toward a MeHg-cysteine complex as the major Hg compound in enterohepatic

circulation. MeHg is secreted in bile primarily as a conjugate of the tripeptide glutathione (Clarkson 1994) which is at least partially degraded to its constituent amino acids in the digestive tract. The breakdown of protein and peptides is aided by the action of peptidases (secreted from intestinal epithelium) on small peptides such as glutathione (Stevens 1990). Presumably, the MeHg remains bound to the cysteine amino acid due to the high affinity of mercurials for SH groups, even at low pH (Jocelyn 1972). The MeHg-cys complex is then transported across the intestinal epithelium to complete the enterohepatic cycle. Some proportion of MeHg of biliary origin will re-enter the body via this process.

Clarkson (1993, 1994) further contends that MeHg-cys is transported across the intestinal epithelium on neutral amino acid transporters by virtue of its structural similarity to the amino acid methionine. This tendency for co-transport of MeHg-cys has been termed "molecular mimicry" (Clarkson 1993), and has been shown to occur at the blood-brain barrier (Clarkson 1993). The putative relationship of molecular mimicry of MeHg-cys to growth rate is intriguing, in that amino acid transporter density in gut epithelium is up-regulated in response to an increase in food supply (Stevens 1990). This up-regulation represents an additional vector for MeHg accumulation via the gut in response to increasing ration.

There are two ways that MeHg-cys could reach the intestinal epithelium: (1) directly from food; and (2) via bile excreted into the gastrointestinal (GI) tract. These two components of supply would reinforce each other, enhancing GI uptake. MeHg supply via food increases in conjunction with food intake, while biliary MeHg-cys output increases in response to the increasing dose of MeHg (dose being the product of dietary MeHg concentration and ration size). Regardless of whether dose increases due to ration size or changes in MeHg concentration, the effect would be the same – an augmentation in supply of MeHg-cys to the intestinal epithelium. When consideration of the up-regulation of amino acid transporters is added to this scenario, it is not inconceivable that higher rates of short term MeHg bioaccumulation could occur. The validity of this "molecular mimicry" phenomenon to MeHg accumulation-growth

interactions in fish remains unproven at this time.

The potential importance of growth-accumulation interactions should be particularly relevant for younger fish, as the larval and juvenile stages have the highest growth rate of any life stage, particularly for top predator fish species whose ultimate size is at least a kilogram in mass. It is of interest that, in some lakes, Hg bioaccumulation rates are extremely rapid in the juvenile period (Norstrom et al. 1976, Fagerström et al. 1974), even for species such as perch, which are typically planktivorous-insectivorous up to 20 g (Lindqvist et al. 1991). That these prey items have relatively low Hg concentrations makes such trends all the more interesting. Given the relatively high growth rates observed for juveniles of top predator fish species (Kamler 1992), the importance of growth rate differences to Hg accumulation merits further study. In addition, it may be reasonable, from a biomonitoring perspective, to focus on younger fish (Parks et al. 1991). This has numerous advantages, both in terms of sampling effort and impact on populations from such effort.

3.0 Elimination/Depuration Issues in Mercury Bioaccumulation

3.1 Introduction

The bioaccumulation of MeHg occurs because it is taken up faster than it is excreted (Hamelink and Spacie 1977). Uptake is thought to be quite efficient, while it is generally accepted that the elimination of MeHg is inefficient and rather slow, although there is some controversy regarding MeHg elimination in the literature (Trudel and Rasmussen 1997, Headon et al. 1996).

In nature, uptake occurs continuously, as does elimination, although these two facets of bioaccumulation are not easily separable in field-exposed organisms. Therefore, the technique of depuration is used to estimate the ongoing process of elimination by ceasing to expose experimental organisms to contaminant and following the subsequent loss of chemical from the organisms. From the depuration behaviour of chemicals, biological half times ($t_{1/2}$'s) can be calculated (Magos 1987, Niimi 1987), which for MeHg are generally between 300 and 1700 days, although some sources suggest somewhat more rapid elimination, between 100 and 170 days (Sharpe et al. 1977, Norstrom et al. 1976).

A second issue of interest to MeHg bioaccumulation is the role of the metalloid selenium (Se) in elimination mechanisms for MeHg in fish. Interactions between Se and heavy metals such as Hg have been recognized for decades. On one level, Se has been shown to protect against Hg toxicity (Craig 1986) . On the other hand, the use of Se to ameliorate elevated Hg levels in aquatic systems (Rudd et al. 1983) is aimed at altering Hg cycling, ultimately reducing rates of accumulation in top predator species. Such a use is not specifically toxicological, as there is no indication that the fish in these systems are under toxic stress from Hg. Rather, they manipulate biogeochemical transfer points in these systems with the end goal of reducing transfer of Hg to human consumers of fish (Lindqvist et al. 1991, Björnberg et al. 1989).

Se has been proposed as a tool for Hg amelioration in aquatic systems on the basis

of evidence from a wide range of sources. Anecdotal evidence includes findings from studies around Canadian metal smelters. Harrison and Klaverkamp (1990) observed an inverse relationship between Hg and Se in fish near the Cu/Zn smelter at Flin Flon, Manitoba, in spite of elevated levels of both elements in the lake sediments. Although they did not measure Se levels in biota, Wren and Stokes (1988) also found there to be an inverse relationship between Hg in biota and sediments around the Sudbury Cu smelter (elevated Se levels are known to exist in lakes around Sudbury (Nriagu and Wong 1983)).

Direct evidence for the suppressing effect of Se on Hg bioaccumulation in fish comes from whole lake Se additions in Sweden (Lindqvist et al. 1991, Paulsson and Lundberg 1989) and the earlier mesocosm studies of Rudd et al. (1980, 1983). The Swedish studies showed dramatic reductions in Hg levels in fish after just one year of Se addition.

In spite of this growing body of evidence for a role for Se in reducing Hg bioaccumulation in fish, the mechanisms responsible for this remain elusive. Currently, the accepted mechanism for Hg-Se interaction in aquatic systems is that of Björnberg et al. (1988), which proposes that Se sequesters Hg in sediments as mercuric selenides. These selenides have an extremely low solubility coefficient ($k_s=10^{-58}$), even lower than that of mercuric sulfide ($k_s=10^{-53}$). The mercuric selenide should be less available for microbial methylation, so that the addition of Se to a lake will ultimately suppress the formation of MeHg and the sediments will therefore serve as a Hg "sink" rather than a "source" as is normally expected in Precambrian shield lakes.

While the theory of Björnberg et al. (1988) certainly has merit, it excludes other possible points of interaction between Hg and Se in the biogeochemical cycle. There are other possible mechanisms which could be operating to alter Hg cycling in lakes. These mechanisms need not be mutually exclusive, and, in fact, probably operate concurrently. A schematic of these mechanisms is presented in Fig. 3.1. For fish, the mechanisms responsible for altering Hg cycling are essentially control points for uptake and depuration of Hg. The numbered arrows represent the potential interaction points for Se and Hg (mechanisms).

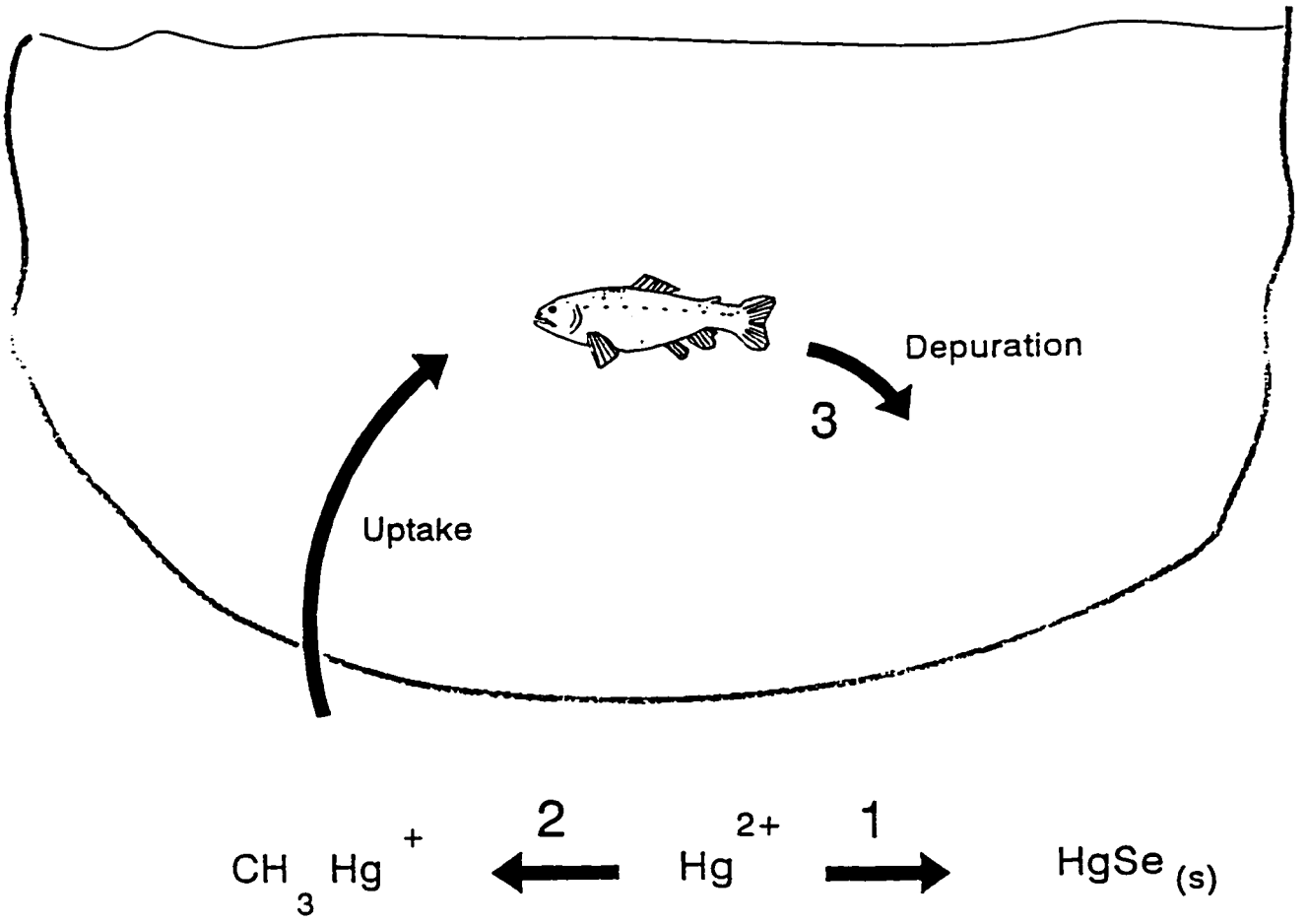


Fig. 3.1 Proposed mechanisms for the sites of interaction between Se and Hg in aquatic ecosystems.

Björnberg's mechanism is **(1)** in Fig. 3.1, with Hg being channelled to the HgSe sink in sediment. A second possible mechanism involves the accumulation of Se in sediments. Lacustrine sediments are sinks for Se (Rudd et al. 1980, Lemly 1985, Lemly and Smith 1987, Andersson et al. 1990). The accumulation of Se in sediments (eg. in lakes near smelters) may increase the Se:Hg ratio, which may in turn shift bacterial methylation from Hg to Se (**(2)** in Fig. 3.1). The methylation of Se by micro-organisms is an important reaction in the environmental Se cycle (Doran 1982). Se methylation is thought to be a microbial detoxification reaction, and is known to occur in lacustrine sediments containing elevated Se concentrations (Chau et al. 1976, Karlson and Frankenberger 1990, Thompson-Eagle and Frankenberger 1990). Methylcobalamin can be utilized by bacteria as a source of methyl groups for Se methylation (McBride and Wolfe 1971). Since this is also the major source of methyl groups for Hg methylation (Craig 1986), elevated Se concentrations in sediments could inhibit Hg methylation by competitive methylation. This mechanism remains to be tested.

The final possible mechanism for Se-Hg interactions involves the clearance (depuration) of Hg in fish in response to increased uptake of Se (**(3)** in Fig. 3.1). This mechanism could consist of two components. First, Se turnover in animals is dose-dependent (Glover et al. 1979). Therefore, in Se-contaminated environments, the turnover of Se in top predator fish species would be expected to increase in response to the increased availability of dietary Se (Se is transferred to top predators via food pathways (Turner and Swick 1983, Lemly and Smith 1987)). Given that Se is known to bind to Hg within biota (Carty and Malone 1979, Coombs and Coombs 1986), the increased turnover of Se could result in the concomitant turnover of Hg in top predators. This implies that the Se acts on MeHg already in fish, possibly involving a coupling of "biochemical" cycles for these elements. Second, the Se-Hg interaction could occur in the gastrointestinal tract (GIT) of fish in an extremely simple manner. It is known that MeHg undergoes a very efficient enterohepatic circulation which accounts for the high propensity for Hg bioaccumulation in vertebrates (Chapter 2). MeHg is excreted from vertebrates as

a complex with the tripeptide glutathione, with the MeHg bound to the sulfhydryl group of the cysteine in glutathione (Chapter 2). The enterohepatic circulation of MeHg is thought to be extremely efficient because the MeHg-cysteine complex (this complex will remain after the action of proteinases and peptidases on protein and peptide groups during digestion) is transported across the gut via the neutral amino acid transporters (Chapter 2). Any dietary factor which could preferentially sequester MeHg away from cysteine would effectively disrupt the enterohepatic circulation of MeHg and thereby enhance depuration.

This mechanism is interpreted as an elimination-dependent parameter in the present context, but it could certainly be viewed as an uptake limiting process as well, in that it certainly applies to MeHg passing through the intestine for the first time as well as to MeHg in the enterohepatic cycle. The uptake-related perspective has been examined previously (Cuvin-Aralar and Furness 1991), but the elimination-related aspects have been overlooked. Under conditions of simultaneous MeHg and Se uptake, the elimination-related interaction would be masked by the presence of ongoing MeHg exposure.

The research presented in this chapter is a test of mechanism **(3)**, the enhancement of MeHg elimination from fish by the addition of Se in the diet. Recent research into the interactions of Se and MeHg in invertebrates (Kelso et al. 1994) overlooked this possibility in its entirety.

3.2 Materials and Methods

3.2.1 MeHg Depuration Experiment

This experiment was undertaken in the Department of Biology wetlab facility at University of Waterloo. Water quality conditions for this facility were mentioned in chapter 2. Immature rainbow trout (5-10 g), raised from eggs, were fed MeHg-spiked food (Martin Trout Chow, 1.5 pt., Martin Feed Mills, Elmira ON). The MeHg-spiked food was the "low" MeHg food from experiment 1 in chapter 2. The food contained MeHg at 9 $\mu g \cdot g^{-1}$ (as Hg). Preparation of the MeHg-spiked food is described in detail in chapter 2. Fish were fed a maintenance ration (approximately 0.5% of body weight per day) for 60 days. Ration size

was not adjusted during this time, so essentially no growth occurred. No fish died in this period, and no fish were emaciated at the start of the depuration phase. Before sampling for the depuration phase occurred, fish were not fed for 14 days to ensure that guts were cleared of food and that exfoliation of intestinal epithelia had probably occurred.

At the end of the 14 day gut clearance period, twenty-one fish were weighed and sampled for muscle Hg. The fish are termed 'initials'. The remaining fish were sampled under light anaesthesia (tricaine methane sulphonate (MS 222) at 35 $mg \cdot L^{-1}$). Weights and lengths were recorded, and each fish was marked with a unique fin clip for identification at the final sampling. Fish were placed in one of ten 20 L glass aquaria (six fish per tank, five aquaria per treatment) which were randomized to either a Se-enriched diet containing sodium selenite (13 $mg \cdot kg^{-1}$, as Se), or a control diet. Rations of 0.5, 1.0, 1.5, 2.0, and 2.5 $mg \cdot g^{-1} \cdot d^{-1}$ were used for both treatments to provide variable growth during the depuration phase. Fish were depurated for 24 days, after which time they were sampled as per the 'initials'. Flow rates of 250 $mL \cdot min^{-1}$ to the aquaria provided 95% water turnover in approximately 2.5 h (Sprague 1969). Individual aquaria were aerated. A minor outbreak of fin rot occurred during the experiment, forcing the removal of four and six fish from the control and Se treatments, respectively.

The control and Se-spiked food was Martin Trout Chow (Martin Feed Mills, Elmira ON). Sodium selenite was added in aqueous form to the trout chow. As in the preparation of MeHg-spiked food, the aqueous selenite solution was added in approximately 10% of the amount of food (v/w) to prevent crumbling of the food. Control food was prepared in the same way, with deionized water substituting for selenite solution.

After one day for gut clearance, the fish were killed by anaesthetic overdose and sampled for muscle Hg levels. Body burdens for Hg were calculated as the product of muscle Hg concentration and body weight (Lockhart et al. 1972).

3.2.2 Data Analysis

Descriptive statistics (mean and standard error) were determined for each sample group, and one-tailed t-tests for differences between initials and depuration groups were

calculated (assuming unequal variances) in the Quattro Pro spreadsheet package (version 6.0 for Windows, Novell Corp., Orem, Utah). Biological half-lives were calculated using the formula of Magos (1987) for a one compartment pharmacokinetic model. This model assumes that Hg in muscle, the slow-clearing compartment in fish, adequately describes overall elimination from the fish. The calculation involves first calculating the elimination constant (k_e).

$$k_e = \frac{\ln B_0 - \ln B_t}{t}$$

where: B_0 and B_t are the body burdens at 0 and t days, respectively.
t is the time of depuration (in days).

Once k_e has been determined, the biological half life ($t_{1/2}$) is determined as:

$$t_{1/2} = \frac{\ln 2}{k_e}$$

3.2.3 Allometric Depuration Calculations. The calculations of Headon et al. (1996) were applied to the 'initial' fish samples to compare experimentally observed elimination with that predicted by the allometric elimination equation of Sharpe et al. (1976). Headon et al. (1996) calculated the allometric elimination constant as follows:

$$k_e = 0.029\, W^{-0.58}$$

where: k_e is the fractional clearance coefficient (day^{-1}).
W is the fish weight (g)

3.3 Results

The mean and standard error of Hg body burden for the 'initials' were 43087 ± 3962 ng. After 25 days of depuration, control and Se-treated fish had 48884 ± 3591 and 40578

± 3456 ng Hg, respectively (mean ± standard error) (Fig. 3.2). Neither of these values were significantly different from the 'initials' (one-tailed t= -1.09, p>t = 0.14 (controls) and t=0.477, p>t=0.318 (Se)). However, the Se-treated body burdens were significantly lower than controls (t=1.684, p>t=0.049) after 25 days of depuration.

Biological half-lives for Hg in the Se-treated depuration groups was 289 d. The $t_{1/2}$ for the 'control' depuration group was set to ∞, as the k_e for this group was -0.005, indicating that Hg burdens were increasing. However, because the mean Hg burden for this group was not actually statistically different from the mean of the 'initals', the most that can be said is that the control depuration group did not lose Hg. The value chosen for $t_{1/2}$ is intended to reflect this fact.

Using the allometric clearance equation (Sharpe et al. 1976, Headon et al. 1996), the predicted $t_{1/2}$ for elimination was 100 ± 84 d (mean ± standard error for n=21 fish sampled at day zero of the depuration period).

3.4 Discussion

Based on the wide range of estimates for biological half-life of MeHg in aquatic organisms (23-2000 days) (Trudel and Rasmussen 1997, Headon et al. 1996) it is very difficult to conclude just what is a realistic range for this parameter. Depending on which values are accepted as "real", the importance of elimination to the overall bioaccumulation process can vary widely. Interpretations vary from that of Norstrom et al. (1977), whose bioaccumulation model utilizes rapid estimates of elimination, to that of researchers who consider elimination of MeHg to be a considerably slower process (Headon et al. 1996).

There are several technical and conceptual reasons for the broad range of elimination estimates in the literature. First, as mentioned in chapter 1, there are semantic issues in the study of MeHg elimination. The actual definitions of contaminant elimination are most commonly stated as (1) "the time required for a body to eliminate half the amount of an administered substance through the normal channels of elimination" (Daniel 1978)

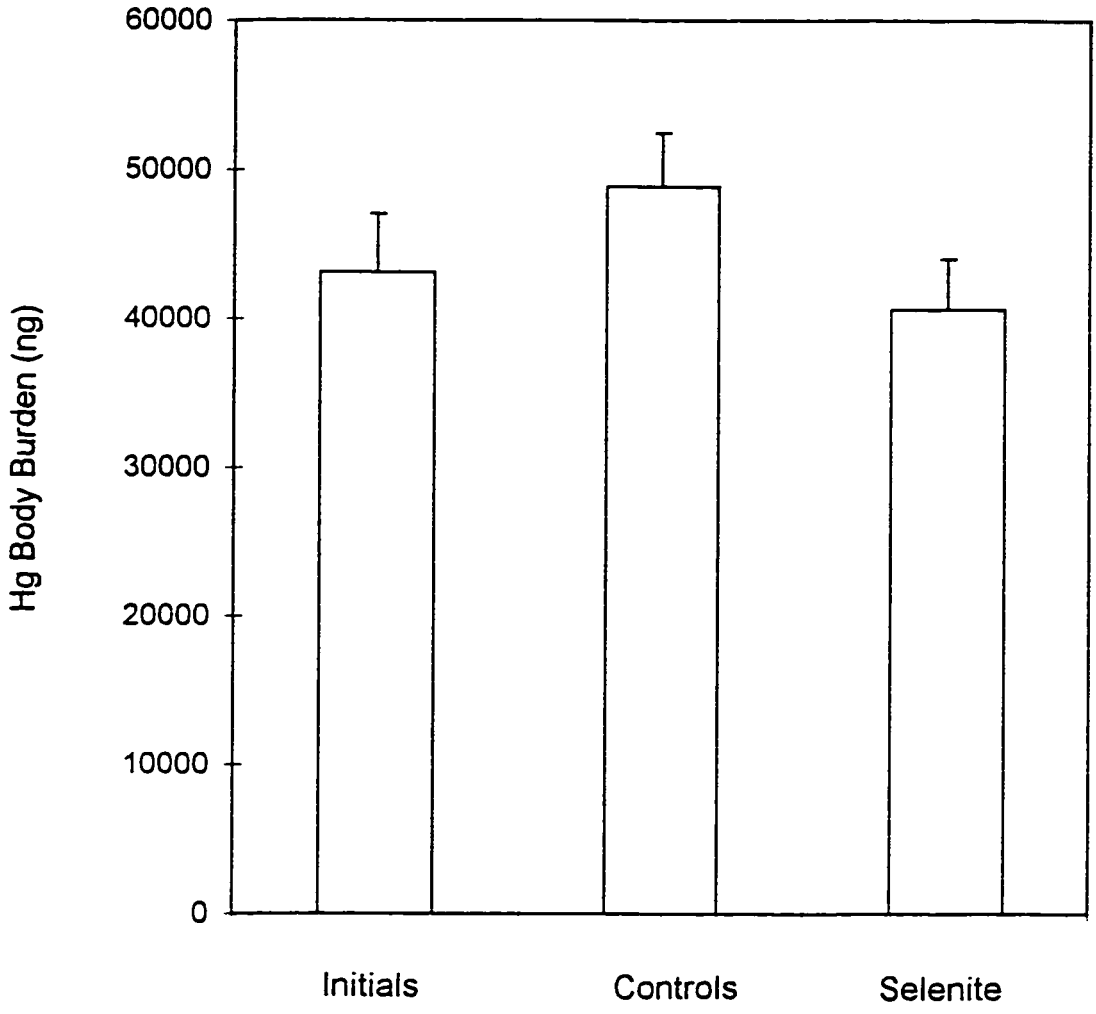


Fig. 3.2 Body burdens (ng per fish) of 'initials', Se-fed (n=24), and control (n=26) fish. Error bars indicate standard errors.

(the preferred definition) or as (2) "the time required to reduce the concentration of a chemical by one-half in a designated medium or organism such as soil, water, and fish" (ASTM 1978). In the MeHg literature, definition (1) is most commonly used, so rarely is this issue actually problematic, although the use of definition (2) has to consider that growth in depuration studies will lead to a "dilution" of MeHg concentrations in fish tissues (Niimi 1987).

3.4.1 Whole-body Estimates of MeHg Elimination in Fish and Implications for Allometry of Hg Elimination

The choice of dosing and MeHg detection methods used for depuration studies are the most important factors having bearing on the estimates of elimination. Studies utilizing single oral doses, usually of ^{203}Hg-labelled MeHg (referred-to as ^{203}MeHg below) (Sharpe et al.1977, DeFreitas et al. 1977, Rodgers and Beamish 1982, Penreath 1976) have characteristics which influence the estimates of elimination. Since these types of experiments predominate in the literature, they merit discussion here. First, although organ distributions of MeHg within the body can be obtained by dissection (Penreath 1976), typically, whole body elimination is determined (Sharpe et al. 1977, DeFreitas et al. 1977, Rodgers and Beamish 1982, Ruohtula and Miettinen 1975). Whole-body estimates of depuration after single doses of ^{203}MeHg therefore include all compartments in the body. As discussed below, this hinders the isolation of 'slow-' and 'fast'-clearing pools of MeHg in fish, and probably accounts in large part for the rapid elimination estimates generated from such studies. This, and other inherent complexities in determining whole-body estimates of depuration from single oral doses, are apparently not appreciated in the literature. For example, although two phases in elimination are well recognized after oral dosing regimes (Trudel and Rasmussen 1997), these compartments or pools are understood only as mathematical entities; corresponding physiological descriptions are mentioned only in passing (Rowan and Rasmussen 1995) or not at all (Trudel and Rasmussen 1997).

The elimination of MeHg in single oral dose depuration studies follows a

characteristic biphasic kinetics (Trudel and Rasmussen 1997). Typically, an initial rapid loss of dose is observed, representing chemical not actually absorbed. This loss does not represent elimination per se, but corresponds to unassimilated dose, measured as the proportion lost by defecation. Following this initial intestinal loss phase, the two phases of true elimination ('fast' and 'slow') have half-lives of days-to-weeks and months-to-years, respectively (Trudel and Rasmussen 1997). The slow phase certainly includes muscle (Rowan and Rasmussen 1995), and probably includes blood, liver, gill, and kidney. In published MeHg depuration studies, the loss of MeHg from these organs suggests that they are, themselves, rapidly eliminating organs (Penreath 1976, Boudou and Ribyere 1983). However, the loss of MeHg from these organs (liver, gill, and kidney) probably occurs primarily to blood, from which it is redistributed to the ultimate storage pool (muscle). Since whole-body determinations of ^{203}MeHg do not distinguish radioactivity from individual organs, the slow-clearing pool includes muscle (truly slow-clearing) as well as liver, blood, gill, and kidney (all rapidly-clearing).

The organs responsible for the 'fast' phase of depuration after a single oral dose have not been identified in previous reviews (Rowan and Rasmussen 1995, Trudel and Rasmussen 1997). From a wide range of literature sources, it seems reasonable that this phase is a pool represented by ^{203}MeHg bound to the gastrointestinal tract (GIT). Consideration of the physiology of the GIT can account to a large degree for the rapid estimates of MeHg elimination found in the literature (Sharpe et al. 1977, deFrietas et al. 1977). In particular, whole-body determinations of ^{203}MeHg necessarily include intestinal ^{203}MeHg as part of the whole-body radioisotope pool. There are four specific considerations in this regard. First, the intestine opens directly to the external environment via the anus, so it is a potential route of excretion. Second, as the site of uptake of MeHg, the GIT can account for a significant portion of the body burden of MeHg in the early stages of such a depuration experiment (Penreath 1976). As discussed below, sloughing (exfoliation) of intestinal epithelial cells into the GIT with any MeHg remaining bound to the cells can be a major source of loss of MeHg in early stages of depuration (via defecation). Third, there are distinct differences in the structure and relative proportion of the GIT to

fish body mass as a function of feeding ecology. Herbivorous species have the most extensive GIT, with carnivores having relatively short GIT's (Steffens 1989, Weatherly and Gill 1987). Obviously, single oral dose depuration studies with herbivorous species will initially see a greater proportion of the body burden remain in the GIT than in carnivores, which would affect whole-body clearance rates. Fourth, developmental stages within individual species are characterized by changes in feeding ecology and increases in size. Allometric changes in the GIT have been noted (Steffens 1989, Denton and Yousef 1976, Weatherley and Gill 1987). Individually, these factors are of variable importance, but as a whole, they provide reasonable grounds for rejecting, or at least questioning, much of the published literature on MeHg depuration, particularly those studies utilizing whole-body determinations of radioactive ^{203}Hg-labelled MeHg after single oral dose exposures.

The loss of MeHg from the GIT via exfoliation of intestinal epithelium after oral dosing has been determined for rats (Norseth and Clarkson 1971). It is thought that MeHg is bound to structural constituents (proteins) of the cells; these constituents are not reabsorbed and are therefore passed through the GIT as fecal output. Such data do not exist for fish, but, as in mammals, the intestinal mucosa of fish represents a "renewing" cell population (Weatherly and Gill 1987) in which cells rapidly proliferate to replace damaged cells. In vertebrates, enterocytes are formed from mitotically active precursor cells in the crypts of the intestinal villi. Over a period of 2-6 days, the enterocytes mature and migrate toward the tip of the villi, where they ultimately are 'sloughed' into the lumen of the GIT (Texter et al. 1968, Lipkin 1985). At the histological level, there are degrees of similarity between the absorptive tissues of fish and higher vertebrates (Kuperman and Kuz'mina 1994, Grau et al. 1992, Stroband 1977, MacLeod 1978). However, it is unclear whether the replacement of intestinal epithelium occurs more slowly in poikilotherms. Presumably this is the case, which could mean that the loss of MeHg bound to the intestinal epithelium may be a significant factor affecting the length of the 'fast' phase of elimination in single oral dose depuration experiments.

In their recent review, Trudel and Rasmussen (1997) examined eighty-one estimates of elimination from thirteen different studies of MeHg depuration in fish. One of

their major conclusions was that MeHg elimination is allometric, with smaller fish eliminating the chemical more rapidly than large fish. Their allometric exponent for clearance (ζ= -0.2) is considerably less than that of Norstrom et al. (1976) (ζ=-0.58). Trudel and Rasmussen (1997) attribute their smaller value to the fact that they only utilized studies longer than 90 days in duration on which to base their calculations. According to Rowan and Rasmussen (1995), it appears that such a time period is required to allow the isolation of slow and fast phases of contaminant depuration. While the conclusions of Trudel and Rasmussen (1997) are a considerable advancement relative to previous interpretations, there are problems in the literature data which compromise the validity of several aspects of the review.

The first aspect of this issue concerns the thirteen references for MeHg elimination used in the review. Twelve of the cited articles utilized single oral doses of ^{203}MeHg to expose the fish coupled with whole-body ^{203}Hg determinations to estimate elimination. These twelve studies provided 77 of the 81 $t_{1/2}$ estimates used in the review. Of these 77 estimates, only 17 used fish larger than 100 g. In fact, the majority of elimination estimates (45) utilized fish less than 20 g in weight. Based on previous reviews in their research group (Rowan and Rasmussen, 1995), Trudel and Rasmussen (1997) chose to break the data into groups from from studies of duration less than or greater than 90 days. It was thought that this would aid in resolving slow and fast phases of elimination. However, by doing so, the data become heavily weighted towards particular size classes of fish. For example, in the >90 day group, only one fish represents the size class of fish less than 20 g. In contrast, the <90 day group had 44 fish in the <20 g class (of 60 fish in total). All 44 of these fish could be reclassified in terms of type of depuration study they represent (ie. single oral dose/whole-body counting), rather than in terms of weight. On this basis, the correlations between elimination rates and weight could well be spurious, and by association, the conclusions regarding the allometry of MeHg elimination are questionable as well. In addition, the differences in elimination with respect to depuration time also reflect a similar bias (Trudel and Rasmussen, 1997), as the <90 day studies all utilized single oral doses of ^{203}Hg-MeHg and whole-body ^{203}Hg determinations to estimate

elimination.

Therefore, the recommendation of Trudel and Rasmussen (1997) that depuration studies should be at least 90 days in duration to adequately resolve slow- and fast-clearing compartments may be true for single-oral-dose exposures and whole-body MeHg determinations of ^{203}Hg. This recommendation has been made in the past (Fowler et al. (1975). However, this interpretation should not necessarily be a requirement for depuration studies in general, and would not be needed for single-oral-dose exposures of ^{203}Hg-MeHg, provided that the GIT is counted separately from the rest of the body. The removal of viscera prior to counting of ^{203}Hg would probably provide the simplest and most conservative means of estimating elimination for single (oral) dose studies using radiolabelled MeHg.

The depuration estimates of Sharpe et al. (1977) and DeFrietas et al. (1977) (derived from single oral dose experiments) are among the most rapid in the literature ($t_{1/2}\approx$100 d), and are probably the most prominent example of the inherent problems with the "single oral dose/whole body counting" type of experiment. The fact that Sharpe et al. (1977) utilized only goldfish (*Carassius auratus*) may reflect the ability of goldfish to eliminate MeHg more rapidly than other fish species (Trudel and Rasmussen 1997). This is not unreasonable, as cyprinids are omnivorous fish with relatively long digestive tracts. Therefore, for a single oral dose of ^{203}MeHg, the initial proportion of MeHg bound to the gastrointestinal tract (GIT) may be greater than for carnivorous fish such as rainbow trout, which have relatively short GITs. In addition, Sharpe et al. (1977) and de Frietas et al. (1977) used goldfish weighing less than 50 g (most fish were less than 17 g) for their research. Steffens (1989) has shown that there is an allometry in intestinal surface area in cyprinids. The weight-specific area ($cm^2 \cdot kg^{-1}$) decreases logarithmically with fish weight, from approximately 1900 $cm^2 \cdot kg^{-1}$ for 2-3 g fish, to 1000 $cm^2 \cdot kg^{-1}$ for 10 g fish, and, finally, to 500 $cm^2 \cdot kg^{-1}$ for 50 g fish. Although, in absolute terms, the mass and length of intestine actually increases in proportion to fish size (Steffens 1989), the actual area for absorption and binding of MeHg decreases. Therefore, if binding of MeHg occurs in GIT epithelium, it could be enhanced in smaller cyprinids of the size used by Sharpe et al. (1977)). This

would further influence the proportion of MeHg bound in the intestinal "fast" pool.

Currently, "single oral dose/whole body counting" depuration studies with ^{203}MeHg constitute the major body of information for elimination estimates of MeHg in fish. As discussed above, there are unappreciated and unresolved technical issues with this approach to elimination. Conceptually, such an approach is imperfect as well. The question of whether depuration even occurs in the environment remains unanswered. It is possible that overwintering periods may offer a natural, seasonal depuration time, although estimates of depuration at low temperature seem to indicate that this is not a likely scenario (Ruohtila and Miettenan 1975).

Although elimination is an ongoing process in fish exposed to MeHg in the environment, elimination is distinct from depuration. Few experiments have actually depurated MeHg-exposed fish under real environmental conditions (Lockhart et al. 1972, Laarman et al. 1976). Such experiments, involving the between-lake transfer of fish, are major undertakings, and therefore not easily funded. The value of the "single oral dose/whole body counting" type of depuration experiment has to be accepted within this framework. Given the current status of the literature, the recommendations of Trudel and Rasmussen (1997) that studies >90 days in duration accurately estimate the long-term clearance of MeHg is reasonable. However, future studies should attempt to circumvent this problem by: (1) dosing fish with more than one meal in oral dosing schemes; (2) if using ^{203}MeHg for single oral dose depuration studies, remove the viscera before counting the radioactivity in the fish; and (3) using intravenous injection (pharmacokinetic) approaches to depuration.

3.4.2 Pharmacokinetic Approaches to MeHg Elimination in Fish

Although free of concerns about proportion of dose actually accumulated, bolus intravenous doses of MeHg are also problematic and may produce questionable estimates of elimination as well. This type of elimination study is approached from a classical pharmacokinetic framework in which clearance from blood is used to estimate whole-body clearance (Schultz and Newman 1997). The assumptions of this approach are difficult to

justify on several fronts, and the validity of classical pharmacokinetic models for clearance (such as that used by Schultz and Newman 1997) must be questioned.

Pharmacokinetic modelling was developed to help understand the behavior of drugs in vertebrates. Typically these drugs are fast-acting and are metabolically degraded on a similar time scale. In contrast, MeHg in fish is not readily demethylated, and is poorly eliminated. As such, MeHg cannot be considered in the same class as pharmacologically active drugs. Schultz and Newman (1997) are flawed in assuming that MeHg is cleared by fish as if it were a drug. Their model assumes that MeHg is cleared from a central compartment (analogous to blood and highly perfused organs such as liver and kidney). This is probably a good representation of how MeHg is, in fact, eliminated from fish. However, the obvious fact that MeHg is actually bound and stored in muscle tissue is neglected by their model. Therefore, the loss of MeHg from blood and plasma is interpreted to indicate elimination from the fish, when in fact, the MeHg is largely transported to muscle. This observation of "redistribution" of MeHg in depuration studies is well-documented in the literature (Giblin and Massaro 1973 , Penreath 1976, Boudou and Ribyere 1983, Wiener and Spry 1996). Schultz and Newman (1997) overlooked this possibility because their previous research examining inorganic Hg elimination demonstrated that $HgCl_2$ did not accumulate in fish muscle (Schultz et al. 1996). Again, it is well-documented that the accumulation patterns of inorganic Hg and MeHg are quite different, with inorganic Hg accumulating in liver and kidney (like other inorganic metals such as Cd, Zn, and Cu), while MeHg accumulates primarily in muscle (Penreath 1976, Boudou and Ribyere 1983, Lindqvist et al. 1991). A more appropriate model for MeHg accumulation and elimination accommodates storage of MeHg in the "peripheral" compartment (primarily muscle) while accepting that elimination does occur from the "central" compartment. Such a model reflects that the loss of MeHg from blood is a result of two factors: (1) elimination from the body, and (2) transfer to muscle (the predominant flux).

The published $t_{1/2}$ estimates for $HgCl_2$ and MeHg in channel catfish (*Ictaluras punctatus*) were 722 and 34 days, respectively (Schultz et al. 1996, Schultz and Newman

1997). These values are opposite to the entire literature on Hg bioaccumulation, and reflect at least two factors in the research of Schultz and colleagues. First, the model used for inorganic Hg depuration (Schultz et al. 1996) overlooked the physical data (the fact that 48% of the injected $HgCl_2$ was eliminated in 156 days) and instead, "due to the multi exponential pattern of elimination *predicted by the model*" (Schultz et al. 1996) (italics mine), the $t_{1/2}$ of 722 days predicted by the model was published. Second, the MeHg depuration estimates (eg. $t_{1/2}$=34 d.) were taken strictly from blood data, with no apparent consideration that MeHg is ultimately stored in a peripheral compartment rather than being excreted. The half-life for MeHg is very clearly wrong, even with respect to the estimates discussed in the previous section.

The use of intravenous dosing for depuration is not without merit. However, for applicability to real environmental situations, depuration should be measured from the 'slow' or 'peripheral' pool of the body. In the literature at present, this approach is lacking.

3.4.3 Muscle Hg Levels for Estimating Elimination

The heavy dependence of Trudel and Rasmussen's (1997) review on the depuration estimates of so few researchers (three literature sources accounted for 56 of the 81 elimination estimates) coupled with the inherent flaws in so many cited articles (all of the <90 day studies used whole body counting) demonstrates that the field of MeHg elimination is not without controversy. There is a clear need for alternate depuration techniques.

The depuration data presented in this chapter used muscle Hg levels in juvenile rainbow trout, with the assumption that this tissue was representative of both the 'slow' clearing pool of MeHg in the body and the largest proportion of the body mass. Lockhart et al. (1972) have shown that the product of muscle Hg concentrations (from muscle biopsies) and body weight is an accurate representation of body burden. Therefore, in this study, the use of body burden (but derived from muscle Hg concentrations) provides estimates of elimination from the 'slow' compartment of the body. Essentially no elimination

of Hg body burden occurred in control fish after 25 days of depuration. The time frame for this study was less than a third of that suggested as being required for estimating the slow phase of elimination by Trudel and Rasmussen (1997). The use of muscle MeHg analysis to estimate elimination would appear to be a conservative approach. In addition to the study of Lockhart et al. (1972), Riisgård and Hansen (1990) also demonstrated the value of muscle Hg for depuration research. In the latter study, no difference in muscle Hg burden was observed, even after periods of less than a month. It would appear that the need for longer depuration periods are a strict requirement only for studies using the methods typical of those cited in Trudel and Rasmussen (1997), as discussed above.

The possibility of using muscle Hg values to estimate elimination in field-exposed fish should have an obvious utility, both in terms of convenience and relevance. As discussed in chapter 2, of the life stages, young fish seem to accumulate Hg most rapidly. In terms of availability, ease of collection and conduction of experiments (including possible transport back to laboratory settings), young fish are obvious choices for environmentally relevant depuration studies. Problems of exposure become moot, as the fish have already been exposed in their environment. Using muscle biopsies for initial and subsequent estimates of Hg burdens (Lockhart et al. 1972), it should be possible to estimate elimination in a more realistic manner than currently available techniques.

3.4.4 The Issue of Allometry of MeHg Elimination

The allometry of MeHg elimination is well entrenched in the Hg bioaccumulation literature (Fagerström et al. 1974, Norstrom et al. 1976, Sharpe et al. 1977, Harris and Snodgrass 1993, Rodgers 1994, Trudel and Rasmussen 1997); the large number of studies using this approach seem to give it credence. The ontogeny of this concept is of considerable interest. Fagerström et al. (1974) are the first to mention this in print. They cited "recent experimental results on mercury by de Freitas (pers. comm.)" who found that MeHg elimination in pike was inversely related to body weight. The citation "endless loop" produced is significant; de Freitas (a co-author on Norstrom's article) was able to cite a previously published paper to support his findings on the allometry of Hg elimination. Upon

examination of the literature, it appears that this entire school of thought developed from elimination estimates from *six* pike (finally published in de Freitas et al. 1977 three years after originally being cited as a pers. comm.). Each successive author since then has cited the previous findings until a comparatively large body of literature exists supporting the allometry of Hg elimination.

There are few studies that do not support allometry of Hg elimination. Headon et al. (1996) and Hamilton (1972) both found that crayfish eliminate MeHg very slowly and do not demonstrate allometric clearance. Headon et al. (1996) simulated clearance using the allometric equation of Sharpe et al. (1976) and found that measured elimination was underestimated by a factor of approximately 25. It is possible that the distinct physiology of crayfish accounts for this lack of allometry and slow clearance. However, the findings of this chapter also bring into doubt the parameter values, if not the concepts, of the MeHg elimination literature. In this experiment, fish were all small in size (between 5 and 35 g initially), yet did not clear any appreciable Hg in the 25 day depuration period. Estimated elimination of MeHg using the allometric elimination equation of Sharpe et al. (1976) gave values for $t_{1/2}$ of approximately 100 d. This value is similar to several literature values cited in Trudel and Rasmussen (1997), but appears to overestimate the actual elimination rate considerably. Given the small size of the fish used in this study, the lack of elimination of MeHg agrees more closely with Headon et al. (1996) than with the elimination literature in general.

3.4.5 A Potential role for Selenium in MeHg Elimination in Fish

The potential of Se as a Hg ameliorative agent in aquatic systems is well-documented in the literature (Rudd et al. 1983, Paulsson and Lundberg 1989, Lindqvist et al. 1991, Cuvin-Aralar and Furness 1991), although the mechanisms for this ameliorative action remains unclear. The majority of literature dealing with Hg-Se interactions considers aspects of toxicity (Rudd et al. 1980, Pelletier 1985), whereas the ameliorative effects of Se on Hg bioaccumulation in aquatic systems seems to suggest merely an alteration in Hg cycling (Björnberg et al. 1988), with Hg toxicity being of little

importance in that regard.

The interaction examined in this research was that of mechanism (3) of Fig. 3.1, namely the enhancement of Hg depuration by Se. In experiment 4, after only 25 days of dietary Se exposure, the body burden of Hg in juvenile rainbow trout was reduced relative to control fish. Although the data are not definitive, this work suggests that Se (as sodium selenite), does in fact alter Hg elimination. The lack of a statistically significant difference between 'initials' and day 25 'selenite' samples reflects the slow elimination processes in these fish.

The absence of a substantive body of evidence corroborating such a finding has much to do with study designs used in previous studies of Hg-Se interactions. For example, some studies examine (1) uptake and interaction directly from water (Davies and Russel 1988) or food (Ringdal and Julshamn 1985), or (2) toxicity interactions from water (Pelletier 1985) or food (Ohi et al. 1976). The lack of an interaction between Hg and Se in some of these studies probably is simply a reflection of the experimental design, including the chemical form of both Se and Hg under study.

In the research presented in this chapter, the interaction under study was strictly that of selenite on MeHg already present in the body. The mechanism by which selenite reduces the Hg burden in depurating fish remains uncertain. Given the known propensity for Hg-Se binding, and given the generally faster rate of elimination of Se relative to Hg (Glover et al. 1979), it is not inconceivable that the binding of MeHg to selenite enhances depuration (possibly by disrupting enterohepatic circulation). Iwata et al. (1982) have demonstrated that sodium selenite degrades MeHg *in vitro* by cleavage of the Hg-carbon bond. This may be a part of the process of the interaction of Se in MeHg depuration.

The overall interaction of Hg and Se in aquatic systems, and the potential of Se for ameliorating Hg accumulation in top predator fish is undoubtedly a complex matter. The three mechanisms proposed for this interaction in Fig. 3.1 may work in concert. The previous use of Se for reducing Hg levels in fish (Paulsson and Lundberg 1989, Lindqvist et al. 1991) were successful in achieving the desired result; Hg levels in fish were dramatically reduced within a year. However, in this case, the medicine was probably

worse than the sickness. The levels of Se in the treated lakes (ca. 10 $\mu g \cdot L^{-1}$) were high enough to apparently cause reproductive failure in several fish populations (Lindqvist et al. 1991, Lemly 1985). This reflects the very small therapeutic window for Se in a Hg amelioration role. Further forays into whole-lake Se addition cannot be justified until the basic science in this area has been undertaken.

4.0 The Measurement of Short-Term Growth in Fish

4.1 Introduction

The measurement of growth is central to both fisheries biology and aquatic ecotoxicology; growth estimates are essential to stock assessment, while growth impairment is a relevant endpoint for assessing contaminant exposure. The need for techniques that provide estimates of recent growth of fish has been noted on several fronts. Adams and McLean (1985) called for indices that reflect growth and respond relatively rapidly to changing environmental conditions, while Norstrom et al. (1976) noted a need for techniques for the measurement of recent growth in bioaccumulation studies. They both concluded that the use of annual growth is a poor substitute for measurements of recent growth in contaminant research.

Growth is simply change in size over time, which can be considered as the ultimate expression of fish health (Adams and McLean 1985). Within a defined time interval, growth can be expressed as a rate. Traditional techniques for growth measurement include: (1) **size-at-age** - where age is determined from calcified tissues such as scales or otoliths; and (2) **mark recapture** - in which tagged or marked fish are recaptured after a defined time interval (Weatherly and Gill 1987). Both these approaches have limitations; older fish are difficult to age accurately and can bias estimates, whereas mark-recapture studies can be costly depending on the spatial scale on which they are undertaken. Although the traditional approaches still have their place, the need for useful markers of recent growth is not trivial (Houlihan et al. 1993). Instantaneous growth, obtained as the growth interval approaches zero (Ricker 1979), is, at present, measurable only via nucleic acid analysis. This technique utilizes the correlation between growth and tissue RNA content or RNA:DNA ratio under the assumption that RNA content will reflect the level of protein synthesis (and therefore growth) occurring in a tissue.

Estimates of fish growth rate by nucleic acid analysis typically use muscle tissue as the source material, as increase in fish mass is largely due to increase in muscle

protein. Whole-body nucleic acid levels are used with larval fish because of difficulties in separating individual tissues from larvae. Since liver, the hub of metabolism in fish, shows increases in RNA levels both after feeding and fasting (McMillan and Houlihan 1992), it is not recommended for growth analysis by nucleic acid techniques (Bulow 1987). Surprisingly, blood has not yet been used for growth estimation, although the characteristics and composition of the erythrocyte cell population in the blood stream should be well suited for this purpose.

Erythrocytes are responsible for gas exchange in vertebrates. There is a defined basal level of erythrocyte synthesis (erythropoiesis) required to replace effete erythrocytes lost from circulation. In growing fish, however, erythropoiesis must be induced in order to support the increased respiratory needs associated with larger body size. This induction of erythropoiesis should be measurable as an increase in the blood RNA concentrations. Such an approach has been utilized in human haematology (Tanke et al. 1980), and can be justified by considering the composition of the erythrocyte cell population, which consists of subpopulations of reticulocytes (immature erythrocytes) and mature erythrocytes. Reticulocytes are released into circulation after erythropoiesis without any hemoglobin, which is synthesized within the cells as they circulate. High levels of RNA are found in reticulocytes relative to mature erythrocytes (Lane and Tharp 1980). Reticulocytes can also be distinguished from mature erythrocytes on the basis of histological appearance (Keen et al. 1989). They are virtually circular, with large nuclei and granular (basophilic) purple-staining cytoplasm with Wright-Geimsa stain, whereas mature erythrocytes are elongated and oval, with smaller nuclei and clear cytoplasm which stains yellowish to pink. Partially mature erythrocytes have intermediate properties. Under conditions of rapid growth, erythropoiesis should be stimulated, giving a concomitant increase in the proportion of reticulocytes and partially mature erythrocytes in the blood relative to the situation under slow growth. These differences should be detectable by both histological examination of blood smears or RNA analysis.

The use of stained blood smears has several possible advantages relative to the

use of nucleic acid analysis for the estimation of growth. The method is simple, requiring only staining equipment and a compound microscope, both of which are readily available and can be used under either laboratory or field conditions. Virtually anyone can learn to do the cell counts in a matter of hours. Perhaps most importantly, because they are stained and preserved, the samples can be kept indefinitely.

There is limited evidence supporting the use of erythrocytes for estimating fish growth, although as early as 1978 it was recognized that the immature erythrocyte stages might be particularly useful indicators of toxic stress (Christensen et al. 1978). Lane and Tharp (1980) demonstrated that starvation reduced the proportion of immature erythrocytes, whereas reduction in blood volume increased this proportion via enhanced erythropoiesis. Bridges et al. (1976) noted that the immature erythrocyte population increased dramatically during the growing season of winter flounder (*Pseudopleuronectes americanus* Walbaum). The use of blood for growth estimation fits well into the overall framework of blood sampling in ecotoxicology, which has the potential to be as useful for biomonitoring as it is in human toxicology and pathology. Blood sampling is non-destructive, enabling repeated sampling of fish over time. When coupled with pharmacokinetic modelling, blood can provide evidence of recent contaminant exposure, with contaminant levels varying as contaminants clear the blood stream. Finally, blood is technically simple to analyse relative to other tissues, due to its fluid nature. This can considerably reduce technical costs in contaminant residue analysis, since the need for tissue digestion and extraction procedures are decreased or eliminated.

This chapter compares the relative value of nucleic acid analysis in blood, muscle, and liver for estimating growth rates in fish. Fluorometric methods for analysing nucleic acids (Karsten and Wollenberger 1972, Clemmesen 1987) were adapted for this work. After tailoring a method for fluorometric assay of nucleic acids, the possibility that erythrocyte subpopulations could be distinguished by extremely simple histological methods prompted a further comparison of the relative advantages of blood smears versus nucleic acid analysis.

4.2 Materials and Methods

4.2.1 Experiment 5

This experiment was designed to manipulate growth rates in fish and compare nucleic acid analysis in blood with that of the more commonly used liver and muscle. In the department of biology wetlab at the University of Waterloo, rainbow trout (150-250 g) were individually tagged by unique fin clips, weighed, and measured prior to the beginning of feeding. Fish (3 per tank) were fed at rations of 0, 5, 10, 15, 20, 25, and 30 mg food (Martin Trout Chow; Martin Feed Mills, Elmira, ON) per gram body weight per day for 14 days. At the higher rations, it was necessary to feed the fish in smaller quantities several times per day. To ensure gut clearance, fish were not fed for 48 h prior to the initial or final sampling.

Flow rates to the 60 L fish tanks were 1 $L \cdot min^{-1}$, providing complete replacement of tank water in approximately 5 hours (Sprague 1969). Tanks were aerated individually with air stones. Water temperature over the course of the experiment never exceeded 0.5° difference from 12°C.

Fish were killed by overdose of anaesthetic (200 $mg \cdot L^{-1}$ tricaine methane-sulfonate). Blood was collected by caudal vein puncture using 26 gauge needles and 5 mL plastic syringes (Becton Dickinson & Co., Lincoln Park NJ) and transferred into heparinized Vacutainer tubes (Becton Dickinson). Heparinized whole blood was stored at -80°C for nucleic acid extraction and analysis. After blood sampling, liver and muscle were removed and frozen at -80°C until nucleic acid extraction and analysis.

4.2.2 Experiment 2

Growth was examined in juvenile rainbow trout as part of a methyl mercury bioaccumulation experiment (chapter 2). Juvenile rainbow trout were lightly anaesthetized (tricaine methanesulfonate at 35 mg/L, with 150 mmol/L NaCl to minimize osmotic and handling stress), weighed, measured, tagged with unique fin clips and transferred into a 20 L glass aquarium. Eight fish were placed in each of eight aquaria, giving 64

fish in four tanks of each of two treatments (dietary MeHg-exposed and controls). Tanks were fed rations of 5, 15, 25, and 35 $mg \cdot g^{-1}$ body weight for either control food (Martin Feed Mills, Elmira, ON) or methyl mercury treated food (24 $\mu g \cdot g^{-1}$). Fish were fed for 20 days, with no adjustment of ration for growth over this period. Two fish developed fin rot in one control tank and were removed after 5 days. Subsequent ration to the tank was corrected for the loss of these fish. No mortalities or overt signs of toxicity were observed in the remaining fish for the duration of the experiment. Sampling was undertaken as in experiment 5, with the addition of the collection of a blood smear for each fish.

4.2.3 Blood Smears

Ten µL of blood was added to the surface of a microscope slide, diluted 500-fold in isotonic saline containing 5 $mmol \cdot L^{-1}$ EDTA, and spread evenly across the slide to distribute the erythrocytes. Smears were air dried, fixed in absolute methanol for 1 min, and redried. Dry fixed smears were immersed in Camco Quick Stain II (Baxter Scientific, McGraw Park, IL) (a buffered combination Wright-Giemsa stain) for 1-2 min and rinsed for 1 min in high purity water. Smears were stored without coverslips.

4.2.4 Nucleic Acid Analysis

The fluorometric methods for nucleic acid analysis are generally considered the most sensitive of those available (Dortch et al. 1983, Bulow 1987). The most widely used of these methods is that of Karsten and Wollenberger (1972), which uses the fluorescent dye ethidium bromide (EB) for detection of RNA and DNA. EB intercalates between the base pairs of double-stranded regions of nucleic acids (including single-stranded RNA which invariably has regions where it folds back on itself resulting in double-stranded regions). To be able to distinguish between fluorescence due to RNA and DNA complexes with EB, the method uses ribonuclease enzyme (RNase) to degrade RNA, giving a total nucleic acid (TNA) fluorescence prior to RNASE digestion, and a DNA fluorescence reading after RNase addition. The RNA value is given by

subtraction.

The time required to perform the Karsten and Wollenberger assay was restrictive, largely because of the requirement for RNASE digestion. Therefore, the method of Clemmeson (1988), which used two fluorochromes, EB, which is not specific to RNA or DNA, and bisbenzimidazole (Hoechst 55238, Sigma, St. Louis MO) (bB), which only fluoresces with DNA. Although two runs on the fluorometer are required, no time-consuming incubations are necessary. This method was the one chosen for subsequent growth experiments, and the specific details of the assay are provided below.

In experiment 5, approximately 50 mg of liver or muscle was placed in a 16×150 mm disposable borosilicate glass test tube (VWR Scientific, Toronto, ON) and homogenized in 2.5 mL of Tris buffer at high speed using a Polytron homogenizer (Brinkmann Instruments, Toronto, ON). Homogenates were mixed by vortex for 1 min with 10 mL of a 25% phenol and 75% chloroform solution. The phenol was prepared from reagent grade phenol saturated with Tris buffer and containing 0.1% 8-hydroxyquinoline as an antioxidant. The Tris-saturated phenol was frozen at -20° C and thawed as needed. Phenol was mixed with chloroform and allowed to separate from the aqueous Tris phase. The addition of chloroform to phenol causes loss of water/buffer from the buffer-saturated phenol. The phases form a fine emulsion and have to be allowed to fully separate or some of the buffer will partition to the sample homogenate, diluting the sample and lowering the final nucleic acid concentration by as much as 20%. Although most phenol-chloroform extractions use a 50:50 solution, the more dilute 25:75 mixture of phenol-chloroform was less viscous and separated better, giving a cleaner aqueous phase and smaller interphase.

Preparation of whole blood for nucleic acid analysis was considerably simpler than the preparation of liver or muscle. Blood (10 µL) was haemolysed at room temperature with 90 µL of 5 mM EDTA in MilliQ water in a 1.5 mL polypropylene microcentrifuge tube. Three hundred µL of Tris was added to the haemolysed and mixed briefly before the addition of 1.0 mL of phenol-chloroform solution. The

microcentrifuge tube was mixed by vortex and centrifuged for 3 minutes at 14,000 rpm in an Eppendorf 5415 benchtop microcentrifuge to separate the phases.

RNA concentrations were determined using a double fluorochrome method adapted from Clemmeson (1988). The major change in the method concerns the differential fluorescence of nucleic acid complexes with the fluorochrome. Dutton et al. (1995) found that fluorometric assay of nucleic acids requires careful optimization. Fluorochromes interact physically with nucleic acids; the failure to recognize this can lead to considerable error in nucleic acid determinations (Dutton et al. 1995).

For DNA analysis, 50 µL of the nucleic-acid- containing aqueous phase was added to wells in a 96 well plastic culture plate. Four "blank" wells per plate, containing 50 µL of Tris buffer, were extracted in the same fashion as the samples. Five wells containing 50 µL of DNA standard were also analysed per plate. After adding 25 µL of bB solution (2.5 $\mu g \cdot mL^{-1}$ in MilliQ water; made fresh from a 1 $mg \cdot mL^{-1}$ stock solution) to each well, the culture plate was tapped to mix the wells, and fluorescence was read using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. This procedure was repeated until fluorescence stopped increasing; initial range finding tests were used so that optimal fluorescence was reached in approximately four plate scans. Each scan was saved as a text file and the files were processed later in a spreadsheet.

TNA analysis was similar, although the sample volume was increased to 100 µL per well to compensate for the lower fluorescence yield of EB-nucleic acid complexes. DNA was used for TNA standard curves. The optimal fluorescence for a sample was converted into units of DNA using a DNA fluorescence standard curve for bB. These DNA values were then converted back to optimized fluorescence values, but for a DNA-EB standard curve. The fluorescence for the amount of DNA in the sample (obtained from bB fluorescence) was subtracted from total TNA fluorescence to give fluorescence due to EB-RNA binding. However, because these fluorescence values were in µg of DNA, they were converted to µg RNA by dividing by 0.46 (Karsten and Wollenberger 1972).

4.2.5 Data Analysis

In experiment 5, growth rates for individual fish were calculated according to the relative growth formula used in chapter 2. RNA concentrations in tissues were regressed against growth rate to assess the relationship between these variables. The regressions were calculated in Quattro Pro 6.0 for windows (Novell Corp. Orem UT).

In experiment 2, blood smears were analysed by counting the cells in five fields of view at 200x magnification. The number of immature erythrocytes was determined by a combination of shape and staining characteristics. Round cells with large nuclei and purple-staining granular cytoplasm were easily scored as immature cells. Mature cells were also easily distinguished by their distinctive oval shape, small oval pink nucleus, and clear pinky-yellow cytoplasm. Cells that were partially mature were scored as either immature or mature depending on the degree to which these characteristics were observed. If the coefficient of variation (standard deviation/mean×100) for the five counted fields exceeded 10%, additional fields were counted until the coefficient of variation was acceptable. The average percentage of reticulocytes for all blood smears were then regressed against the corresponding growth rates to assess the relationship between the variables.

4.3 Results

4.3.1 Comparison of RNA Concentrations in Liver, Muscle, and Blood

RNA concentrations in liver, muscle, and blood displayed a linear response to growth rate (Fig. 4.1). Blood and muscle RNA levels were low for fish that were starved and losing weight, and increased with increased growth rate. Under conditions of weight loss, blood RNA levels were most stable of the three tissues examined. Muscle RNA levels, although more variable under conditions of weight loss, were less variable in growing fish than blood RNA (Fig. 4.1). In contrast, liver RNA levels were elevated after 21 days of starvation, and were inversely related to growth rate for positive growth rates.

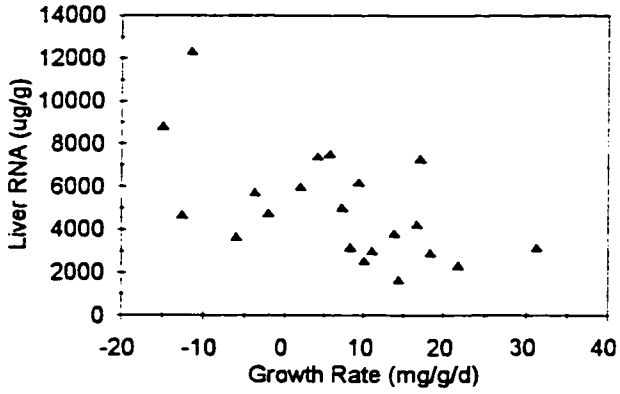
4.3.2 Reticulocyte Enumeration on Whole Blood Smears

The presence of immature erythrocytes in peripheral blood is easily detectable under Wright-Giemsa staining. With approximately 500-fold dilution, 100 µL of diluted blood spread over a standard microscope slide provides approximately 100 cells per field at 200x magnification. Two counts, one for the total number of erythrocytes, and one for the number of reticulocytes, are conducted for each field of view. By counting at least five fields, an average percentage of reticulocytes in the blood can be easily determined.

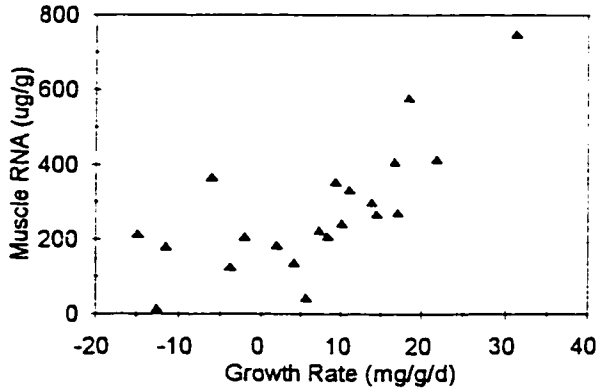
Since this technique was incorporated into an experiment that included MeHg exposure (chapter 2), the effect of MeHg on growth had first to be compared. As seen in Fig. 4.2, the growth yield per unit ration was essentially the same, although several MeHg-exposed fish showed considerable loss of weight over the course of the experiment. Whether this was due to Hg toxicity or due to the development of a feeding hierarchy in the tanks is not known. Nonetheless, it seems reasonable to pool the reticulocyte counts for both controls and MeHg-exposed fish based on the overall growth pattern for the two treatments.

The reticulocyte count as a function of growth rate for the pooled data is presented in Fig. 4.3. The regression equation for '% reticulocytes' in blood as a function of growth rate was; (% reticulocytes) = 0.548(Growth rate ($mg \cdot g^{-1} \cdot d^{-1}$)) + 3.305 ($r^2$=0.720).

a)



b)



c)

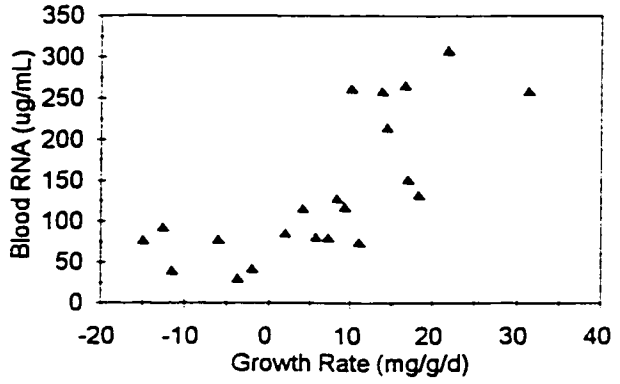
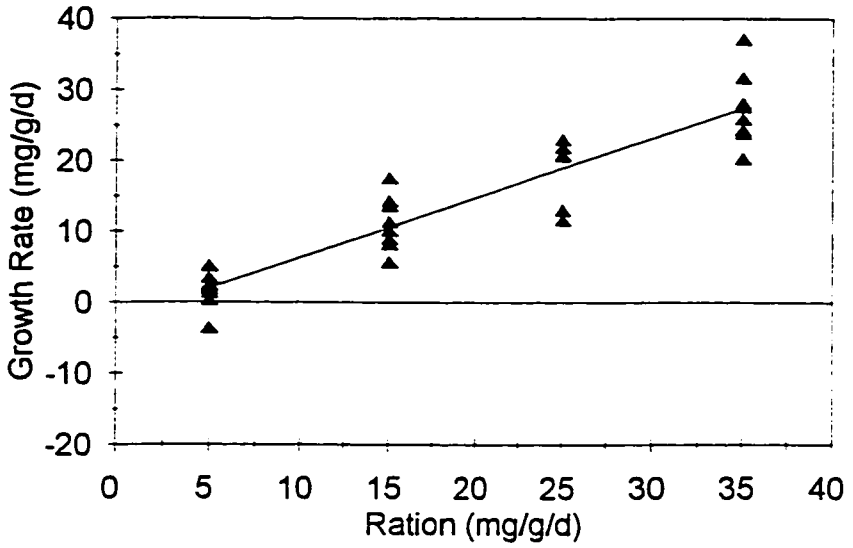


Fig. 4.1 RNA concentrations as a function of growth for liver (a), muscle (b), and blood (c). Regressions of (R) on growth rate (G) were: R= -124(G) + 5912 (r^2=0.335) (liver); R=10.1(G) + 212 (r^2=0.514) (muscle); R=5.45(G) + 103 (r^2=0.566) (blood).

a)



b)

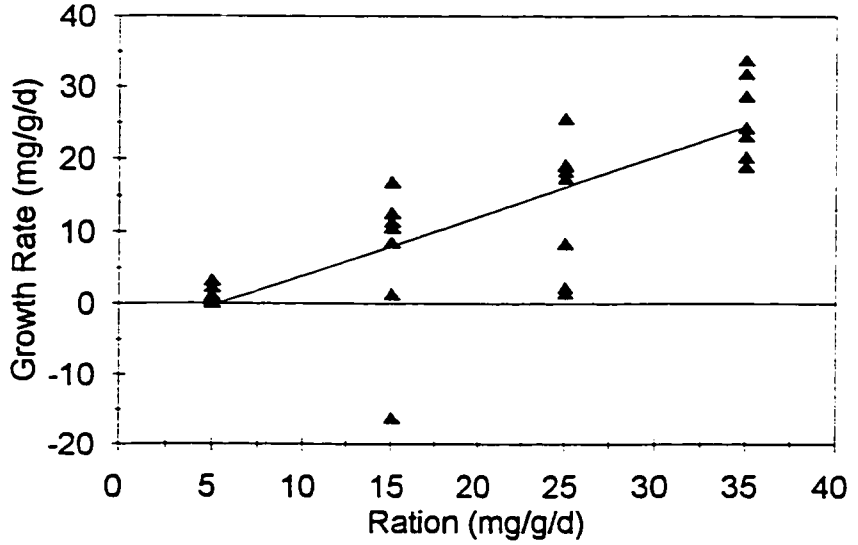


Fig. 4.2. Growth yield of fish for (a) control food and (b) MeHg-spiked food. Individual data points and regression lines are plotted. Regression equations for growth rate (G)($mg \cdot g^{-1} \cdot d^{-1}$) as a function of ration (R) ($mg \cdot g^{-1} \cdot d^{-1}$) were: G=0.847(R) - 2.103 (r^2=0.860) (controls); and G= 0.822(R) - 4.30 (r^2=0.632).

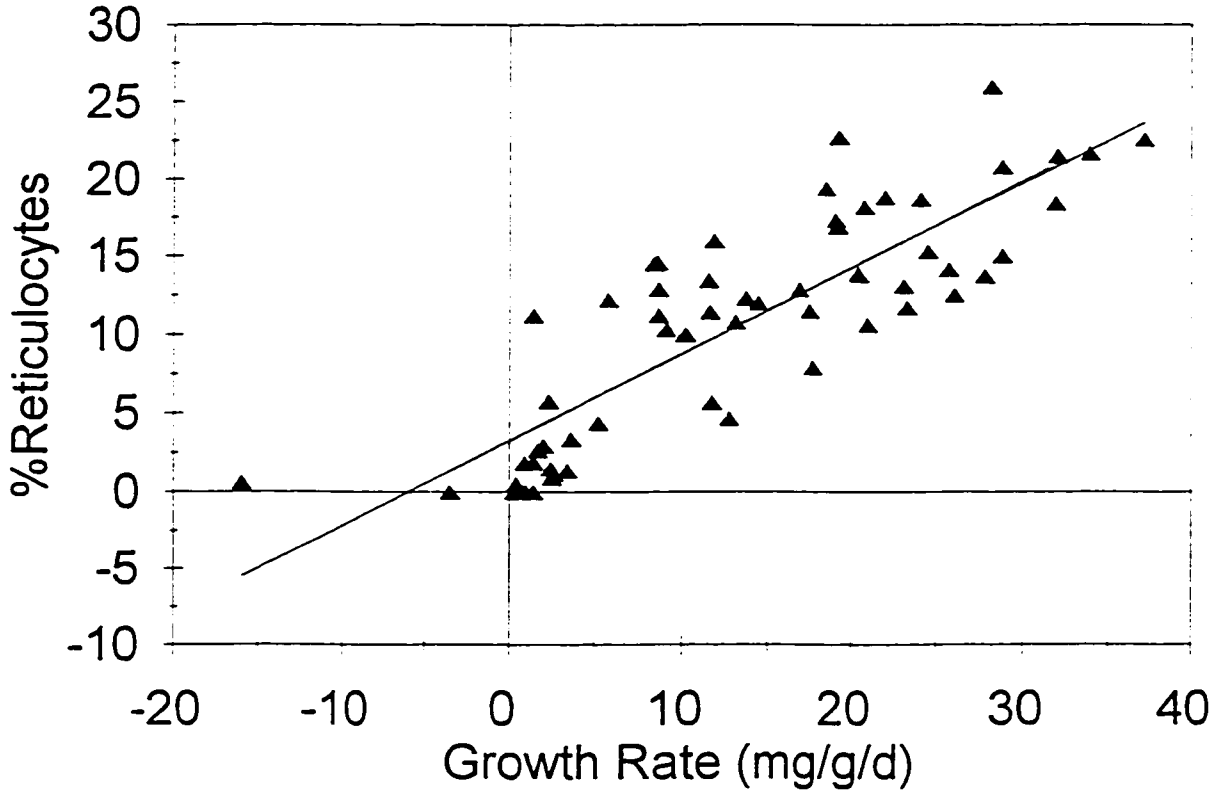


Fig. 4.3 Plot of % reticulocytes as a function of growth rate for control and MeHg-fed fish in experiment 2. Total sample size was 61 fish. Regression line plotted in addition to data points.

4.4 Discussion

The findings for liver nucleic acids are interesting; they suggest that in conjunction with either blood or muscle nucleic acid analysis, liver nucleic acids provide a more complete picture of growth. The high levels of RNA found in the liver of fish starved for 21 days are consistent with other reports for salmonids. McMillan and Houlihan (1992) found that protein synthesis in rainbow trout liver was elevated after 6 days of fasting, while muscle protein synthesis was negligible. They concluded that "survivability" of fish is attributable to the "white muscle-liver axis", whereby amino acids from muscle are utilized by liver to maintain critical body functions. In relation to this, Loughna and Goldspink (1984) found that a decrease in protein synthesis in fasting rainbow trout corresponded with both a reduction in muscle RNA content and an increase in muscle protein degradation in fish starved for up to 56 days.

In terms of data quality, muscle would seem to be the most suitable tissue for use in RNA analysis. However, other considerations contribute to the value of any technique. For example, the preparation of tissue is important from the standpoint of cost. Muscle is a structural tissue and must be physically destroyed before RNA extraction can occur. In contrast, blood RNA analysis requires the destruction only of erythrocyte membranes (it is primarily erythrocyte nucleic acids that are of concern), which can be achieved simply by haemolysing the blood in hypotonic solution. The nucleic acids are easily extracted from this matrix. A second consideration concerns the use of non-destructive sampling techniques. Blood is easily obtained from fish without killing them. Muscle sample collection is more invasive, and although biopsy techniques have existed for many years (Lockhart et al. 1972), blood sampling is the best means of non-destructive sampling. In appropriate situations (eg. small lakes where fish can be marked and resampled), this provides some potential for the use of repeated measures analysis of the same individual over time. Where repeated measures analysis is not viable, the technical considerations of blood analysis are still considerable relative to the sampling of muscle or other organs or tissues. The major obstacle to the

sampling of blood for nucleic acid analysis, and hence growth rate estimation, is simply that there is no precedent for it.

There are no direct reports of prior research on the quantification of erythropoiesis as an indicator of growth, although there is limited anecdotal corroboration. Bridges et al. (1976) observed an increase in the percentage of immature erythrocytes (10 ± 4% of the circulating erythrocytes) in winter flounder sampled during the warmest (active growing) period in June. The rest of the year, immature erythrocytes accounted for between 0 and 3% of the erythrocytes. This suggests that erythropoiesis is stimulated in conjunction with periods of high growth. Härdig and Höglund (1984) examined seasonal haematological changes in Baltic salmon (*Salmo salar*), and found elevated levels (15%) in April to June relative to 7-10% in the fall and winter months. Some researchers have discussed immature erythrocytes in passing (Williams and Warner 1976, Setka and Cech 1994), while others equate an increase in the total number of erythrocytes to an increase in erythropoiesis (Murad et al. 1990) without consideration of immature cell numbers. Surprisingly, as early as 1978 it was proposed that immature erythrocytes "have been largely ignored although they might be particularly useful indices of toxic stress" (Christensen et al. 1978).

Erythropoiesis in fish has been examined primarily with respect to three factors, blood loss, starvation, and hypoxia. **Bleeding** (10 to 15% of blood volume) has been reported to stimulate erythropoiesis, with increases in immature erythrocytes in peripheral blood (Lane 1979, Lane and Tharpe 1980); **severe bleeding** (60-75% of blood volume) caused an increase in immature erythrocytes from 1-2% to 36-55% (Schindler and deVries 1986). **Starvation** is a known erythropoietic depressant (Lane and Tharp 1980), but in conjunction with blood loss, starved fish undergo a more vigorous erythropoietic response than fed fish (Lane and Tharp 1980). **Hypoxic effects** on erythropoiesis are not as dramatic as for bleeding, with documented increases in immature erythrocytes from 0.83 to 2.32% of the total erythrocyte population after brief but severe hypoxia (Murad et al. 1990).

All four of the known erythropoietic factors (blood loss, temperature, hypoxia, and

starvation) can be linked to growth in fish. The findings of Bridges et al. (1976) implicate temperature in erythropoietic stimulation, which corroborates the literature on erythropoiesis. Of course, temperature can be linked to both hypoxia and growth, since as temperature increases in spring, the oxygen carrying capacity of water decreases, from 13 $mg \cdot L^{-1}$ at 0°C to 10 $mg \cdot L^{-1}$ at 15°C and 8.7 $mg \cdot L^{-1}$ at 22°C (Wetzel 1983). Furthermore, with the increasing temperature due to more direct solar radiation on north temperate regions in spring, phytoplankton productivity increases, giving spring and summer maxima for various algal groups (Wetzel 1983). Since this is followed by increases in growth for young planktivorous fish, it becomes a question of determining the proximate cause for erythropoietic stimulation. Temperature and oxygen supply (and demand) will be temporally overlapping with growth, so it is not surprising that their effects have not been isolated.

Regardless of the interactions between these erythropoietic stimuli, it is reasonable to consider growth as an agent with components due to blood loss and hypoxia. This may be particularly true for young fish undergoing rapid growth. Okeyo and Hassler (1985) monitored growth of smallmouth bass in a northern California reservoir and documented weight changes of 222 $mg \cdot g^{-1} \cdot d^{-1}$ (total weight change from 0.5 g to 8.5 g over 73 days). This sort of dramatic weight increase probably represents a significant constraint on physiological oxygen supply. In fact, such rapid growth could be equated to situations where some degree of blood loss has occurred. It is therefore not unreasonable to suggest that rapid growth might be an erythropoietic stimulant. In the experiments of this chapter, weight gains were on the order of 0 to 40 $mg \cdot g^{-1} \cdot d^{-1}$ and the percentage of immature erythrocytes varied between 0 and 25% of the circulating erythrocytes (Fig. 4.3). Growth rates like those observed by Okeyo and Hassler (1985) are considerably higher; the percentage of immature erythrocytes could be of obvious interest for the use of this method.

The good relationship between blood RNA concentrations and growth in experiment 5 suggested that erythropoiesis might in fact be a suitable means for detecting growth in fish. However, the fluorometric NA analytical techniques require

specialized equipment which is ill-suited for field situations. In contrast, the use of stained blood smears for estimating fish growth requires only a compound microscope and a couple of Coplin jars, and is easily adapted for field research. Because of its simplicity and low cost, this technique has great potential for fisheries ecology and ecotoxicology.

Although there is no doubt that immature and mature erythrocyte populations can be distinguished by flow cytometry, as has already been demonstrated with human reticulocytes (Tanke et al. 1980), this approach has not yet been used in fisheries research, where flow cytometry studies have focussed on white blood cells (Morgan et al. 1993). Flow cytometry has been used to examine the status of fish erythrocytes after repeated bleeding, which was found to increase the proportion of cells in G_2 /M phase of the cell cycle (Kendall et al. 1992, Fisher et al. 1994). This is thought to indicate the stimulation of erythropoiesis, and it would appear that flow cytometry should be the definitive tool for verifying the value of reticulocyte enumeration as a growth analysis tool. As with fluorometric NA analysis, the capital costs for flow cytometry are considerable; this technique is probably less applicable for general use than the simple blood smear technology. However, from a statistical basis, flow cytometry should be better than microscopy, as it is possible to count thousands of cells per sample without difficulty, in comparison with the several hundred cells which can be easily enumerated manually.

The need for suitable methods for estimating recent growth in fish is well-documented (Houlihan et al. 1993). To be routinely useful, a method should provide useable data at minimal expense in the widest variety of settings. The initial use of blood smear reticulocyte counts seems to meet these requirements, and therefore should be of considerable value. Where available, flow cytometric analysis could be of great utility, but in remote locations or if a flow cytometer is unavailable, the microscopic enumeration technique should be more reliable. Regardless of the technical approach chosen, this technique requires further testing in the field.

5.0 General Discussion: Issues of Methyl Mercury Bioaccumulation

The bioaccumulation of Hg in fish has aspects of both practical and theoretical interest. Mercury concentrations in fish represent a widespread potential human health problem (Clarkson 1990, Mason et al. 1995, Weiner and Stokes 1990, Lindqvist et al. 1991), although even this perception is not without controversy (Eyl 1973). Regardless of the perspective held on human health, all indications point to increasing levels of Hg in the environment, and in particular, aquatic life, with trends in the northern hemisphere showing dramatic increases in the number of fish consumption advisories (Watras et al. 1994, Lindqvist et al. 1991, Anonymous 1996).

Hg bioaccumulation and biomagnification are responsible for the threat to human health, in that once Hg is in an aquatic ecosystem, these phenomena are inevitable. Therefore, both from a practical human health perspective and as an overall indication of environmental health, the study of Hg bioaccumulation by fish can be of considerable value. Bioaccumulation studies can provide: (1) **monitoring information** - what are the temporal trends in a lake? Is the problem changing over time? (Borgmann and Whittle 1992); (2) **spatial comparisons** - there is a need to identify problem lakes and regions (Watras et al. 1994, Cabana et al. 1994). In turn, the spatial trends can often be linked to factors such as regional depositional trends or characteristics of regional watershed types. However, because of the complexity of aquatic ecosystems, the identification of key physiological mechanisms underlying bioaccumulation are best studied under controlled experimental conditions in the laboratory, with subsequent corroborative research being conducted in field situations. An alternative approach to understanding mechanisms of Hg accumulation is via mathematical modelling. At present, there is considerable focus on the modelling of Hg in fish and aquatic systems.

The use of modelling in the study of Hg bioaccumulation arises for several reasons, not the least of which is cost. In a modelling exercise, real or fabricated data can be inputted to the model and various scenarios can be examined without the expense of physical sampling and analysis. However, the value of a model's output is

dependent on the characteristics of the model and the quality of the data. In this sense, models can be characterized in several ways, the most fundamental distinction being between descriptive and explanatory models (Bossel 1994). Descriptive models essentially mimic systems without concern over the underlying concepts, while explanatory models have a structure that corresponds closely to that of the real system. In explanatory models, the system parameters can often be measured directly or are available from literature sources (Bossel 1994).

In Hg bioaccumulation models, there is some question as to whether the currently popular models such as the bioenergetics-based model of Norstrom et al. (1976) are truly explanatory or just descriptive. Rodgers (1994) considers the bioenergetics-based models useful for *describing* Hg accumulation (italics mine). This, of course, is already available from monitoring data, and given that model output must be corroborated with real-world data anyway, the value of such modelling approaches is questionable.

The bioenergetics-based models of Hg bioaccumulation (Norstrom et al. 1976, Harris and Snodgrass 1992, Rodgers 1994) are extremely detailed, complicated, and conceptually inaccessible. In addition, these models are heavily parameterized (Hamelink and Spacie 1977); there are 15 parameters in the model, many of which cannot be easily measured and must therefore be approximated or derived from literature values. Rodgers (1994) considers it serendipitous that Norstrom et al. (1976) were able to fit their model so well, given that their original data for aqueous Hg are probably 10-100 times higher than actual values (due to analytical limitations of the time period). Of course, the fit of the Norstrom model was not serendipitous at all, since the other model parameters were manipulated to ensure correspondence with actual data. The underlying tenor of the bioenergetics modelling approach is clearly stated by Norstrom et al. (1976) in the statement that "These points do not arise from a precise understanding of metabolic processes, but they appear to be an adequate *description* of fish bioenergetics in terms of providing a basis for writing the *required mathematical equations*." (italics mine) Although Norstrom et al. (1976) consider that "for a pollutant

bioaccumulation model to have general and easy applicability, it must be able to use information that can be readily derived from relatively unsophisticated field data", their own model is far from such a model.

There are indications that several aspects of the bioenergetics-based models of Hg bioaccumulation are clearly in error. For example, both in this thesis (chapter 3) and in the published literature (Headon et al. 1996), data suggest that the elimination component of these models overestimates Hg elimination. Therefore, if the model outputs accurately simulate real data (Norstrom et al. 1976, Harris and Snodgrass 1994, Rodgers 1994), the actual values of the uptake component of bioaccumulation must be overestimated to account for this. If the findings of Headon et al. (1996) are valid, actual elimination is up to 15-fold slower than the estimates used in the bioenergetics models. Therefore, uptake estimates must be overestimated by the same amount (by a factor of 15). Altering any or all of the ten parameters unique to the uptake equation could achieve this correction. Therefore, if the model outputs "fit" real data, they do so by "curve fitting", not necessarily by representation of real phenomena.

The currently available Hg accumulation models are so frequently used because the alternative, biomonitoring, is generally seen as too restrictive and expensive. However, until issues such as allometric elimination are clarified, it would appear that modelling is not as useful as the direct study and documentation of Hg bioaccumulation in aquatic systems. It seems logical that since models all ultimately require physical data for comparison with actual trends, it would be better to simply find biomonitoring approaches that are simple and inexpensive.

One alternative approach involves the use of young-of-year or, in general, early age classes of top predator fish species. The use of immature fish as biomonitors of Hg exposure and accumulation has been documented elsewhere (Parks et al. 1991, Slotton et al. 1995), and is substantiated on several grounds. First, young top predator fish can accumulate Hg quite rapidly. Lakes can be distinguished on the basis of early life stage Hg accumulation, with some lakes showing the greatest accumulation in the youngest fish, as seen in Ottawa River perch (Norstrom et al. 1976), lake tout from Lake Muskoka

(MacCrimmon et al. 1983), or the pike of Lake Kyrksjön (Fagerström et al. 1974). At the other extreme, lakes are characterized by a slow Hg accumulation pattern, as seen in the lake trout of Tadenac and Bella Lakes (MacCrimmon et al. 1983). In Bella Lake, Hg accumulation is slow over all age classes. The Hg accumulation trends in Tadenac Lake trout are between those of Muskoka and Bella, with a low accumulation rate up to age five, after which accumulation increases dramatically (due to a shift in diet from invertebrates to smelt). The accumulation rate for Tadenac trout actually parallels that of Muskoka after age five, but because of low accumulation rates at earlier ages, the Hg levels in Tadenac trout never equal those of Muskoka trout of the same age. The differences in accumulation profiles for these lakes could, therefore, be discerned from sampling immature fish.

In this comparison, the differences in accumulation in fish from these two types of lakes is probably not specifically due to food web structure, although the nature of trophic Hg transfer can also affect the MeHg dose received by top predators (Cabana et al. 1994). In this discussion, since the immature trout feed primarily on invertebrates regardless of lake, the rapid accumulation in fish from the "rapid accumulation" lakes is likely a reflection of Hg supply to the lake or supply of MeHg to the base of the food web (via higher lakewide Hg methylation rate). Watras and Bloom (1992) believe that Hg accumulation rates are dictated primarily by supply, so the higher accumulation rate in L. Muskoka fish probably reflects higher Hg concentrations in their diet. This in turn suggests that, in addition to young fish, aquatic invertebrates could be used to identify "problem" lakes or changes in Hg accumulation trends over time. Both types of survey could be accomplished with considerably less expense than adult fish surveys. There can be no doubt that there is value in studying adult fish, and in very large lakes, capturing of adults in spawning migrations can be easier than capturing juveniles, which are effectively "needles in a haystack". However, in the great majority of lakes (smaller lakes and streams) the use of minnow traps, seining, or electroshocking for collection of juveniles could provide inexpensive, viable alternatives for sampling fish populations in the study of Hg accumulation. It is clear from the literature that effort toward the

sampling of immature fish has been decidedly overlooked.

There are several advantages to the study of young fish: (1) Sample size can be larger than for older fish, providing better data; (2) Reproductive considerations can be ignored; (3) Due to the young age of the fish (one year or less), the exposure history can be ignored, as dietary shifts are minimal over this period; (4) The small size and natural dosing of young fish should facilitate depuration estimates (to clarify natural elimination estimates once and for all); and (5) because of the extremely high growth rates in young-of-year fish, histological reticulocyte counts should be useful for estimating growth rates (for establishing growth-bioaccumulation relationships, a logical extension of this research).

The issues of point (4) require clarification. The relatively low dosing rates (in comparison to the experiments in this thesis) mean that redistribution issues in field depuration estimates with young fish are less likely to be an issue than in most laboratory-based depuration studies. Secondly, the small size of the fish means that they can be either caged on site or easily transported back to the laboratory for depuration studies. Sequential muscle biopsies (Lockhart et al. 1972, Lettner and Isley 1994) could offer a method for time-course studies of MeHg dynamics in the "slow-clearing" compartment of Hg storage in fish.

The possibility of using minnow species for Hg biomonitoring has also been addressed to some degree (Peterson et al. 1996), but is probably not as useful as sampling young age classes of top predator species. Minnows achieve ultimate body sizes that are considerably smaller than top predators. Therefore, the growth rates observed at early ages will be expected to be proportionately less than for the larger fish. Since it is the rapid early growth that is suggested to be the underlying advantage to sampling immature fish, the use of minnows runs counter to this proposition. It remains to be seen whether minnows can be used to advantage for Hg biomonitoring.

Relative to the other issues studied and discussed (or skirted) in this thesis, the question of Se-Hg interactions and the potential for ameliorating Hg bioaccumulation in fish is at present, of purely academic interest. The complexities of the interactions

(Cuvin-Aralar and Furness 1991) and the problems inherent in adding Se to aquatic systems (Lindqvist et al. 1991) justifiably preclude the use of this technique for Hg amelioration in the near future. The data reported on Se-Hg interactions in this thesis, although sparse, suggest a mechanism of depuration which has previously been overlooked (Bjornberg et al. 1989, Cuvin-Aralar and Furness 1991, Kelso et al. 1993). In conjunction with the growth-related aspects of this research and other recently published research (Mason et al. 1995, 1996) it would appear that even after twenty-five years of research, considerably more research is needed before it can be said that Hg bioaccumulation in fish is well understood.

6.0 References

Adams, S.M. and R.B. McLean. 1985. Estimation of largemouth bass, *Micropterus salmoides* Lacepe, growth using the liver somatic index and physiological variables. *J. Fish Biol.* 26: 111-126.

Andersson, I., H. Parkman, and A. Jernelov. 1990. The role of sediments as sink or source for environmental contaminants–a case study of mercury and chlorinated organic compounds. Limnologica 20: 347-359.

Anonymous. 1996. Fish advisories rise; mercury a major cause. *Environ. Sci. Technol.* 30: 331A.

ASTM 1978. Criteria and rationale for decision making in aquatic hazard evaluation (third draft). In: J. Cairns, K.L. Dickson, A.W. Maki (Eds.). *Estimating the hazard of chemical substances to aquatic life.* ASTM STP 657, Philadelphia. PA. pp. 202-237.

Barber, M.C., L.A. Suárez, and R.R. Lassiter. 1991. Modelling bioaccumulation of organic pollutants in fish with an application to PCBs in Lake Ontario salmonids. *Can. J. Fish. Aquat. Sci.* 48: 318-337.

Barron, M.C. 1995. Bioaccumulation and bioconcentration in aquatic organisms. In: *Handbook of Ecotoxicology.* D.J. Hoffman, B.A. Rattner, G.A. Burton, Jr., and J. Cairns Jr. (Eds.). Lewis Publishers, Boca Raton, pp. 652-666.

Björnberg, A. L. Hakanson, and K. Lundbergh. 1988. A theory on the mechanisms regulating the bioavailability of mercury in natural waters. Environ. Pollut. 49: 53-61.

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

Borgmann, U. and D.M. Whittle. 1992. Bioenergetics and PCB, DDE, and mercury dynamics in Lake Ontario lake trout (*Salvelinus namaycush*): a model based on surveillance data. *Can. J. Fish. Aquat. Sci.* 49: 1086-1096.

Bossel, H. 1994. *Modeling and Simulation.* A.K. Peters, Wellesley, MA. 484 pp.

Boudou, A. and F. Ribyere. 1983. Contamination of aquatic biocenoses by mercury compounds: an experimental ecotoxicological approach. In: J.O. Nriagu (Ed.). *Aquatic Toxicology.* Wiley-Interscience, N.Y. pp. 73-116.

Brett, J.R. and T.D.D. Groves. 1979. Physiological energetics. In: *Fish Physiology, Vol. VIII.* Academic Press, N.Y. pp. 280-352.

Bridges, D.W., J.J. Cech, Jr., and D.N. Pedro. 1976. Seasonal hematological changes in winter flounder, *Pseudopleuronectes americanus. Trans. Amer. Fish. Soc.* 106: 596-600.

Bulow, F.J. 1987. RNA-DNA ratios as indicators of growth in fish: a review. In: R.C. Summerfelt and G.E. Hall (Eds.), The Age and Growth of Fish. The Iowa University Press, Ames, Iowa. pp. 45-64.

Cabana, G., A. Tremblay, J. Kalff, and J.B. Rasmussen. 1994. Pelagic food chain structure in Ontario lakes: a determinant of mercury levels in lake trout (*Salvelinus namaycush*). *Can. J. Fish. Aquat. Sci.* 51: 381-389.

Canfield, P.J., N. Quartararo, D.L. Griffin, G.N. Tsoukalas, and S.E. Cocaro. 1994. Haematological and biochemical reference values for captive Australian snapper, *Pagrus auratus. J. Fish Biol.* 44: 849-856.

Carty, A.J. and S.F. Malone. 1979. The chemistry of mercury in biological systems. In: J.O. Nriagu (Ed.). The Biogeochemistry of Mercury in the Environment. Elsevier/ North Holland Biomedical Press, Amsterdm, pp. 433-479.

Chau, Y.K., P.T.S. Wong, B.A. Silverberg, P.L. Luxon, and G.A. Bengert. 1976. Methylation of selenium in the aquatic environment. Science. 192: 1130-1131.

Christensen, G.M., J.T. Fiandt, and B.A. Poeschel. 1978. Cells, proteins, and certain physical-chemical properties of brook trout (*Salvelinus fontinalis*) blood. *J. Fish Biol.* 12: 51-60.

Clarkson, T.W. 1973. Excretion and absorption of methyl mercury after polythiol resin treatment. *Arch. Environ. Health* 26: 173-176.

Clarkson, T.W. R. Hamada, and L. Amin-Zaki. 1984. Mercury. In: J.O. Nriagu (Ed.) *Changing Metal Cycles and Human Health.* Springer-Verlag, Berlin, pp. 285-309.

Clarkson, T.W. 1990. Human health risks from methylmercury in fish. *Environ. Toxicol. Chem.* 9: 957-961.

Clarkson, T.W. 1993. Molecular and ionic mimicry. *Ann. Rev. Toxicol. Pharmacol.* 32: 545-571.

Clarkson, T.W. 1995. The toxicology of mercury and its compounds. In: *Mercury Pollution. Integration and synthesis.* C.J. Watras and J.W. Huckabee (Eds.). Lewis Publishers, Boca Raton, pp. 621-628.

Clemmesen, C.M. 1988. A RNA and DNA fluorescence technique to evaluate the nutritional condition of individual marine fish larvae. *Meeresforsch.* 32: 134-143.

Craig, P.J. 1986. *Organometallic Compounds in the Environment.* Wiley, N.Y. 368 pp.

Cuvin-Aralar, M. L.A. and R.W. Furness. 1990. Tissue distribution of mercury and selenium in minnows *Phoxinus phoxinus*. *Bull. Environ. Contam. Toxicol.* 45: 775-782.

Cuvin-Aralar, M. L.A. and R.W. Furness. 1991. Mercury and selenium interaction: a review. *Ecotox. Environ. Safety* 21: 348-364.

Daniel, N., Ed. 1978. *McGraw-Hill Dictionary of Scientific and Technical Terms.* McGraw-Hill, New York.

Davies, I.M. and R. Russell. 1988. The influence of dissolved selenium compounds on the accumulation of inorganic and methylated mercury compounds from solution by the mussel *Mytilus edulis* and the plaice *Pleuronectes platessa*. *Sci. Total Environ.* 68: 197-205.

deFreitas, A.S.W., M.A.J. Gidney, A.E. McKinnon, and R.J. Norstrom. 1977. Factors affecting whole-body retention of methyl mercury in fish. Proc. 15th Annual Life Sciences Symposium at Richland, Washington. U.S. Energy Research and Development Administration. pp. 441-451.

Denton, J.E. and M.K. Yousef. 1976. Body composition and organ weights of rainbow trout, *Salmo gairdneri*. *J. Fish Biol.* 8: 489-499.

Doran, J.W. 1982. Microorganisms and the biological cycling of selenium. In: K.C. Marshall (Ed.) Adv. Microb. Ecol. Vol. 6, pp. 1-32.

Dortch, Q., T.L. Roberts, J.R. Clayton, Jr., and S.I. Ahmed. 1983. RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. *Mar. Ecol. Prog. Ser.* 13: 61-71.

Dutton, M.D., R.J. Varhol, and D.G. Dixon. 1995. Technical considerations of the analysis of nucleic acids with ethidium bromide. *Anal. Biochem.* 230: 353-355.

Eyl, T.B. 1973. Tempest in a teapot. In: *Mercury Poisoning: I.* E. Mayz (Ed.). MSS Information Corp., N.Y. pp. 115-119.

Fagerström, T., B. Åsell, and A. Jernelöv. 1974. Model for accumulation of methyl mercury in northern pike *Esox lucius*. *Oikos* 25: 14-20.

Fisher, S.K., C.E. Dallas, C. Jagoe, M.H. Smith, I.L. Brisbin, Jr., and R.K. Chesser. 1994. Sources of error associated with sample collection and preparation of nucleated blood cells for flow cytometric analysis. *Cell Biol. Toxicol.* 10: 145-153.

Fowler, S.W., J. La Posa, M. Heyraud, and W.C. Renfro. 1975. Effect of different radiotracer labelling techniques on radionuclide excretion from marine organisms. *Mar. Biol.* 30: 297-304.

Giblin, F.J. and E.J. Masaro. 1973. Pharmacodynamics of methyl mercury in the rainbow trout (*Salmo gairdneri*): tissue uptake, distribution and excretion. *Toxicol. Appl. Pharmacol.* 24: 81-91.

Glover, J., O. Levander, J. Parizek, and V. Vouk. 1979. Selenium. In: L. Friberg, G.F. Nordberg, and V.B. Vouk (Eds.). Handbook on the Toxicology of Metals. Elsevier/New Holland Medical Press. Amsterdam. pp. 555-578.

Gobas, F.A.P., X. Zhang, and R. Wells. 1993. Gastrointestinal magnification: the mechanism of biomagnification and food chain accumulation of organic chemicals. Environ. Sci. Technol. 27: 2855-2863.

Grau, A., S. Crespo, M.C. Sarasquette, and M.L. Gonzalez de Canales. 1992. The digestive tract of the amberjack (*Seriola dumerili*, Risso): a light and scanning electron microscope study. *J. Fish Biol.* 41: 287-303.

Hamelink, J.L. and A. Spacie. 1977. Fish and chemicals: the process of accumulation. *Ann. Rev. Pharmacol. Toxicol.* 17: 167-177.

Harris, R.C. and W.J. Snodgrass. 1993. Bioenergetic simulations of mercury uptake and retention in walleye (*Stizostedion vitreum*) and yellow perch (*Perca flavescens*). *Water Poll. Res. J. Can.* 28: 217-236.

Harrison, S.E. and J.F. Klaverkamp. 1980. Metal contamination in liver and muscle of northern pike (*Esox lucius*) and white sucker (*Catastomas commersoni*) and in sediments from lakes near the smelter at Flin Flon, Manitoba. Environ. Toxicol. Chem. 9: 941-956.

Headon, C.M., R.J. Hall, and G. Mierle. 1996. Dynamics of radiolabelled methylmercury in crayfish (*Orconectes virilis*). *Can. J. Fish. Aquat. Sci.* 53: 2862-2869.

Hoar, W.S. 1983. *General and Comparative Physiology.* Prentice-Hall, Englewood Cliffs, N.J. 851 pp.

Houlihan, D.F., E. Mathers, and A. Foster. 1993. Biochemical correlates of growth rate in fish. In: *Fish Ecophysiology.* J.C. Rankin and F.B. Jensen (Eds.). Chapman and Hall, London. pp. 45-71.

Iwata, H., T. Masukawa, H. Kito, and M. Hayashi. 1982. Degradation of methylmercury by selenium. *Life Sci.* 31: 859-866.

Jocelyn,P.C. 1972. *Biochemistry of the SH Group.* Acad. Press, London. 404 pp.

Kamler, E. 1992. *Early Life History of Fish. An Energetics Approach.* Chapman and Hall. London. 267 pp.

Karlson, U. and W.T. Frankenberger, Jr. 1990. Volatilization of selenium from agricultural evaporation pond sediments. Sci. Total Environ. 28: 119.

Karsten, U. and A. Wollenberger. 1972. Determination of DNA and RNA in homogenized cells and tissues by surface fluorometry. *Anal. Biochem.* 46: 135-148.

Keen, J.E., A.M. Calarco-Steele, and A.H. Houston. 1989. The circulating erythrocytes of rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 94A:699-711.

Kelso, J.R.M., K.M. Murphy, K.I. Adare, A.J. Niimi, and C.-J.C. Jackson. 1994. *Potential Mercury Amelioration Methods in Aquatic Ecosystems. Can. Tech. Rep. Fish. Aquat. Sci.* No. 1993. 20 pp.

Kendell, C., S. Valentino, A.B. Bodine, and C.A. Luer. 1992. Flow cytometric DNA analysis of nurse shark, *Ginglymostoma cirratum* (Bonaterre) and clearnose skate, *Raja eglanteria* (Bosc) peripheral red blood cells. *J. Fish Biol.* 41: 123-129.

Kuperman, B.I. and V.V. Kuz'mina. 1994. The ultrastructure of the intestinal epithelium in fishes with different types of feeding. *J. Fish Biol.* 44: 181-193.

Laarman, P.W., W.A. Willford, and J.R. Olson. 1976. Retention of mercury in the muscle of yellow perch (*Perca flavescens*) and rock bass (*Ambloplites rupestris*). *Trans. Amer. Fish. Soc.* 2: 296-300.

Lane, H.C. 1979. Some haematological responses of normal and splenectomized rainbow trout *Salmo gairdneri* to a 12% blood loss. *J. Fish Biol.* 14: 159-164.

Lane, H.C. 1980. The response of the haemoglobin system of fed and starved rainbow trout, *Salmo gairdneri* Richardson, to bleeding. *J. Fish Biol.* 16: 405-411.

Lane, H.C., J.W. Weaver, J.A. Benson, and H.A. Nichols. 1982. Some age related changes of adult rainbow trout, *Salmo gairdneri* Rich., peripheral erythrocytes separated by velocity sedimentation at unit gravity. *J. Fish Biol.* 21: 1-13.

Lane, H.C. and T.P. Tharp. 1980. Changes in the population of polyribosomal containing red cells of peripheral blood of rainbow trout, *Salmo gairdneri* Richardson, following starvation and bleeding. J. Fish. Biol. 17: 75-81.

Lanno, R.P. 1990. *The Chronic Toxicity of Thiocyanate to Rainbow Trout and Fathead Minnow.* Ph.D. Thesis, University of Waterloo. 78 pp.

Lemly, A.D. 1985. Toxicology of selenium in a freshwater reservoir. Implications for environmental hazard evaluation and safety. Ecotox. Environ. Safety 10: 314-338.

Lemly, A.D. and G.J. Smith. 1987. Aquatic cycling of selenium: implications for fish and wildlife. U.S. Fisheries and Wildlife Service leaflet 12. Washington, D.C., 10 pp.

Lettner, J.K. and J.J. Isley. 1994. A liver and muscle biopsy technique for electrophoretic evaluation of largemouth bass. *Prog. Fish-Culturist.* 56: 288-290.

Lindqvist, O. K. Johansson, M. Aastrup, A. Andersson, L. Bringmark, G. Hovsenius, L. Hakanson, A. Iverfeldt, M. Mieli, and B. Timm. 1991. Mercury in the Swedish environment - recent research on causes, consequences and corrective measures. Water Air Soil Pollut. 55: 1-261.

Lipkin, M. 1985. Growth and development of gastrointestinal cells. *Ann. Rev. Physiol.* 47: 175-197.

Lock, R.A.C. 1975. Uptake of methylmercury by aquatic organisms from water and food. In: *Sublethal Effects of Toxic Chemicals on Aquatic Animals*. J.H. Koeman and J.J.T.W.A. Strik (Eds.). Elsevier, Amsterdam. pp. 61-80.

Lockhart, W.L., J.F. Uthe, A.R. Kenney, and P.M. Mehrle. 1972. Methylmercury in northern pike (*Esox lucius*): distribution, elimination, and some biochemical characteristics of contaminated fish. *J. Fish. Res. Bd. Canada*, 29: 1519-1523.

Loughna, P.T. and G. Goldspink. 1984. The effects of starvation upon protein turnover in red and white myotomal muscle of rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* 23: 223-230.

MacCrimmon, H.R., C.D. Wren, and B.L. Gots. 1983. Mercury uptake by lake trout, *Salvelinus namaycush*, relative to age, growth, and diet in Tadenac Lake with comparative data from other precambrian shield lakes. *Can. J. Fish. Aquat. Sci.* 40: 114-120.

MacLeod, M.G. 1978. Effects of salinity and starvation on the alimentary canal anatomy of the rainbow trout *Salmo gairdneri* Richardson. *J. Fish Biol.* 12: 71-79.

Magos, L. 1987. The absorption, distribution, and excretion of methyl mercury. In: *The Toxicity of Methyl Mercury*. C.U. Eccles and Z. Annau (Eds.). Johns Hopkins University Press. Baltimore. pp. 24-44.

Martin, R. 1996. *Gel Electrophoresis: Nucleic Acids*. Bios Scientific Publishers. Oxford. pp. 90-91.

Mason, R.P., J.R. Reinfelder, and F.M.M. Morel. 1995. Bioaccumulation of mercury and methylmercury. *Wat. Air Soil Poll.* 80: 915-921.

Mason, R.P., J.R. Reinfelder, and F.M.M. Morel. 1996. Uptake, toxicity, and trophic transfer of mercury in a coastal diatom. *Environ. Sci. Technol.* 30: 1835-1845.

McBride, B.C. and R.S. Wolfe. 1971. Biosynthesis of dimethylarsine by methanobacterium. *Biochem.* 10: 4312-4317.

McKim, J.M., G.F. Olson, G.W. Holcombe, and E.P. Hunt. 1976. Long-term effects of methylmercuric chloride on three generations of brook trout (*Salvelinus fontinalus*): toxicity, distribution, and elimination. *Can. J. Fish. Aquat. Sci.* 33: 2726-2739.

Morgan, J.A.W., T.G. Pottinger, and P. Rippon. 1993. Evaluation of flow cytometry as a method for quantification of circulating blood cell populations in salmonid fish. *J. Fish Biol.* 42: 131-141.

Morrison, K.A. and N. Thérien. 1995. Changes in mercury levels in lake whitefish (*Coregonus clupeaformis*) and northern pike (*Esox lucius*) in the LG-2 reservoir since flooding. *Water Air Soil Poll.* 80: 819-828.

Munn, M.D. and T.M. Short. 1997. Spatial heterogeneity of mercury bioaccumulation by walleye in Franklin D. Roosevelt Lake and the upper Columbia River, Washington. *Trans. Amer. Fish. Soc.* 126: 477-487.

Niimi, A.J. 1983. Biological half-lives of chemicals in fishes. *Rev. Environ. Contam. Toxicol.*, Vol. 99. Springer-Verlag, N.Y. pp. 1-46.

Niimi, A.J. 1987. Physiological effects of contaminant dynamics on fish. In: J.O. Nriagu (Ed.). *Aquatic Toxicology*. Wiley-Interscience, N.Y. pp. 207-246.

Norseth, T. and T.W. Clarkson. 1971. Intestinal transport of ^{203}Hg-labeled methyl mercury chloride: role of biotransformation in rats. *Arch. Environ. Health.* 22: 568-577.

Norseth, T. 1973. Biliary complexes of methylmercury: a possible role in organ distribution. In:*Mercury, Mercurials, and Mercaptans.* M.W. Miller and T.W. Clarkson (Eds.). Charles C. Thomas Publishing. Springfield IL. pp. 247-263.

Norstrom, R.J., A.E. McKinnon, and A.S.W. deFreitas. 1976. A bioenergetics-based model for pollutant accumulation by fish. Simulation of PCB and methylmercury residue levels in Ottawa River yellow perch (*Perca flavescens*). *Can. J. Fish. Aquat. Sci.* 33: 248-267.

Nriagu, J.O. and H.K. Wong. 1983. Selenium pollution of lakes near the smelters at Sudbury, Ontario. *Nature.* 301: 55-57.

Ohi, G., S. Nishigashi, H. Seki, Y. Tamura, T. Maki, H. Konno, S. Ochiai, H. Yamada, Y. Shimamura, I. Mizoguchi, and H. Yagyu. 1976. Efficacy of selenium in tuna and selenite in modifying methylmercury intoxication. *Envir. Res.* 12: 49-58.

Okeyo, D.O. and T.J. Hassler. 1985. Growth, food, and habitat of age 0 small-mouth bass in Clair Engle Reservoir, California. *Calif. Fish. Game* 71: 76-87.

Parks, J.W., C. Curry, D. Romani, and D.D. Russell. 1991. Young northern pike, yellow perch and crayfish as bioindicators in a mercury contaminated watercourse. *Environ. Monit. Assess.* 16: 39-73.

Paulsson, K. and K. Lundberg. 1989. The selenium method for treatment of lakes for elevated levels of mercury in fish. *Sci. Total Environ.* 87/88: 495-507.

Penreath, R.J. 1976. The accumulation of mercury from food by the plaice *Pleuronectes platessa* L. *J. Exp. Mar. Biol. Ecol.* 25: 51-65.

Peterson, M.J. and G.R. Southworth. 1996. Monitoring mercury in fish in a stream system recieving multiple industrial inputs. *Environ. Monit. Assess.* 40: 91-105.

Phillips, D.J.H. 1993. Bioaccumulation. In: *Handbook of Ecotoxicology. Vol. 1.* P. Calow (Ed.). Blackwell, London. pp. 378-396.

Pickering, A.D. 1986. Changes in blood cell composition of the brown trout, *Salmo trutta* L., during the spawning season. *J. Fish Biol.* 29: 335-347.

Quentel, C. and A. Obach. 1992. The cellular composition of the blood and haematopoietic organs of turbot *Scophthalmus maximus* L. *J. Fish Biol.* 41: 709-716.

Ricker, W.E. 1979. Growth rates and models. In: *Fish Physiology, Vol. VIII.* Academic Press, N.Y. pp. 677-743.

Ringdal, O. and K. Julshamn. 1985. Efect of selenite on the uptake of methylmercury in cod (*Gadus morhua*). *Bull. Environ. Contam. Toxicol.* 35: 335-344.

Riisgård, H.U. and S. Hansen. 1990. Biomagnification of mercury in a marine grazing food-chain: algal cells *Phaeodactylum tricornutum*, mussels *Mytilus edulis* and flounders *Platichthys flesus* studied by means of a stepwise-reduction-CVAA method. *Mar. Ecol. Prog. Ser.* 62: 259-270.

Rodgers, D.W. 1994. You are what you eat and a little bit more: bioenergetics-based models of

methylmercury accumulation in fish revisited. In: *Mercury Pollution. Integration and synthesis.* C.J. Watras and J.W. Huckabee (Eds.). Lewis Publishers, Boca Raton, pp. 427-439.

Rodgers, D.W. and F.W.H. Beamish. 1982. Dynamics of dietary methyl mercury in rainbow trout, *Salmo gairdneri.* Aquat. Toxicol. 2: 271-290.

Rowan, D.J. and J.B. Rasmussen. 1995. The elimination of radiocaesium from fish. *J. Appl. Ecol.* 32: 739-744.

Rudd, J.W.M., M.A. Turner, B.E. Townsend, A.L. Swick, and A. Furitani. 1980. Dynamics of selenium in mercury-contaminated freshwater experimental ecosystems. Can. J. Fish. Aquat. Sci. 37: 848-857.

Rudd, J.W.M., M.A. Turner, A. Furutani, A.L. Swick, and B.E. Townsend. 1983. The English-Wabigoon river system: I. A synthesis of recent research with a view towards mercury amelioration. Can. J. Fish. Aquat. Sci. 40: 2206-2217.

Ruohtula, M. and J.K. Miettinen. 1975. Retention and excretion of ^{203}Hg-labelled methylmercury in rainbow trout. *Oikos.* 26: 385-390.

Schindler, J.F. and U. de Vries. 1986. Scanning cytophotometry of carp, *Cyprinus carpio* L., erythrocyte populations: the influence of short-term hypoxic environment and the recovery period following severe bleeding. *J. Fish Biol.* 28: 741-752.

Schintu, M., F. Jean-Caurant, and J.-C. Amiard. 1992. Organomercury determination in biological reference materials: application to a study on mercury speciation in marine mammals off the Faröe Islands. *Ecotox. Environ. Safety* 24: 95-101.

Schmitz, R.J. 1996. *Introduction to Water Pollution Biology.* Gulf Publishing, Houston. p. 54.

Schultz, I.R., E.L. Peters, and M.C. Newman. 1996. Toxicokinetics and disposition of inorganic mercury and cadmium in channel catfish after intravascular administration. *Toxicol. Appl. Pharmacol.* 140: 39-50.

Schultz, I.R. and M.C. Newman. 1997. Methyl mercury toxicokinetics in channel catfish (*Ictalurus punctatus*) and largemouth bass (*Micropterus salmoides*) after intravascular administration. *Environ. Toxicol. Chem.* 16: 990-996.

Setka, J.D. and J.J. Cech, Jr. 1994. Blood and muscle characteristics of leopard shark (*Triakis semifasciata*) and brown smoothhound (*Mustelus henlei*). *Calif. Fish Game* 80: 89-96.

Sharpe, M.A., A.S.W. deFreitas, and A.E. McKinnon. 1977. The effect of body size on methylmercury clearance by goldfish (*Carassius auratus*). *Env. Biol. Fish.* 2: 177-183.

Sijm, D.T.H.M., W. Selnen, and A. Opperhuizen. 1992. Life-cycle biomagnification study in fish. *Environ. Sci. Technol.* 26: 2162-2174.

Slotton, D.G., J.E. Reuter, and C.R. Goldman. 1995. Mercury uptake patterns of biota in a seasonally anoxic northern California reservoir. *Water Air Soil Poll.* 80: 841-850.

Soldatov, A.A. 1996. The effect of hypoxia on red blood cells of flounder: a morphologic and autoradiographic study. *J. Fish Biol.* 48: 321-328.

Sprague, J.B. 1969. Measurement of pollutant toxicity to fish I: bioassay methods for acute toxicity. *Wat. Res.* 3: 793-821.

Steffens, W. 1989. *Principles of Fish Nutrition.* Ellis Horwood Ltd., Chicester. 384 pp.

Stevens, B.R. 1990. Amino acid transport in intestine. In: *Mammalian Amino Acid Transport: Mechanisms and Control.* M.S. Kilberg and D. Häussinger (Eds.). Plenum Press, N.Y. pp. 149-164.

Stow, C.A. and S.R. Carpenter. 1994. PCB accumulation in Lake Michigan caho and chinook salmon: individual-based models using allometric relationships. *Environ. Sci. Technol.* 28: 1543-1549.

Stroband, H.W. J. 1977. Growth and diet dependent structural adaptations of the digestive tract in juvenile grass carp (*Ctenopharyngodon idella*, Val.). *J. Fish Biol.* 11: 167-174.

Takahashi, H. and K. Hirayama. 1971. Accelerated eliminated elimination of methyl mercury from animals. *Nature* 232: 201-202.

Tanke, H.J., I.A.B. Nieuwenhuis, G.J.M. Koper, J.C.M. Slats, and J.S. Ploem. 1980. Flow cytometry of human reticulocytes based on RNA fluorescence. *Cytometry*. 1: 313-320.

Texter, E.C., C.-C. Chou, H. C. Laureta, and G.R. Vantrappen. 1968. *Physiology of the Gastrointestinal Tract.* Mosby, St. Louis., 262 pp.

Thomann, R.V. 1989. Bioaccumulation model of organic chemical distribution in aquatic food chains. *Environ. Sci. Technol.* 23: 699-707.

Thompson-Eagle, E.T. and W.T. Frankenberger, Jr. 1990. Volatilization of selenium from evaporation pond water. *J. Environ. Qual.* 19: 125-131.

Trudel, M. and J.B. Rasmussen. 1997. Modelling the elimination of mercury by fish. *Environ. Sci. Technol.* 31: 1716-1722.

Watras, C.J. and N.S. Bloom. 1992. Mercury and methylmercury in individual zooplankton: implications for bioaccumulation. *Limnol. Oceanogr.* 37: 1313-1318.

Watras, C.J., N.S. Bloom, R.J.M. Hudson, S. Gherini, R. Munson, S.A. Claas, K.A. Morrison, J. Hurley, J.G. Weiner, W.F. Fitzgerald, R. Mason, G. Vandal, D. Powell, R. Rada, L. Rislov, M. Winfrey, J. Elder, D. Krabbenhoft, A.W. Andren, C. Babiarz, D.B. Porcella, and J.W. Huckabee. 1994. Sources and fates of mercury and methylmercury in Wisconsin lakes. In: *Mercury Pollution. Integration and synthesis.* C.J. Watras and J.W. Huckabee (Eds.). Lewis Publishers, Boca Raton, pp. 153-177.

Weatherley, A.H., and H.S. Gill. 1983. Relative growth of tissues at different somatic growth rates in rainbow trout *Salmo gairdneri* Richardson. *J. Fish Biol.* 22: 43-60.

Weatherley, A.H., and H.S. Gill. 1987. *The Biology of Fish Growth.* Academic Press, London. 443 pp.

Wetzel, R.G. 1983. *Limnology. 2nd Ed.* Saunders, N.Y., 763 pp.

Weiner, J.G. and D.J. Spry. 1997. Toxicological significance of mercury in freshwater fish. In: *Interpreting Concentrations of Environmental Contaminants in Wildlife Tissues.* Lewis Publishers, Chelsea, Mich. (In Press) 39 pp.

Williams, R.W. and M.C. Warner. 1976. Some observations on the stained blood cellular elements of channel catfish, *Ictalurus punctatus*. *J. Fish Biol.* 9: 491-497.

Wren, C.D. and P.M. Stokes. 1988. Depressed mercury levels in biota from acid and metal-stressed lakes near Sudbury, Ontario. *Ambio*. 17: 28.

Appendix 1. Arithmetic Manipulation of Growth-Ration and Hg accumulation-Growth Relationships.

The underlying basis of the calculations such as estimated Hg dose and assimilation efficiency in chapter 2 (section 2.3.4) are based on the fact that Hg exposure was via the diet. Therefore, both growth and Hg bioaccumulation should be a function of food consumption. These relationships have been determined experimentally, and are expressed as linear regressions in Table 2.1.

Equation (1) Growth rate as a function of ration: Growth Rate = 0.824(Ration) - 4
(both ration and growth rate are in units of $mg \cdot g^{-1} \cdot d^{-1}$)

Equation (2) Hg accumulation rate as a function of Growth rate:
Hg accumulation rate = 2.635(Growth rate) + 87.127
(Hg accumulation rate is in units of $ng \cdot g^{-1} \cdot d^{-1}$; growth rate is in units of $mg \cdot g^{-1} \cdot d^{-1}$)

Growth rate is common to both equations, and therefore links them. The equations can be solved arithmetically to derive food consumption, MeHg consumption, total dose of MeHg received, body burden, and MeHg uptake efficiency.

Step1: The linear regression equation for growth as a function of ration can be used to calculate the amount of food consumed in a given simulation based on assumed growth rates (alternatively, the growth rate can be derived by assuming a certain ration).

Step2: Once growth rate is determined, the regression equation for Hg accumulation as a function of growth rate can be used to calculate Hg concentrations in muscle of fish.

Calculated Quantities

Body Weight: Growth rate × time (days) + initial weight (g) *(The experiment from which these equations were derived was a 20 day exposure; these calculations assume 20 days of exposure as well. Initial weight was set at 10 g.)*

Food Consumption: Ration × time × initial body weight *(For this calculation, daily corrections in ration size to accomodate increases in body weight were not included, as no such corrections were made in the original experiment.)*

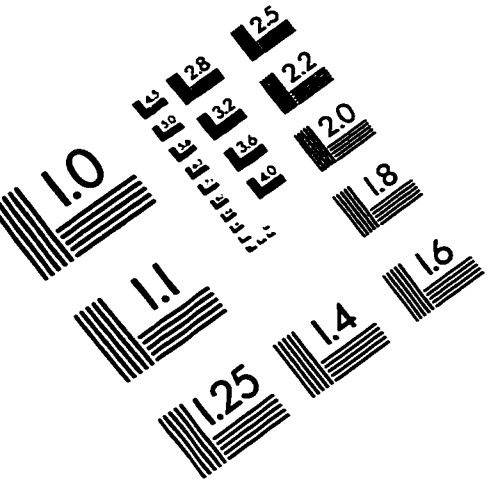
Dose: Food consumption × [Hg] in food *(Food [Hg] was 24 $\mu g \cdot g^{-1}$)*

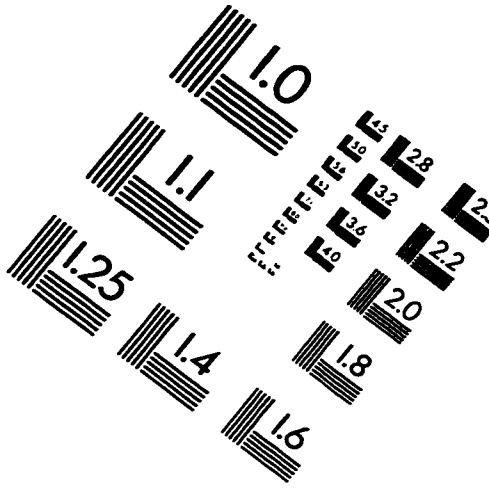
Muscle [Hg]: Hg accumulation rate × time

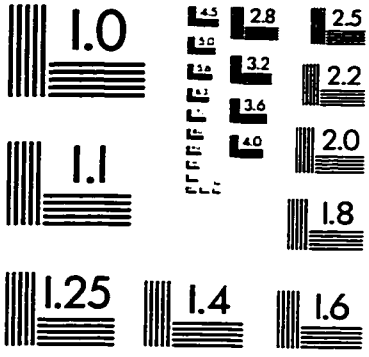
Body Burden: muscle [Hg] × Body Weight *(For calculations of body burden, the approach of Lockhart et al. (1972) was used. They found that the product of muscle Hg concentration and body weight gave estimates of body burden equivalent to that produced by measuring Hg in whole body homogenates.)*

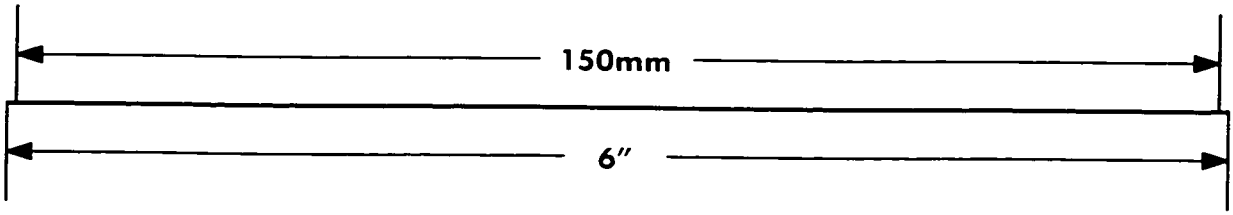
Accumulation Efficiency: Body burden ÷ dose

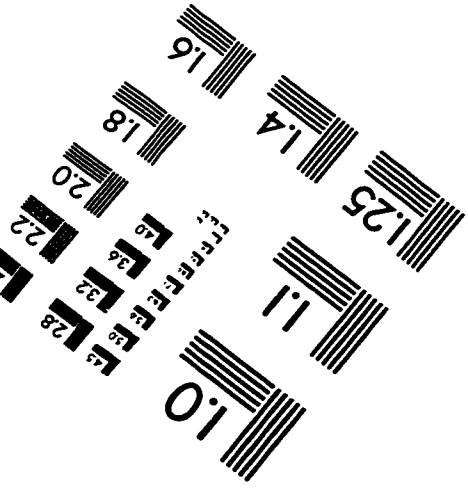
IMAGE EVALUATION TEST TARGET (QA-3)

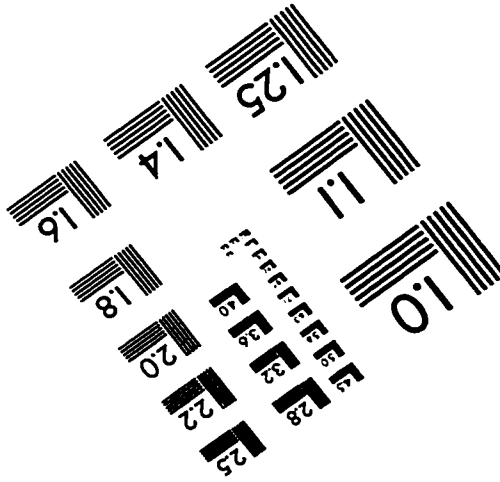












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