UNIVERSITY OF MANITOBA

AN EXAMINATION OF HEAVY METALS, METALLOTHIONEIN, AND CADMIUM BIOACCUMULATION IN FISH EXPOSED TO HEAVY METALS IN THE ENVIRONMENT AND THE LABORATORY

by

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ABSTRACT

Field and laboratory experiments examined the effects of heavy metal exposure on metallothionein (MT), Cu, Zn, and Cd concentrations in liver and kidney of freshwater fish. In 1986, populations of white sucker (Catostomus commersoni) and northern pike (Esox lucius) were sampled from eight lakes near the Cu/Zn smelter at Flin Flon, Manitoba, Canada. Cu and Zn concentrations in pike and sucker liver were lower than in previous studies in 1976 and 1982. These reductions in liver metal concentrations (and the absence of hepatic MT induction in both species) corresponded to lower metal concentrations in recent sediments, and appeared to reflect reductions in heavy metal deposition near the smelter since a 251 m stack began operation in 1974. In contrast to liver, Cu and Zn concentrations in kidney were elevated relative to fish from reference lakes. It is currently not known whether renal metal concentrations are decreasing over time in the fish populations near Flin Flon.

Cd concentrations in northern pike liver and kidney were statistically related to sediment Cd concentrations, but Cd was not bioaccumulating in fish from any lakes near the smelter. A laboratory experiment indicated that Cd bioaccumulation in rainbow trout (Oncoryhnchus mykiss) liver was a function of the ratio of Cu and Zn to Cd in the diet. In future, the potential for Cd-metal interactions to minimize Cd

toxicity in the environment should not be overlooked.

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CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The production of pollution by human activity has been an intermittent problem in human societies for centuries (Matthews 1986), but since the industrial revolution, efforts towards removing pollutants from the biosphere have not kept pace with the increasing quantities of wastes produced by a growing world population (Forstner and Wittman 1981). Of the many pollutants produced by human activity, metals, particularly the transition metals are important pollutants of the environment (Forstner and Wittman 1981, Forstner and Prosi 1979).

Transition metals have the physical properties of high thermal and electrical conductivy, high reflectivity, and the characteristic tendency to form highly coloured coordination compounds. With the exception of scandium, titanium, and yttrium, the densities of the transition metals are all greater than 5.0 g/cm^3, which classifies them as heavy metals (Nieboer and Richardson 1980). Transition metals occur in most geographical regions on earth in trace quantities and several are required by biota as nutrients in trace quantities (Frieden 1984), hence the term trace metal is often used synonymously with transition metal or heavy metal.

Unlike most organic compounds, metals cannot be degraded by normal processes in the environment. When they are released to the environment as industrial wastes they accumulate in the

biosphere. Aquatic systems are particularly susceptible to metal pollution because they act as sinks for metals, which accumulate in sediments. From these sinks (and during the sedimentation process itself), metals may be mobilized into aquatic food webs, where they exert toxic effects (Forstner and Wittman 1981).

The need for assessment of contaminant effects on ecosystems is increasing with the increasing pollutant load to the biosphere (Neff 1985, Haux and Forlin 1988). Defining the appropriate methods for this task is an important issue in aquatic ecotoxicology.

Traditionally, the determination of effects of metallic pollutants on ecosystems has relied upon extrapolation from standardized laboratory bioassays to predict effects under field conditions (Levin et al. 1989). The inadequacy of laboratory tests for this purpose (Kimball and Levin 1985, Cairns 1986) is partly offset by the ease and economy of application of these tests and by the large body of experience which has developed around them. In spite of the limitations of standardized laboratory toxicity tests, their development has led to dramatic improvements in water quality and ecosystem protection (Levin et al. 1989). However, because laboratory toxicity tests are retrospective, not prospective (Nriagu 1988), it has been recognized that early-warning indicators for determining metal effects <u>in situ</u> are are also required (Neff 1985, Larsson et al. 1985, Haux and Forlin 1988, Nriagu

1988).

The biochemical strategy of ecotoxicology has been developed to fulfill such a need, based upon the concept that biologic responses to a toxicant originate within individual organisms at the cellular level (Larsson et al. 1985, Haux and Forlin 1988), whereas the effects of toxicants at higher levels of organization (eg. the whole animal or population level) occur after longer latency periods (during which these cellular alterations can be observed). The early biologic responses to toxicant exposure have been called biological markers or biomarkers (Henderson et al. 1989, Cullen 1989), or biological indicators (Sindermann 1988, Adams et al. 1989). Ideally, a biomarker would be chemical-specific and reversible, detectable in trace quantities, available by non-invasive techniques, inexpensive to analyze, and quantitatively relatable to a toxicant exposure (Haux and Forlin 1988, Henderson et al. 1989).

Metallothionein (MT) is a cysteine-rich metal-binding protein which has received great attention as a biomarker of exposure to the transition metals from Groups IB and IIB of the periodic table (Klaverkamp et al. 1984, Neff 1985, Hamilton and Mehrle 1986, Engel and Roesjadi 1987, Engel 1988, Haux and Forlin 1988, Sindermann 1988).

Metallothionein was first discovered as a cadmium-binding protein in equine renal cortex (Margoshes and Vallee 1957), during early research into mammalian cadmium bioaccumulation

(Kagi and Kojima 1987). Since its discovery, MT has been found to be ubiquitous in the animal kingdom (Palmiter 1987). Vertebrate MT has been characterized as a 6000-7000 molecular weight protein having 60 amino acid residues, of which 20 are thiolate cysteine residues (Fowler et al. 1987). The primary structure of MT has a characteristic arrangement of cysteine in cys-x-cys sequences which are responsible for the metal-binding properties of MT. The high thiol content and metal-binding tendencies of the protein are acknowledged in its name, metallothionein (Kagi and Schaeffer 1988).

Several physiological roles have been proposed for MT: as metal donor to apoenzymes of copper- and zinc-requiring enzymes; as a pool of cysteine for sulfur metabolism; as an anti-oxidant; and as a metal detoxification molecule (Hamer 1986). Because MT was discovered due to the fact that it binds cadmium, it is not surprising that much research into MT function has focused on the role of MT in cadmium detoxification (Hamer 1986). In the literature, MT is often considered to be strictly a cadmium-binding protein, which implies a specific physiological role for MT in cadmium metabolism. However, the increased release of cadmium into the biosphere is a phenomenon associated with human industrial activity (Nriagu 1988), so it is quite unlikely that MT has evolved specifically to detoxify a metal which has only recently become concentrated in the biosphere (Waalkes and Goering 1990). The central function of MT appears to be the

regulation of zinc and copper metabolism, of which detoxification of these two metals is an implicit part. The fact that MT is capable of reducing cadmium toxicity seems to be incidentally related to the chemical similarity between zinc and cadmium (Waalkes and Goering 1990).

Fish are the most numerous vertebrate taxa (approximately 20,000 species) (Zarafullah et al. 1989). They are important as experimental organisms for studying and monitoring environmental metal contamination because of the large number of species available to study, their widespread distribution, and because the aquatic ecosystems in which they live act as sinks for metals.

Metallothionein has received extensive attention as a monitoring tool for assessing stress in fish due to heavy metal exposure (Klaverkamp et al 1984, Neff 1985, Hamilton and Mehrle 1986, Engel and Roesijadi 1987, Haux and Forlin 1988). The primary justification for using MT as a monitoring tool is its inducibility (Palmiter 1987). The de novo synthesis (induction) of MT in target organs is a direct response to metal assimilation by these organs (Scheuhamer and Templeton 1990). Consequently, elevated MT concentrations have been utilized as a measure of MT induction in aquatic animals (Olafson et al. 1979, Deniseger et al. 1990).

However, at least four limitations of MT as a biomarker of environmental metal pollution have been identified and need to be addressed. First, seasonal variation in MT concentra-

tions have been observed in rainbow trout (Haux et al. 1987), corresponding to sexual differences during the reproductive cycle. This variation may reduce the use of MT to distinguish between true responses to metal exposure and normal seasonal patterns of MT induction (Engel 1988). Second, MT can be induced by at least 26 chemical compounds or physiological conditions in addition to exposure to copper, zinc, and cadmium (Waalkes and Goering 1990), so that elevated MT concentrations in fish organs do not necessarily imply exposure to metals. For example, hepatic MT in rainbow trout was induced after exposure to No. 2 fuel oil, indicating that MT induction does occur after non-metal contaminant exposures (Steadman et al. 1991). Therefore, MT may be more suitable as a general biomarker rather than a presumed metal-specific biomarker. Third, a dose-response relationship between MT and environmental metal exposure has not been consistently demonstrated to date (Haux and Forlin 1988). Fourth, any research on MT requires a method for measuring MT rapidly, accurately, and inexpensively. Research on MT has been hindered by the lack of a consistent, validated method for measuring MT (Haux and Forlin 1988). Among the methods available for measuring MT, a metal saturation assay using cadmium (Onasaka and Cherian 1982, Eaton and Toal 1982) has become widely used. However, the cadmium saturation assay is inhibited by copper (Eaton 1985) due to the fact that copper binds more strongly to MT than does cadmium (Neilson et al.

1985). In fish species which have characteristically high hepatic copper concentrations (such as rainbow trout), MT may be underestimated by the cadmium saturation assay (as demonstrated by Lauren and McDonald (1987)). Therefore, a metal saturation assay which is not inhibited by copper would be useful.

The research presented in this thesis addresses two of these limitations concerning the use of metallothionein as a bioindicator of metal exposure in fish. Chapter II documents the development of a rapid and reliable mercury saturation assay for measuring MT concentrations in fish. This assay was then used to measure MT in liver and kidney of fish collected from eight lakes near the copper/zinc smelter at Flin Flon, Manitoba, Canada (Chapter III). The eight lakes were located on a gradient of metal deposition near the smelter, and provided an opportunity to test for a dose-response relationship between MT and metals in sediment. Chapter IV reports an experiment examining cadmium bioaccumulation in rainbow trout liver. The experiment was stimulated by two findings from the research conducted near the Flin Flon smelter: (1) cadmium bioaccumulated in fish from northern Ontario lakes far removed from any point sources of cadmium deposition, while cadmium did not bioaccumulate in fish from lakes near the Flin Flon smelter; and (2) of the variables measured in the Flin Flon research, only tissue cadmium concentrations were related to cadmium concentrations in sediment.

CHAPTER II

A NEW MERCURY SATURATION ASSAY FOR MEASURING METALLOTHIONEIN IN FISH

INTRODUCTION

Metallothioneins (MTs) are a family of low-molecular-weight, cysteine-rich proteins which bind metals from groups IB and IIB of the periodic table (Kagi and Nordberg 1979, Hamer 1986, Kagi and Kojima 1987). These proteins appear to have evolved for intracellular zinc and copper regulation (Hamer 1986). MT synthesis is induced in animals by exposure to these, and other, heavy metals, and a capacity for the inducible synthesis of MT has been demonstrated for most tissues and organisms studied to date (Palmiter 1987). MT induction and its correlation with toxicity acclimation in fish (Klaverkamp et al. 1984, Klaverkamp and Duncan 1987) indicates that this regulatory function includes the detoxification of zinc, copper, mercury, and cadmium. MT has, therefore, been proposed as a promising biochemical indicator of heavy metal exposure in aquatic organisms (Klaverkamp et al. 1984, Neff 1985, Hamilton and Mehrle 1986, Haux and Forlin 1988).

Since MT has no easily detectable function other than metal binding properties, MT analysis is limited to immunological methods or metal binding assays (Kikuchi et al. 1990). The immunological methods include radioimmunoassay (RIA) (VanderMallie and Garvey 1979, Hogstrand and Haux 1989), and enzyme-linked immunosorbent assay (ELISA) (Thomas et al. 1986). Metal binding assays include gel filtration chromatog-

raphy and metal summation (Klaverkamp and Duncan 1987, Noel-Lambot et al. 1979), and metal saturation assays utilizing either cadmium (Onasaka and Cherian 1982, Eaton and Toal 1982, Payan et al. 1988, Klein et al. 1990), silver (Scheuhamer and Cherian 1986), or mercury (Piotrowski et al. 1973, Zelazowski and Piotrowski 1977, Patierno et al. 1983, Lobel and Payne 1987). Electrochemical methods such as polarography (Olafson and Sim 1979) are similar to metal binding assays because they utilize the binding and release of metals under variable oxidation-reduction conditions.

The metal saturation methods are popular because they are simple and rapid (Dieter et al. 1987). These methods detect MT by replacing the metals bound (in situ) to the cysteinyl thiol residues of MT with a displacing metal of higher binding affinity for these thiol residues. The MT concentration is estimated from the amount of displacing metal remaining in solution (ie. all of the displacing metal is assumed to be bound only to MT), using known metal-binding stoichiometries for MT, and (or) by calibration with an MT standard curve.

The majority of metal saturation methods of MT analysis have been developed for use with mammalian tissues; their use with fish tissues has not been evaluated adequately. Some piscine MTs have a much higher copper content than mammalian MTs (Klaverkamp and Duncan 1987, Olsson and Haux 1985). Because copper is more strongly bound to MT than is cadmium (Scheuhamer and Cherian 1986, Eaton 1985, Rupp and Weser

1978), the cadmium saturation assay would be expected to underestimate piscine MT, as demonstrated by Lauren and McDonald (1987). Therefore, the use of cadmium saturation methods to analyze piscine MT may be inappropriate.

The silver saturation method (Scheuhamer and Cherian 1986) may also be limited by two major constraints. First, silver precipitates with halides, so high halide concentrations must be avoided by the method (Cherian 1988). Organs involved in osmoregulation in fish (ie. gills and kidney) are likely sites of chloride interference in the method, particularly after metal exposure, which elevates chloride concentrations in gill (Heath 1987). Second, the stoichiometry of 17.4 g atom/mole, observed by Scheuhamer and Cherian (1986) is considerably higher than previously documented estimates of binding stoichiometry for silver and MT (Neilson et al. 1985). The possibility that silver may bind non-specifically to amino acid residues other than cysteine has not been examined.

The major drawback of MT metal saturation methods is that the detection of thiol groups by metal binding is inherently non-specific. The thiol residues of MT will bind at least 18 different metals (Neilson et al. 1985). These same metals also bind to thiol groups of other proteins and peptides (Jocelyn 1972), both cytosolic and membrane-bound. Such non-specific metal-thiol binding has been documented for the thiol-containing peptides cysteine and glutathione (GSH) (Eaton and Toal 1982, Eaton 1985), and represents a major potential source of

interference in MT metal saturation assays.

The present study was initiated to develop an alternative metal saturation assay for piscine MT. Mercury was chosen as the displacing metal because it is the metal which is most strongly bound to MT (Neilson et al. 1985, Elinder and Nordberg 1985), and to thiols in general, particularly at low pH and even in the presence of chloride (Jocelyn 1972). These chemical characteristics of Mercury have permitted the development of the first metal saturation assay for piscine MT which has been proven to displace copper from MT. This assay is much more rapid to perform than either the cadmium or silver saturation assays.

MATERIALS AND METHODS

The Mercury saturation assay was evaluated in four experiments: (1) the specificity of the assay was tested; (2) the displacement of native MT-bound metals by mercury was evaluated; (3) the effects of sample preparation procedures on MT analysis were determined; and (4) the mercury saturation assay was compared with the original metal saturation assay for MT analysis (Piotrowski et al. 1973). Experiment 4 was necessary to clearly distinguish this method from the mercury saturation assay of Piotrowski et al. (1973), which overestimates MT (Dieter et al. 1987).

Defining Assay Parameters

The assay was performed using a dilution series for each sample. In pseudocytosol experiments (described in the "Defining Assay Specificity" section), samples were sequentially diluted by 1/3, 1/9, and 1/18, providing four replicate determinations. The dilution series used for testing sample preparation procedures (using rainbow trout liver) consisted of 1/1, 1/3, 1/9, 1/27, and 1/81 dilutions. These additional dilutions were used to determine the extent to which samples could be diluted without loss of accuracy.

After preparation of samples (described in the "Assessing Sample Preparation Procedures" section), a dilution series was prepared for each sample in 1.5 mL polypropylene microcentrifuge tubes. For the 18-fold dilution series, 200 µL of 0.9% (w/w) NaCl (saline) was added to the 1/3 and 1/9 dilution tubes, and 100 µL of saline was added to the 1/18 dilution tube. Then, 300 µL of sample was added to the microcentrifuge tube which contained undiluted sample, of which 100 µL was transferred sequentially, after mixing, through the dilution series to leave each replicate tube with 200 µL of appropriately-diluted sample. The same technique was used for the 81-fold dilution series. All aqueous solutions were made with distilled, deionized, and carbon-filtered water (Millipore MilliQ system).

Metal displacement was initiated by adding 200 µL of

^{203}Hg-labelled $HgCl_2$ (containing 10,000 cpm and 10 µg (49.9 nmol) of stable mercury) in 10% (w/w) trichloroacetic acid (TCA) to each dilution replicate. Mercury was provided in excess for samples containing up to 7.13 nmol MT (assuming a binding stoichiometry of 7 nmol mercury per nmol MT (Hamer 1986, Neilson et al. 1985, Elinder and Nordberg 1985).

After at least 1 minute of incubation, the metal-displacement step was terminated by adding 400 µL of 50% (w/w) egg albumin (egg white) in 0.9% NaCl to each assay tube. (The egg white solution was prepared by thorough mixing of egg whites from chicken eggs using a Polytron homogenizer at low speed.) The egg white is used to bind excess, non-MT-bound mercury. The acid-denatured egg white-mercury complex was removed from the TCA solution by centrifugation at 10,000g for 2 min, after mixing the assay tubes by vortex. An Eppendorf 5412 benchtop centrifuge (at room temperature) was used for all 10,000g centrifugation.

With each set of samples, total activity vials and blank vials were also assayed. Each total activity vial contained 200 µL of ^{203}Hg-labelled mercury only, and was required in order to determine the specific activity of the ^{203}Hg used, while each blank vial had sample replaced by 200 µL of 0.9% NaCl. The blanks were necessary to verify that sufficient egg white was present to bind and remove all mercury from the assay tubes when no MT was present in the samples.

The TCA supernatants were removed from the assay tubes by

pipette, and transferred to clean microcentrifuge tubes for the determination of ^{203}Hg activity by gamma counting (LKB-Wallac 1282 Compugamma). The mercury-binding capacity of the heat-treated sample supernatant was calculated as follows:

nmol Hg bound / mL of sample =

[cpm(sample)-cpm(blank)]/[cpm(total)]

x [10 µg Hg/(0.2 mL sample)]

x [1 nmol Hg/0.20059 µg Hg]

x [Dilution (eg. 1,3,9,18,27, or 81)]

The binding capacity per gram of tissue was then calculated by multiplying this value by the initial sample dilution (ie. the volume (mL) of supernatant produced per gram of tissue).

Evaluating Assay Specificity

To demonstrate that the assay is specific for the detection and quantification of MT, relationships between the mercury-binding behavior of egg white, GSH, and MT were assessed by four experimental runs using the mercury saturation assay. First, the mercury-binding capacity of egg white was determined by adding aliquots of 50% egg white solution (in 20 µL increments up to 400 µL) to assay tubes containing 200 µL of mercury in 10% TCA (having 49.9 nmol mercury).

Second, the same sequence of egg white addition was applied to 200 µL aliquots of 50 µg/mL standards of MT, in order to establish whether mercury could be stripped from MT by egg white. Third, the ability of 400 µL of 50% egg white solution to compete with GSH for mercury was assessed on samples containing GSH ranging from 0.5 mM to 10 mM (in 0.5 mM increments). Fourth, the effect of GSH on the binding of mercury by MT was evaluated using the same range of GSH concentrations, with MT added to a concentration of 50 µg/mL in each vial.

The use of egg white solution for the removal of excess mercury was compared with that removed by bovine hemoglobin and bovine serum albumin. Egg white contains approximately 10% protein (Powrie and Nakai 1986), so the 50% egg white solution contains approximately 5% protein. Therefore, 5% solutions of hemoglobin and bovine serum albumin were used for comparison. To 1.5 mL microcentrifuge tubes containing 200 µL of 0.9% NaCl and 200 µL of mercury in 10% TCA (having 49.9 nmol mercury), aliquots of the three protein solutions were added in 50 µL increments up to 400 µL. The proteins denatured on contact with the TCA, were mixed by vortex, and centrifuged for 2 min at 10,000g to precipitate the denatured protein. The supernatants were removed by pipette and the ^{203}Hg concentration was determined.

To test the ability of the assay to measure MT in complex samples of thiol-containing proteins and peptides, a pseudo-

cytosol was prepared to provide mixtures of known composition. The concentration of components in the pseudocytosol was based upon their maximum known concentration ranges in biological tissues, and contained approximately 41 mg of total solid per mL. The pseudocytosol was composed of bovine hemoglobin (26 mg/mL (400 μM)), bovine albumin (12.43 mg/mL (188 μM)), catalase (0.474 mg/ mL (2 μM)), aldolase (0.496 mg/mL (3 μM)), glutathione (GSH) (1.592 mg/mL (5.18 mM)), and cysteine (0.0402 mg/mL (330 μM)). All pseudocytosol constituents were purchased from Sigma Corp., St. Louis, Mo, USA.

The ability of the mercury saturation assay to detect a known quantity of MT from this complex sample was tested in a factorial experiment consisting of two concentrations of pseudocytosol (20 and 40 mg/mL) and two concentrations (10 and 20 μg/mL) of rabbit liver MT (Sigma Corp., St. Louis, Mo., USA). MT was estimated by the analysis of an 18-fold dilution series from each treatment combination.

As a further test of the ability of the assay to detect non-specific mercury binding to low-molecular-weight acid-soluble thiols, dilution series were run on GSH and MT at concentrations corresponding to pseudocytosol treatment levels. Therefore, the treatments for the experiment were 2.53 and 5.06 mM GSH, and 10 and 20 μg MT /mL, respectively. An 18-fold dilution series of the mercury saturation assay was performed on each treatment combination from this factorial experiment.

Gel Filtration Chromatography

The cytosolic distribution of copper, zinc, and cadmium was evaluated by gel filtration chromatography, using a 60 cm x 0.9 cm column of Sephadex G-50 (fractionation range 30,000-1500 kD for proteins) eluted at a flow rate of 0.38 mL/min. Fractions were collected at 3 min intervals. The column was calibrated with ovalbumin (MW 43,000) (void volume, V_o), ribonuclease A (MW 13,700), rabbit liver MT-II (MT elution volume, V_{MT}), and glutathione (total column volume, V_T).

Two buffers were used for gel filtration chromatography. A 10 mM phosphate buffer, pH 7.4 (Gomori 1955) was used for fractionation of supernatants which had not been subjected to the mercury saturation assay, while the acidic (pH 1.45) TCA supernatant from the mercury saturation assay was eluted with a KCl/HCl buffer, pH 1.5 (Gomori 1955) in order to assess the displacement of copper, zinc, and cadmium from MT.

Evaluating Metal Displacement

Rainbow trout (Oncorhynchus mykiss) used in other experiments described here were not exposed to cadmium, so in order to demonstrate the ability of the mercury saturation assay to displace cadmium as well as zinc and copper, a supernatant from one cadmium-injected rainbow trout was used.

The fish (all rainbow trout used in this study were Sundalasora strain, supplied by the Rockwood Aquaculture Research Centre, Gunton, MB) had been injected intraperitoneally with 8.9 µmol cadmium/kg of body weight (1 mg/kg) 11 days prior to sampling. The supernatant was prepared by homogenizing the liver in 10 parts (w/w) of 0.9% NaCl and then centrifuging the homogenate at 10,000g for 10 min. Individual fractions of the chromatographed supernatant were analyzed for cadmium, copper and zinc, providing estimates of <u>in situ</u> metals bound to MT and other cytosolic constituents. These data were pooled into a high-molecular-weight fraction (consisting of the fractions eluted between the void volume (V_o) and the MT elution volume (V_{MT})), a MT fraction, and a low-molecular-weight fraction (consisting of the fractions between V_{MT} and the total elution volume (V_t)). The chromatographed TCA supernatant from the mercury saturation assay analysis of this sample was also pooled into a high-molecular-weight fraction, a MT fraction, and a low-molecular-weight fraction. The presence of metals in the MT fraction was evidence of the incomplete displacement of copper, zinc, and cadmium by mercury.

Metals were analyzed by flame atomic absorption spectrophotometry on a Varian AA-5 atomic absorption spectrophotometer with deuterium background correction, using an air-acetylene flame.

Comparison of the Assay with the Method of Piotrowski et al. (1973)

The liver from a 467g female rainbow trout was homogenized in 5 parts of 0.9% NaCl as described previously. A mercury-binding curve (Piotrowski et al. 1973, Kotsonis and Klaassen 1977) was obtained by diluting the homogenate with 0.9% NaCl and adding 200 µL of ^{203}Hg in 10% TCA (containing 49.9 nmol mercury), to obtain mercury-to-tissue ratios between 0.3 and 7 µmol mercury/g tissue. Six replicate assay tubes for each mercury-to-tissue ratio were incubated for 10 min, centrifuged at 10,000g for 5 min to precipitate denatured proteins, and the ^{203}Hg concentration in the supernatants was determined. MT concentrations were estimated from the plateau regions of the two binding curves according to the methods of Piotrowski et al. (1973) and Kotsonis and Klaassen (1977). In order to demonstrate that the Piotrowski assay overestimates MT concentrations, 400 µL of 50% egg white solution was added to each TCA supernatant from the Piotrowski assay, and the centrifugation procedure was repeated, removing non-MT-bound mercury from the TCA supernatants.

These MT estimates were compared with those produced by the mercury saturation assay. The sample for the mercury saturation assay was prepared from the same homogenate analyzed by the Piotrowski assay. The homogenate was diluted by half, centrifuged for 10 min at 10,000g, and the

supernatant was collected for MT analysis.

Assessing Sample Preparation Procedures

To provide uniform sample material for this experiment, livers from three immature (approximately 200 g) rainbow trout were homogenized in 4 parts (w/w) of 0.9% NaCl and pooled. Subsamples of the pooled homogenate were used to evaluate eight sample preparation procedures, denoted as follows: homogenate (HGT); supernatant from homogenate centrifuged at 10,000g for 10 min (S10); supernatant from homogenate centrifuged at 30,000g for 30 min (Sorvall RC 5B at 4°C) (S30); and supernatants produced by heat denaturation of homogenate in a stirred saltwater bath at 100°C for 0.5, 1.0, 2.0, 6.0, and 10.0 min (HDS½, HDS1, HDS2, HDS6, and HDS10, respectively). Following the heat denaturation step, the samples were cooled on ice for 5 min, mixed by vortex (to aid in the formation of a solid pellet after heat denaturation), and centrifuged at 10,000g for 10 min to produce the final heat-denatured supernatants.

Standardizing the Assay

To enable interlaboratory comparison, commercially available (Sigma Corp.) rabbit liver MT-II was assayed by the

mercury saturation assay. In the portions of this research that used this commercially prepared MT (ie. pseudocytosol experiments), the MT estimates have been left in μg units rather than molar units, to permit easy comparison with product specifications. In the portions of this research using rainbow trout MT, estimates are based upon the well-characterized metal-binding ratios for MT. Each mole of MT binds 7 moles of zinc, cadmium, or mercury, and 12 moles of silver or copper. Molar MT concentrations were calculated as follows (using metal summation from the gel filtration fractions which co-eluted with the V_{MT} for rabbit liver MT-II);

nmol MT = (nmol Cd/7) + (nmol Zn/7) + (nmol Cu/12)

or as

nmol MT = (nmol Hg/7)

for samples analyzed by the mercury saturation assay.

Statistical Analyses

Statistical estimates, such as 95% confidence intervals, used methods described in Steel and Torrie (1980). The multiple comparison procedure used was Tukey's procedure (Steel and Torrie 1980).

RESULTS

Standardizing the Assay

The product specifications for rabbit liver MT-II (Sigma Corp., St. Louis, Mo.; Lot #46F-9555) were 18 µg (275.3 nmol) zinc and 64.4 µg (572.9 nmol) cadmium per mg. Therefore, 917.6 µg of protein (apometallothionein) was contained in each mg of MT. Assuming that the metal-binding sites of MT are fully metal-saturated with cadmium and zinc, the calculated molecular weight of the MT-II apometallothionein was 7573. Analysis of the MT-II by the mercury saturation assay provided a mercury binding capacity of 878.7 ± 54.9 nmol per mg of MT (104% of the product specifications), and a molecular weight estimate of 7310 for apometallothionein.

Estimates of the cadmium and zinc content of the MT-II by flame AAS were 97 %, and 66% of product specifications, respectively. The low estimates of zinc may reflect the loss of zinc as a result of its low binding affinity to apometallothionein. Nevertheless, the mercury saturation estimates and cadmium analysis agreed closely with product specifications, indicating that the mercury saturation assay accurately measured the commercially prepared MT standards, and therefore, that mercury completely displaced cadmium and zinc from MT.

Evaluating Specificity of the Assay

The assay was standardized with respect to the quantity of mercury used for displacement (49.9 nmol mercury per vial), and as little as 50 µL of 50% egg white solution was sufficient to remove all of the mercury from solution (Fig. 1). In comparison, at least 200 µL of hemoglobin or serum albumin was required to remove all the mercury from solution. Due to its greater effectiveness, egg white was adopted for removal of non-MT-bound mercury. Increasing the quantity of egg white from 40 to 400 µL did not strip mercury from 50 µg/mL MT standards (Fig. 2A). Therefore, 400 µL of egg white solution was adopted for removing non-MT-bound mercury.

In the absence of MT, GSH concentrations above 1 mM interfered with the removal of mercury by 50% egg white solution (Fig. 2B), but in the presence of MT (50 µg/mL), this effect was only slight, and only evident at GSH concentrations greater than 5.5 mM. These results indicate that the assay is a competitive binding assay, such that the binding of mercury by GSH can be minimized by diluting the sample relative to the amount of egg white solution used; this dilution of MT relative to egg white prevented mercury-GSH binding without inhibiting mercury-MT binding. Therefore, the use of a dilution series is essential for detecting interference in the assay.

If there is no dilution-dependent effect on mercury binding

Fig. 1. Testing the effectiveness of bovine hemoglobin (open triangle), bovine serum albumin (filled square), and egg white (filled triangle) in removing mercury from solution. All protein solutions had approximately 5% protein content, and were added in 50 µL aliquots to 10% TCA solution containing 249 nmol mercury/mL.

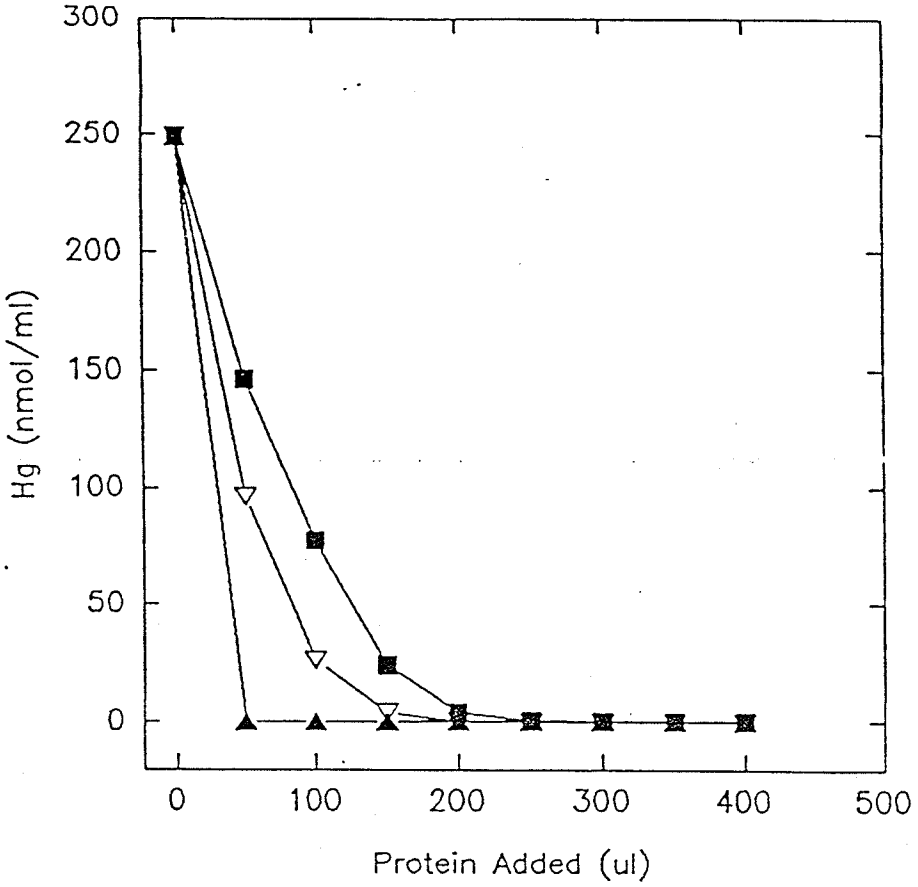
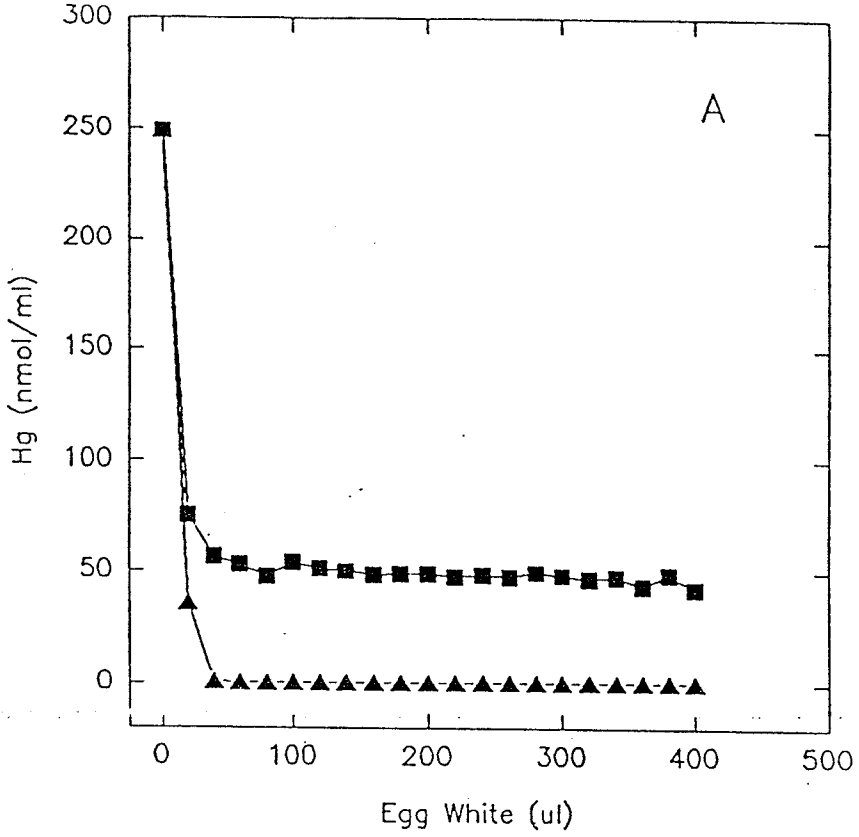
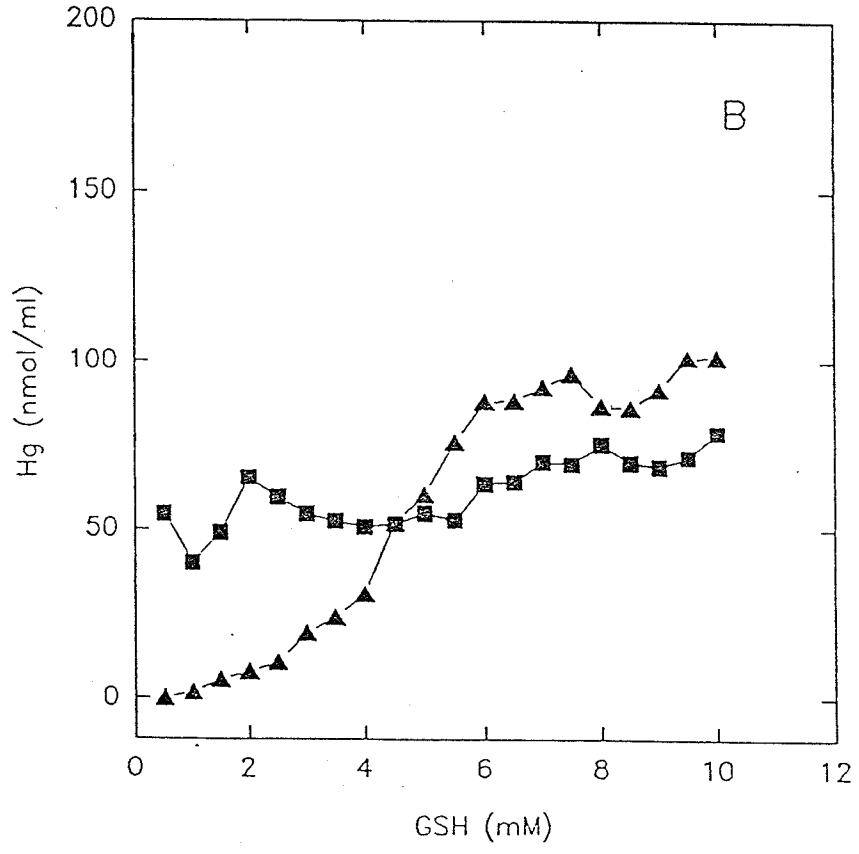

300
250
200
150
100
50
0
Hg (nmol/ml)
0
100
200
300
400
500
Protein Added (ul)

Fig. 2. Testing egg white for the removal of mercury.

A. Increasing volumes of 50% egg-white solution were used to determine its ability to remove mercury from solution in the absence of MT (triangle), and in the presence of 50 μg MT/mL (square).

B. Increasing concentrations of GSH were used to determine its effect on the ability of 400 μL of 50% egg-white solution to remove mercury from solution in the absence of MT (triangle), and in the presence of 50 μg MT/mL (square).


A
Hg (nmol/ml)
300
250
200
150
100
50
0
0
100
200
300
400
500
Egg White (ul)


B
Hg (nmol/ml)
200
150
100
50
0
0
2
4
6
8
10
12
GSH (mM)

in the assay, this is revealed by an approximately zero slope for the dilution-corrected mercury-binding curve. This is the case for samples from the pseudocytosol-MT experiment, which indicate that estimates of MT did not vary with dilution when present alone (Fig. 3A), or when present in a pseudocytosol (Fig. 3B,C).

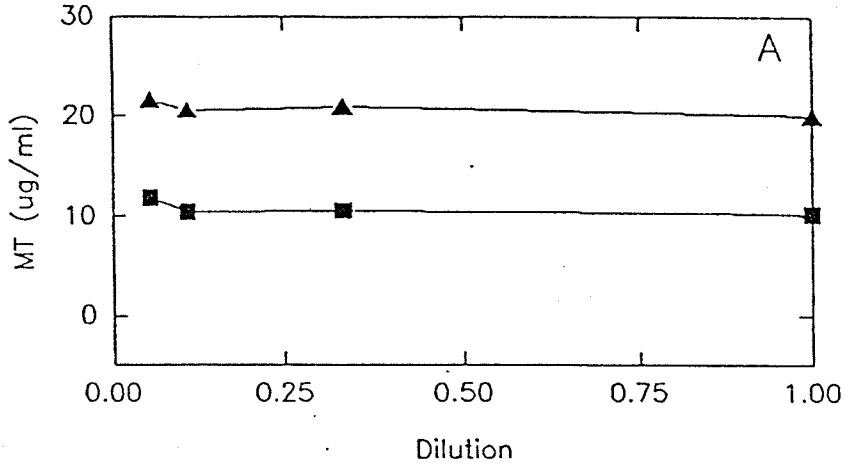
The dilution-corrected mercury-binding curve is necessary to illustrate the potential for systematic, dilution-dependent trends in mercury binding. The four dilution-corrected MT estimates from a dilution series would normally be presented as a mean and confidence interval, and the near-zero slopes of Fig. 3 correspond to a small confidence intervals for the dilution series (Fig. 4). The estimates of 10 and 20 μg MT/mL standards of rabbit liver MT-II were not significantly different from product specifications, and in all MT-pseudocytosol treatment combinations the MT estimates were not significantly different from either product specifications or the standards. Furthermore, MT estimates from all treatment levels of pseudocytosol were statistically distinguished from one another, and the mercury binding in pseudocytosol that did not contain MT was not significantly different from background (Fig. 4).

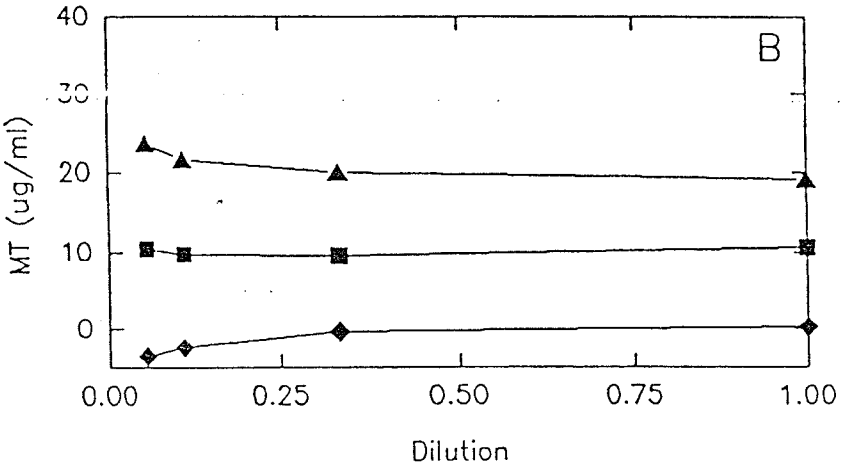
The potential for interference in the assay by acid-soluble thiols was determined in a factorial experiment identical to the MT-pseudocytosol experiment, except that the pseudocytosol was replaced by its GSH component only (2.53 and 5.06 mM GSH).

Fig. 3. Dilution-corrected mercury-binding curves for pseudocytosol containing Sigma rabbit liver MT-II. Each data point represents the MT estimate of an individual dilution replicate.

A. Sigma rabbit liver MT II standards (10 and 20 µg/mL).
B. Pseudocytosol (20 mg/mL).
C. Pseudocytosol (40 mg/mL).

0 µg MT/mL - (diamond); 10 µg/mL - (square); 20 µg/mL - (triangle).


A
30
20
10
0
MT (ug/ml)
0.00
0.25
0.50
0.75
1.00
Dilution


B
40
30
20
10
0
MT (ug/ml)
0.00
0.25
0.50
0.75
1.00
Dilution

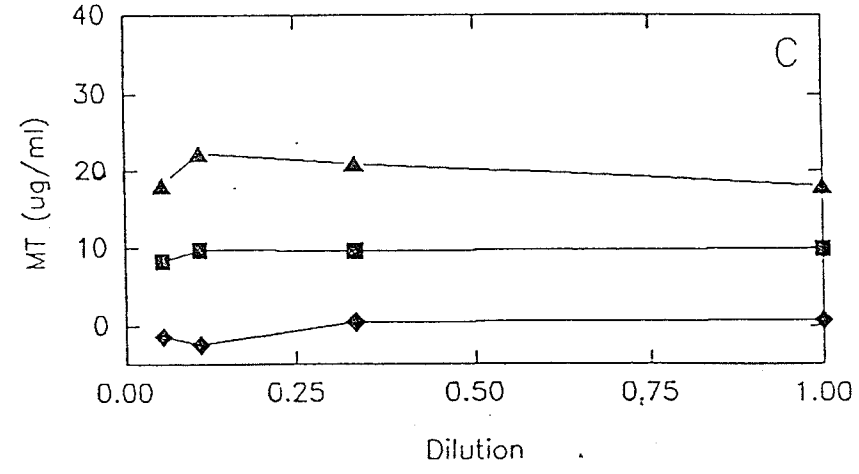
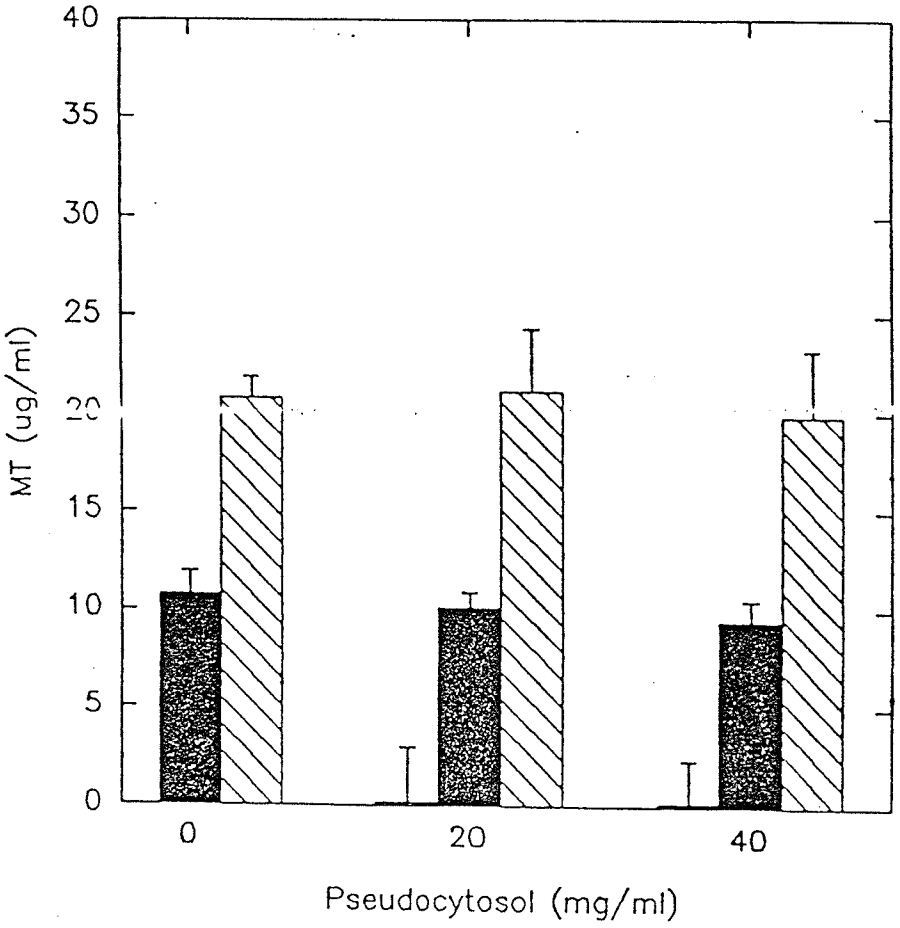

C
40
30
20
10
0
MT (ug/ml)
0.00
0.25
0.50
0.75
1.00
Dilution

Fig. 4. Means and 95% confidence intervals for MT estimates from a dilution series of pseudocytosol containing Sigma rabbit liver MT-II. MT standards are indicated as 0 mg pseudocytosol/mL. The four dilution-corrected data points from the binding curves in Fig. 3A are represented by the group of bars at the left, while the data from Fig. 3B and Fig. 3C are in the center and right bars, respectively.

0 µg MT/mL - (open bar); 10 µg MT/mL - (filled bar); 20 µg MT/mL - (hatched bar).


40
35
30
25
20
15
10
5
0
MT (ug/ml)
0
20
40
Pseudocytosol (mg/ml)

Systematic, dilution-dependent interference was observed among the mercury-binding curves, which had non-zero slopes, (Fig. 5B,C). The importance of dilution in a competitive binding assay is readily seen, in that the MT estimates approached target values in the 1/9- and 1/18-diluted replicates.

Again, the dilution-corrected mercury-binding curves were necessary to illustrate the systematic nature of interference in the assay. When presented in a conventional manner, as a mean and confidence interval, the dilution-dependent interference is revealed by reduced precision and accuracy of the assay (Fig. 6). The loss of precision is indicated by confidence intervals for MT which were too large to statistically distinguish between MT treatment levels when GSH was present. The loss of accuracy can be seen from the elevated MT estimates in all treatment combinations, and is particularly evident in GSH samples that did not contain MT.

Assessing Metal Displacement

Using gel filtration chromatography, it was possible to demonstrate zinc, cadmium, and copper displacement from MT by the mercury saturation assay (Fig. 7). Although zinc, cadmium, and copper were present in the TCA supernatant, they eluted from the Sephadex G-50 column predominantly in low-molecular-weight fractions (Fig. 7B). A small proportion

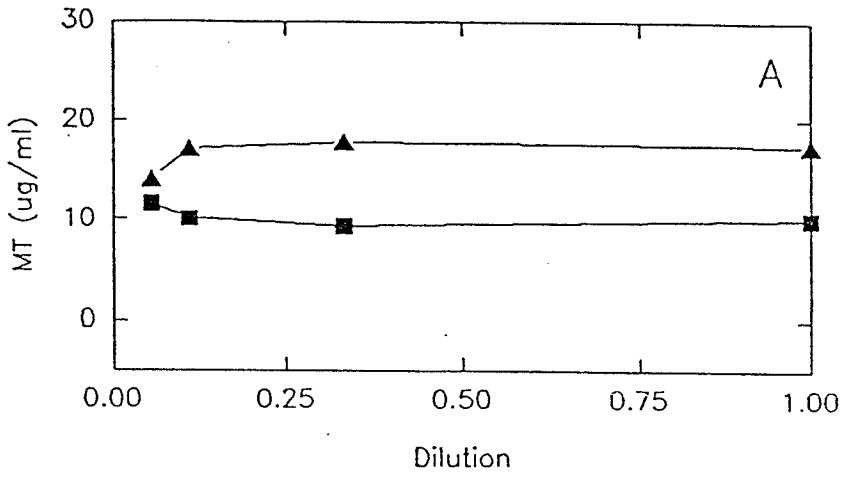
Fig. 5. Dilution-corrected mercury binding curves for samples containing GSH. Each data point represents the MT estimate of an individual dilution replicate.

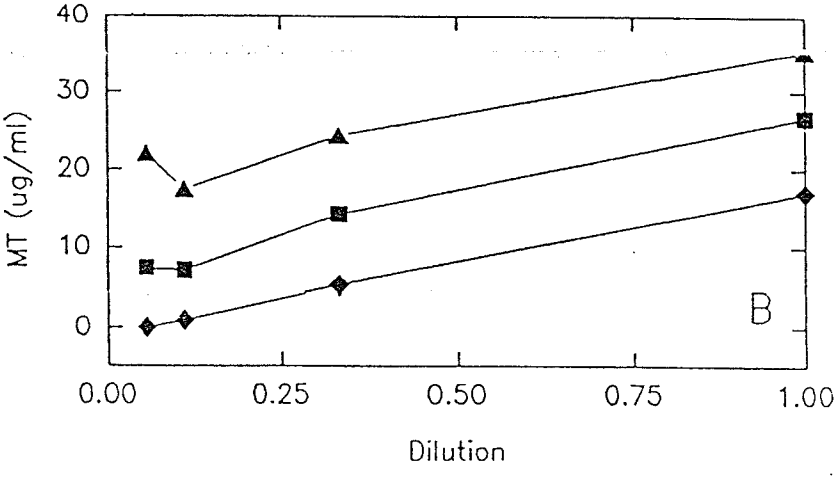
A. Sigma rabbit liver MT II standards.

B. MT standards in the presence of 2.53 mM GSH.

C. MT standards in the presence of 5.06 mM GSH.

0 μg MT/mL - (diamond); 10 μg MT/mL - (square); 20 μg MT/mL - (triangle).


30
20
10
0
MT (ug/ml)
A
0.00
0.25
0.50
0.75
1.00
Dilution


40
30
20
10
0
MT (ug/ml)
B
0.00
0.25
0.50
0.75
1.00
Dilution

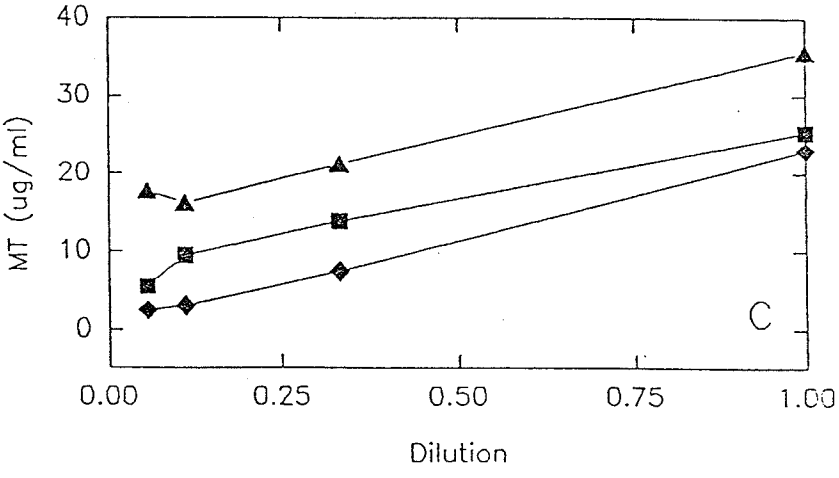

40
30
20
10
0
MT (ug/ml)
C
0.00
0.25
0.50
0.75
1.00
Dilution

Fig. 6. Means and 95% confidence intervals for MT estimates from a 4-replicate dilution series of Sigma rabbit liver MT II standards in the presence of GSH. The four dilution-corrected data points from the binding curves in Fig. 5A are represented by the group of bars at the left, while the data from Fig. 5B and Fig. 5C are in the center and right bars, respectively.

0 μg MT/mL - (open bar); 10 μg MT/mL - (filled bar); 20 μg MT/mL - (hatched bar).

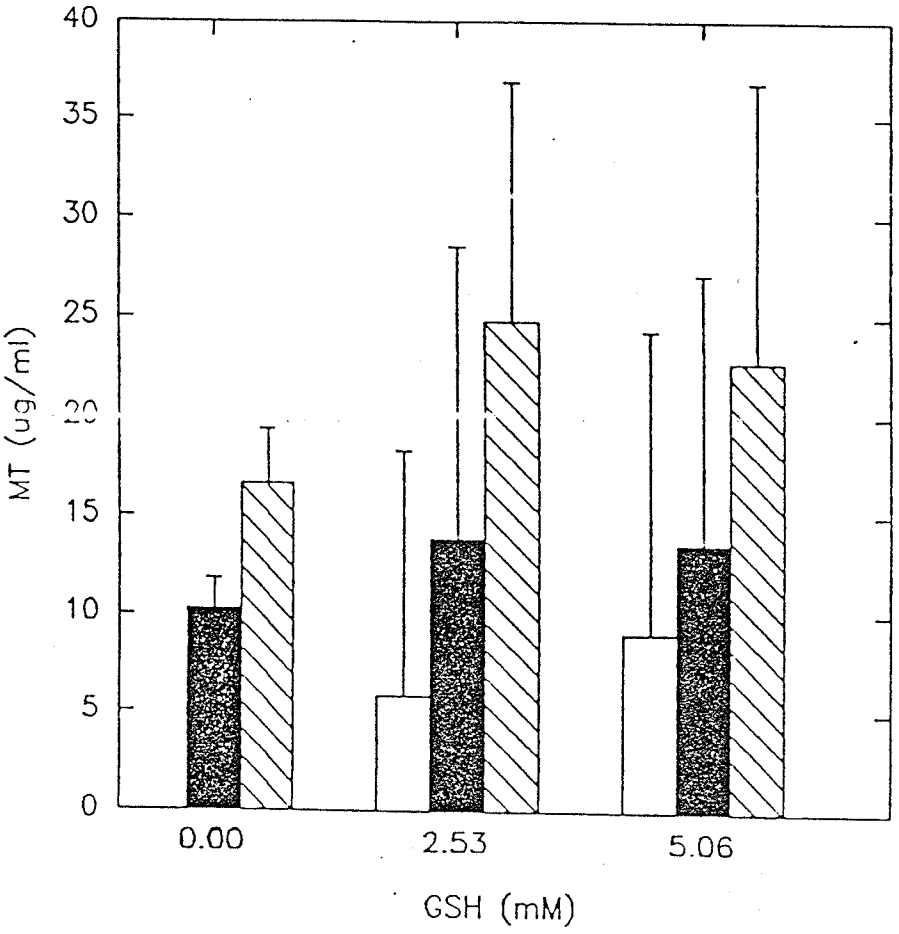
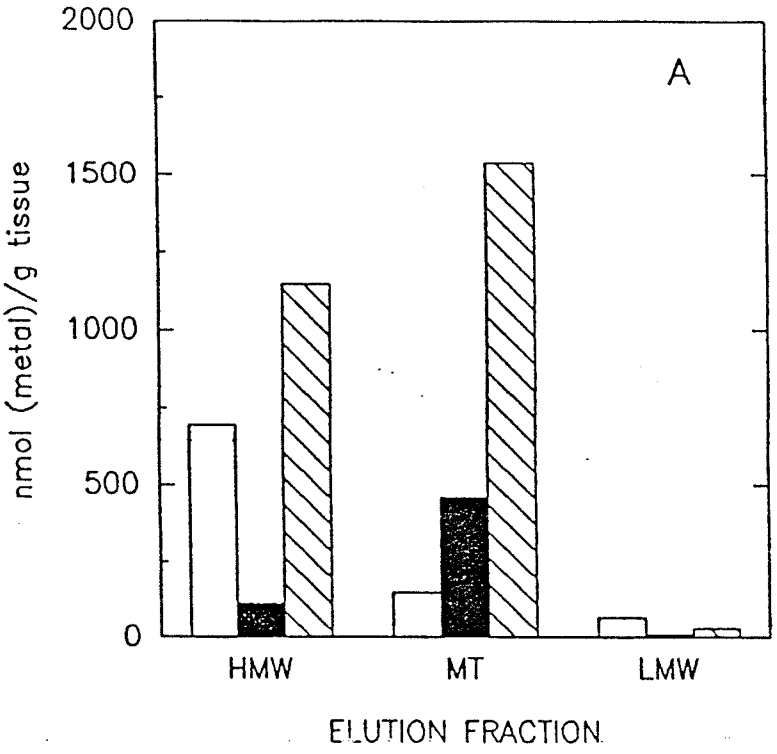
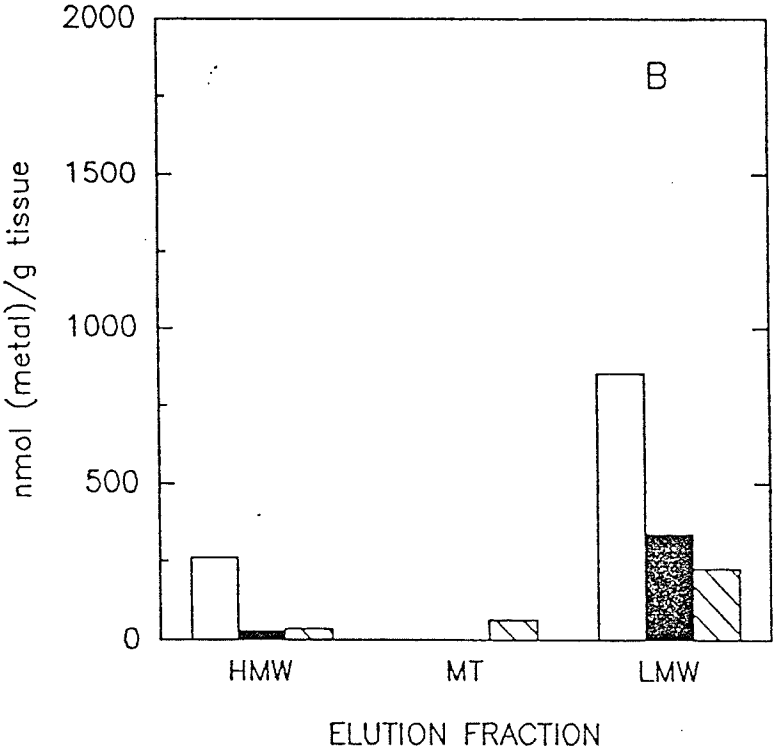

40
35
30
25
20
15
10
5
0
MT (ug/ml)
0.00
2.53
5.06
GSH (mM)

Fig. 7 Metal content (nmol/g tissue) in cytosolic fractions of rainbow trout liver (cadmium-exposed) before (A) and after (B) analysis of MT by the mercury saturation assay. The cytosols were fractionated on a Sephadex G-50 column into high-molecular-weight (HMW;fractions 9-13), metallothionein (MT;fractions 14-20), and low-molecular-weight (LMW; fractions 21-32) components. Values have been corrected back to a per gram of tissue basis using the tissue dilution factor (g of supernatant per g tissue) and the column loading rate (g of supernatant applied to the Sephadex G-50 column).

Open bar - zinc; Filled bar - cadmium; Hatched bar - copper.


2000
1500
1000
500
0
nmol (metal)/g tissue
A
HMW
MT
LMW
ELUTION FRACTION


2000
1500
1000
500
0
nmol (metal)/g tissue
B
HMW
MT
LMW
ELUTION FRACTION

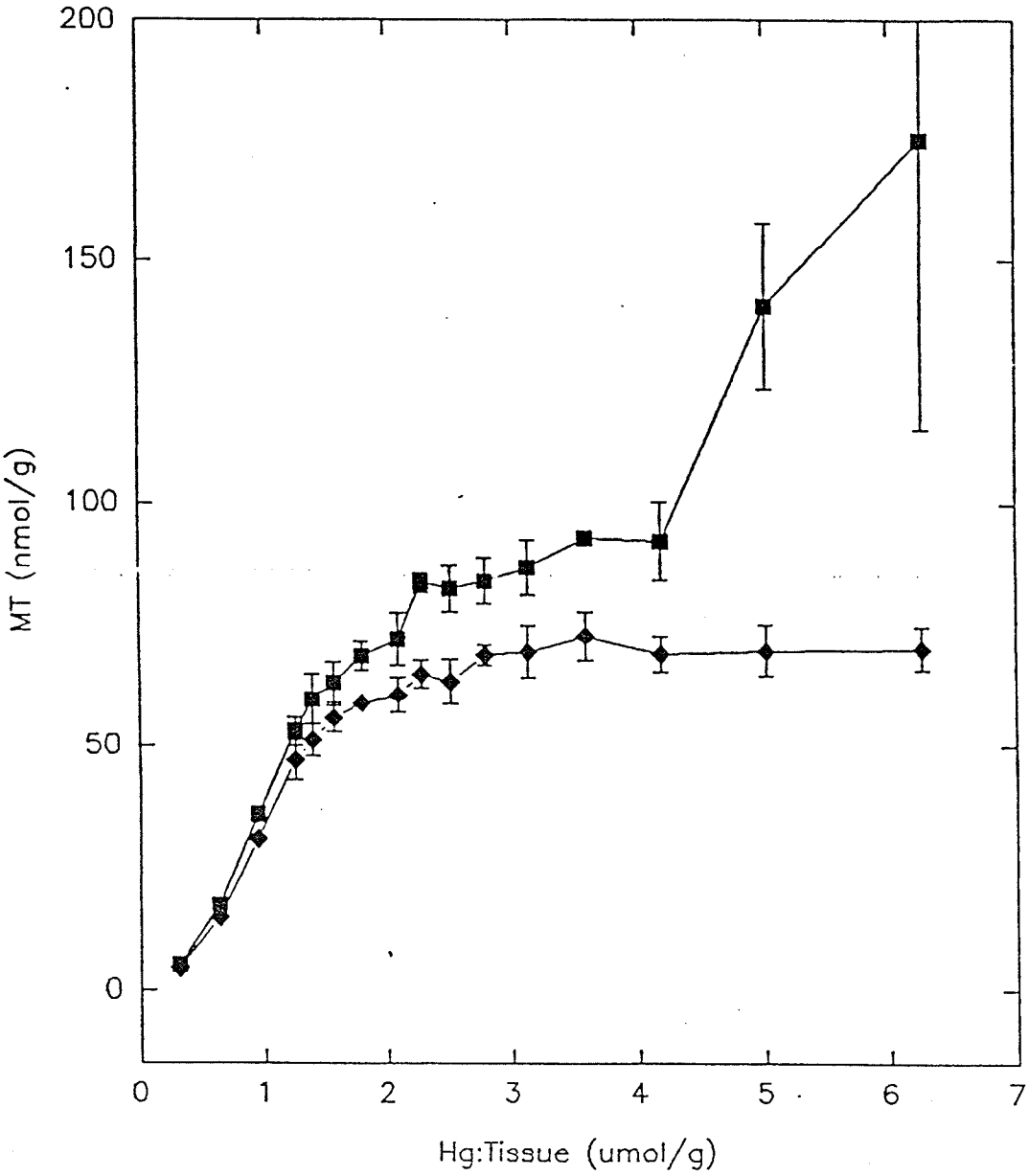
(4.2%) of the original 1538 nmol Cu/g tissue which was contained in the MT fraction remained in the MT fraction of the TCA supernatant. No cadmium or zinc remained in the MT fraction of the TCA supernatant.

Comparing this Assay with the Piotrowski Mercury Saturation Assay

Rainbow trout hepatic MT concentration was estimated from a mercury-binding curve (Fig. 8) similar to that described by Piotrowski et al. (1973) and Kotsonis and Klaassen (1977). The mean MT concentration of 87.13 nmol/g was estimated from the six points comprising the plateau of the binding curve (ie. between 2.25 and 4.25 µmol mercury/g tissue). After treatment with 50% egg white, the MT concentration was 68.01 nmol MT/g (estimated from the same six data points). The smaller MT estimate, and the fact that the plateau region of the binding curve extended to the right after egg white treatment, indicate that it was excess mercury which was removed by the egg white.

The MT estimate produced by the mercury saturation assay was 45.38 nmol MT/g ± 6.24 (SEM). MT estimates from the Piotrowski assay and the modified Piotrowski assay were in close agreement with this value where the two binding curves diverge (at 1.5 µmol mercury/g tissue; Fig. 8), but when MT concentrations were estimated from the plateau regions of the

Fig. 8. Mercury-binding curves for rainbow trout homogenate. Tissue homogenates were incubated with variable quantities of mercury in 10% TCA, samples were centrifuged, and TCA supernatants were assayed for mercury (square). After mercury analysis, 400 µL of 50% egg white solution was added to all TCA supernatants, which were then recentrifuged and reassayed for mercury (diamond).


200
150
100
50
0
MT (nmol/g)
0
1
2
3
4
5
6
7
Hg:Tissue (umol/g)

mercury-binding curves, the respective estimates were 192 and 150% of the MT estimate produced by the mercury saturation assay.

Assessing Sample Preparation Procedures

In the absence of cadmium exposure, the rainbow trout MT was essentially a copper-containing thionein. Copper eluted in both high-molecular-weight and MT fractions after centrifugation (Fig. 9A), but after heat treatment, a large quantity of copper also eluted in the low-molecular-weight fractions (Fig. 9B).

In the S10 and S30 supernatants, zinc was present in high-molecular-weight and MT fractions (Fig. 10A), but after 2 or 10 minutes of heat denaturation, zinc was lost from the MT fractions, and was greatly reduced in the high-molecular-weight fractions (Fig. 10B).

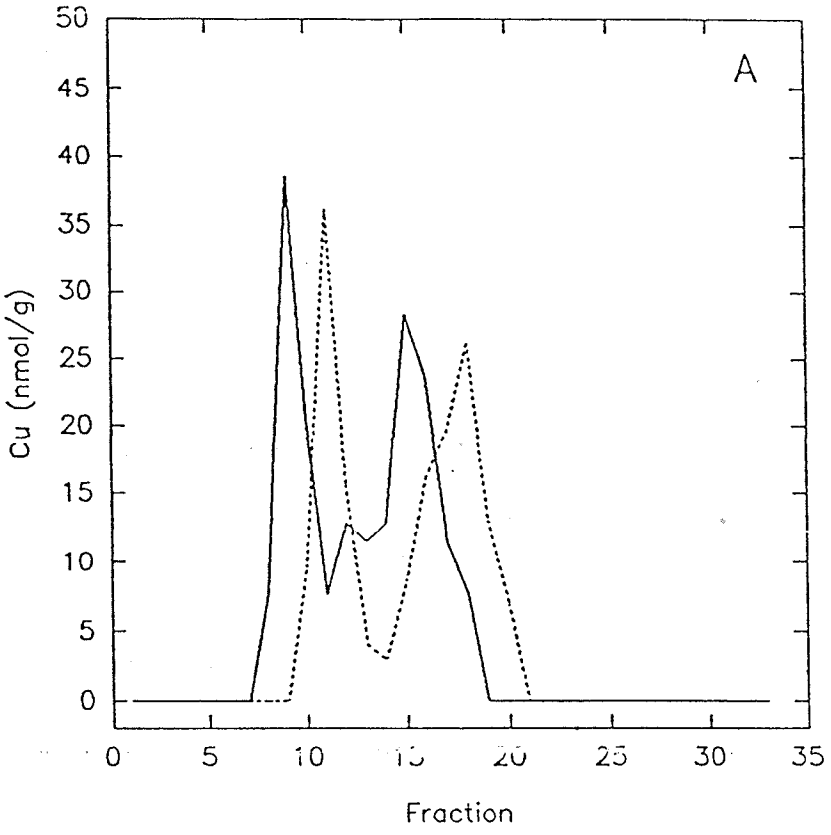
The estimates of MT concentration in rainbow trout liver produced by the various sample preparation methods are presented in Table 1. The lowest estimates of MT were produced by molar summation of copper and zinc eluting from the gel filtration column in fractions corresponding to the elution volume of rabbit liver MT (V_{MT}). In the four supernatants analyzed by metal summation (S10, S30, HDS2, and HDS10), the estimates varied between 6.89 and 9.05 nmol/g.

Supernatants from all eight sample preparation protocols

Fig. 9. Distribution of copper in supernatants generated by different sample preparation procedures. Metal concentrations in each fraction have been corrected to a per gram of tissue basis based on the tissue dilution factor (g of supernatant per g tissue) and the column loading rate (g of supernatant applied to the Sephadex G-50 column). MT eluted between fractions 14 and 20.

A. Centrifuged supernatants: (solid line) S10; (broken line) S30.

B. Heat-treated supernatants: (dotted line) HDS2; (broken line) HDS10.


A
Cu (nmol/g)
Fraction
0
5
10
15
20
25
30
35
40
45
50

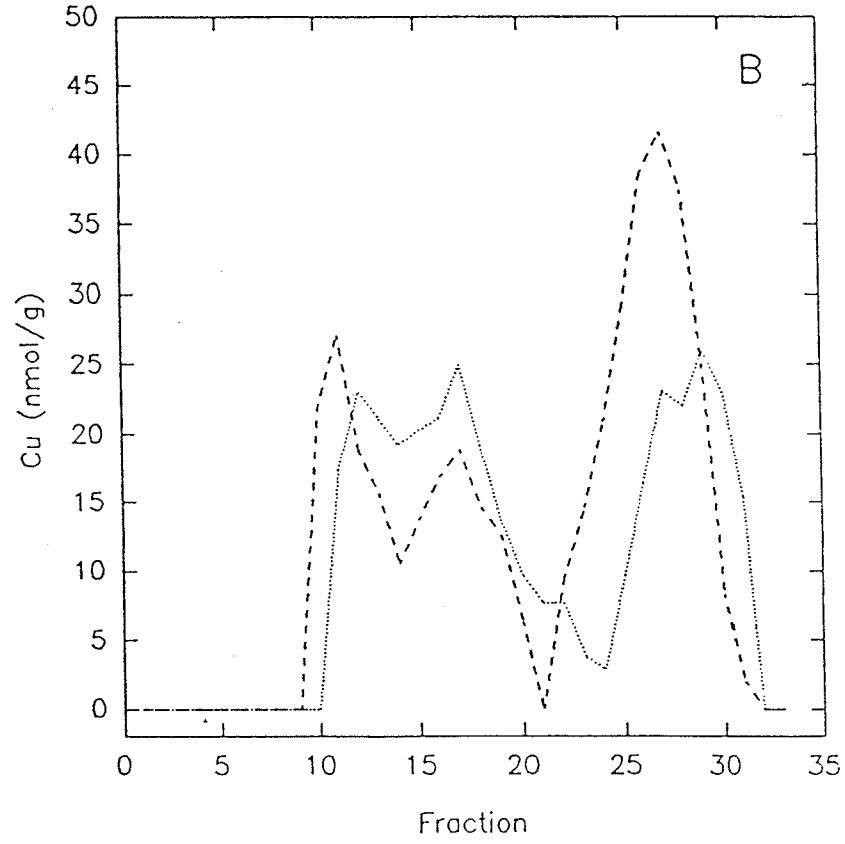
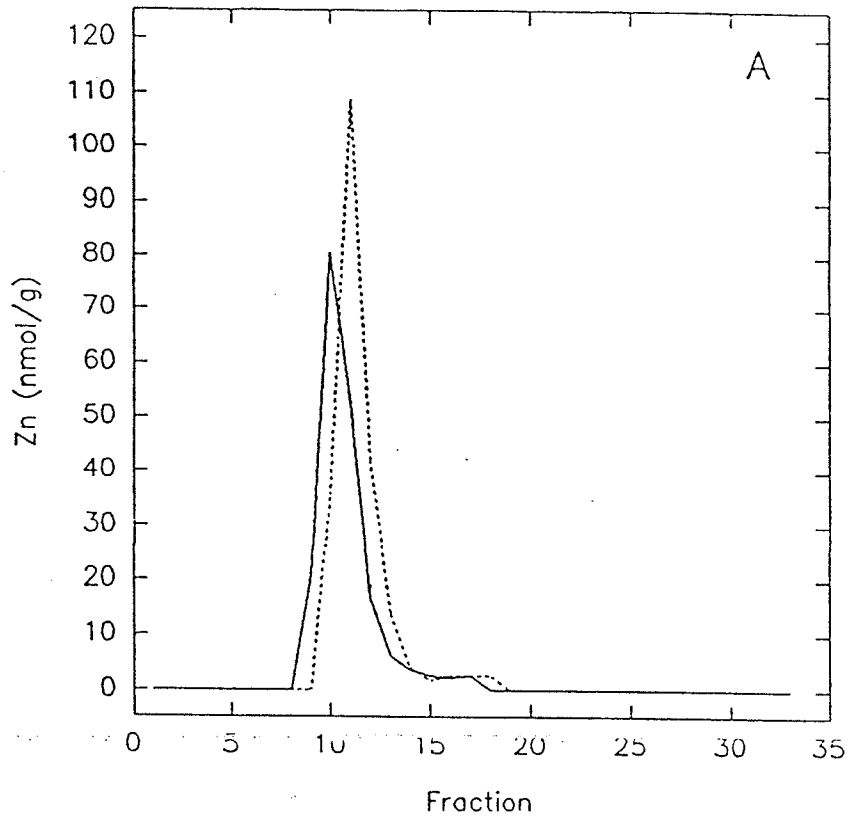
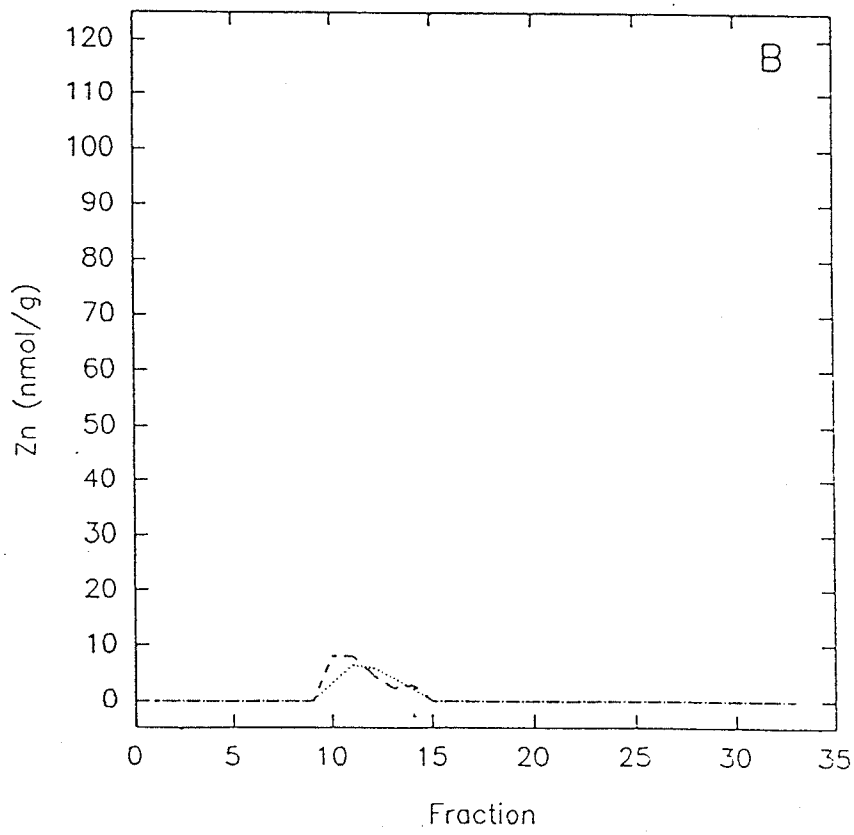

B
Cu (nmol/g)
Fraction
0
5
10
15
20
25
30
35
40
45
50

Fig. 10. Distribution of zinc in supernatants generated by different sample preparation procedures. Metal concentrations in each fraction have been corrected to a per gram of tissue basis (See Fig. 9). MT eluted between fractions 14 and 20.

A. Centrifuged supernatants: (solid line) S10; (broken line) S30.

B. Heat-treated supernatants: (dotted line) HDS2; (broken line) HDS10.


A
Zn (nmol/g)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Fraction


B
Zn (nmol/g)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Fraction

were analyzed by the mercury saturation assay, and 3 statistically distinct groupings of MT estimates resulted (Table 1). Of the protocols tested, only the HDS1 estimate encompassed all of the metal summation results. However, the S10, S30, and HDS½ estimates were not significantly different from that of HDS1. With increased time of heat denaturation, MT estimates increased, but the highest (and most variable) estimate of MT was derived from tissue homogenate.

DISCUSSION

Criteria for validating MT metal saturation assays include specificity, sensitivity, accuracy, reproducibility, linearity (Spry and Wood 1989), rapidity, and low cost (Eaton and Toal 1982).

In MT metal saturation assays, MT is detected by the binding of cadmium, silver, or mercury to its cysteinyl thiol residues. For a MT metal saturation assay to be specific, it is essential to minimize the binding of displacing metal to non-MT thiols in the final assay supernatant.

The interfering effects of such non-specificity on metal saturation assays has been documented for both cadmium (Eaton

Table 1. Estimates of MT concentration (nmol MT/g tissue) produced by different sample preparation methods. Each value represents the mean ± SD for a 4-replicate dilution series. Means with the same letter are not significantly different. Sample abbrev-iations: HGT (liver homogenate); S10 (supernatant from a 10 min centrifugation at 10,000g); S30 (supernatant from a 30 min centrifugation at 10,000g); HDS½-10 (heat-denatured supernatants prepared by heat-treatment for 0.5-10 minutes).

Sample	Metal Summation Estimate	^{203}Hg Estimate	
HGT	-	27.32 ± 14.76	a,b
S10	8.06	11.27 ± 2.03	b,c
S30	8.89	14.50 ± 4.02	a,b,c
HDS½	-	10.92 ± 2.22	b,c
HDS1	-	9.01 ± 2.53	c
HDS2	6.89	18.95 ± 1.91	a
HDS6	-	20.87 ± 2.44	a
HDS10	9.05	15.20 + 1.74	b

1985), and mercury (Zelazowski and Piotrowski 1973, Dieter et al. 1987). In the development of this mercury saturation assay, as elsewhere (Eaton and Toal 1982), GSH (alone and in combination with enzymes and other thiol-containing compounds in a pseudocytosol) was used to evaluate interference. In this assay, a dilution-series approach was used to detect such interference. Samples containing MT and GSH were diluted up to 18-fold and incubated with an excess (but constant) amount of mercury, followed by the addition of 50% egg white solution in sufficient (but constant) quantity to remove all mercury from solution. Over the range of dilution, the egg white-to-sample ratio increased 18-fold, the binding of mercury by GSH was reduced, and interference was detectable as a non-zero slope of the mercury-binding curve (Fig. 5).

The concentrations of GSH and cysteine in the pseudocytosol were chosen because they probably represent the maximum concentrations found in liver (Cooper 1983, Meister and Anderson 1983). When MT was assayed in the presence of GSH, interference was observed (Figs. 5 and 6).

However, when the concentration of GSH which interfered with the assay was included in a pseudocytosol containing several thiol-rich proteins and peptides (including MT), no interference was observed (Figs. 3 and 4). The lack of interference of GSH when present in the pseudocytosol suggests that interference due to the binding of mercury to acid-soluble thiols such as GSH is not likely to be a problem in

complex samples such as tissue cytosols.

The requirement of linearity of calibration curves (Spry and Wood 1989) should be restated more stringently as a requirement for directly proportional linearity. The relationship between tissue dilution and metal-binding capacity of a sample must be directly proportional, so (for example) when a sample is diluted by a factor of 18, the resultant binding capacity should also be 1/18 that of the undiluted sample. Spry and Wood (1989), and Eaton and Toal (1982) have demonstrated linearity in cadmium displacement assays, but the fact that the linear relationships were not directly proportional shows that the MT estimates were dilution-dependent. However, other researchers (Steadman et al. 1991) found the cadmium saturation assay to be non-linear (with respect to dilution) when used to analyze rainbow trout MT. In both cases, the lack of directly proportional linearity is probably due to incomplete displacement of copper from MT by cadmium (Eaton 1985). Copper is extremely important in this respect, since it is the most prevalent metal bound by rainbow trout MT (Olsson and Haux 1985).

The sensitivity of metal saturation methods of MT analysis is generally thought to be poor. They have been considered to be suitable only for measuring induced concentrations of MT (Waalkes et al. 1985), for screening analysis (Dieter et al. 1987), or for providing ballpark estimates of MT (Shaikh and Nolan 1987). In contrast, the RIA is thought to be the most

sensitive method for MT detection (Cherian 1988), with a useful detection limit of 1-300 ng of MT (VanderMallie and Garvey 1979, Hogstrand and Haux 1989). This mercury saturation assay detected 111 ng of MT (in the 1/18 dilution of a 10 µg/mL MT standard), well within the working range of the RIA. Coupled with the simplicity of the mercury method, this degree of sensitivity is adequate for most environmental research requiring MT analysis.

A characteristic of the mercury saturation assay of Piotrowski et al. (1973), the overestimation of MT (Dieter et al. 1987), has been reaffirmed here (Fig. 8). This characteristic overestimation of MT by the Piotrowski mercury saturation assay relates specifically to the strategy used in the assay. By failing to add exogenous cleanup proteins such as hemoglobin or egg albumin, acid-soluble mercury-binding ligands remain in solution, to be quantified as if they were MT. By adding exogenous proteins, the interfering TCA-soluble ligands are stripped of mercury, thereby minimizing this source of overestimation. At the same time, these cleanup proteins do not appear to strip mercury from MT, enabling an accurate MT estimate (Figs. 2,8).

The two mercury-binding curves in Fig. 8 are very similar to the left of the plateau regions (mercury-to-tissue ratios below 1.5 µmol/g), and, where the curves diverge, the MT estimates agree closely with the MT estimate from the mercury saturation assay presented here. However, the mercury-binding

curves plateau at mercury-to-tissue ratios greater than 1.5 µmol mercury/g, and it is from the plateau region that MT concentrations are estimated in the Piotrowski assay. In this experiment, the MT concentrations from the Piotrowski assay before and after egg white treatment were, respectively, 192 and 150% greater than the mercury saturation MT estimate, demonstrating the tendency of the method to overestimate MT.

The displacement of the metals bound by MT in vitro by the displacing metal is essential to verify the effectiveness of a MT metal saturation assay. The demonstration of copper displacement has not been undertaken in most MT metal saturation assays described in the literature. Scheuhamer and Cherian (1986) documented cadmium displacement by silver in their silver saturation assay, but copper displacement was only 59%. Klein et al. (1990) demonstrated copper displacement to be approximately 62% in their cadmium saturation assay. In methods development for this mercury saturation assay, the MT used was predominantly a copper-MT from rainbow trout liver of high copper concentration (6877.1 nmol Cu/g tissue). The copper content which eluted in the MT fractions upon gel filtration chromatography accounted for 1538.3 nmol of the total hepatic copper (Fig. 7). After mercury saturation, only 65.2 nmol of copper could be accounted-for in the MT fraction of chromatographed TCA supernatant, indicating that 96% of the copper had been displaced. Similar levels of copper displacement have not been documented in the literature. Coupled with

the complete displacement of cadmium and zinc by the mercury saturation assay, this high degree of copper displacement further supports the value of mercury as a displacing metal.

Several additional considerations favor the use of the mercury saturation assay over other methods of MT detection. At low pH, mercury is the most specific thiol-combining agent (Jocelyn 1972, Fuhr and Rabenstein 1973, Stricks and Kolthoff 1953). Therefore, it is an obvious choice as a displacing metal for quantifying MT. Because copper, cadmium, and zinc can be removed from MT at low pH (Rupp and Weser 1978, Pulido et al. 1966), their replacement by mercury is favored. A second benefit of using acidic conditions for the assay is that the removal of excess mercury is simplified to one rapid step. Egg white solution is added to the TCA-containing assay tubes, where it denatures on contact and is separated from mercury-MT by centrifugation. Egg white is used because it is readily available, simple to prepare, and more effective than hemoglobin at removing mercury from solution. In contrast, cadmium and silver saturation assays are conducted at circum-neutral pH. The removal of excess cadmium or silver is achieved by 3 additions of blood hemolysate followed by heat treatment and centrifugation after each addition. These procedures are cumbersome and time-consuming in comparison with the mercury saturation assay.

Rapidity and technical ease of performance are important aspects of metal saturation assays for MT analysis. Eight such

assays have been compared on the basis of these two considerations (Table 2). Technical ease is evaluated in terms of steps which must be undertaken to complete the analysis; fewer steps indicate greater technical ease. Adding a reagent to a vial, transfering a sample between vials, mixing a sample, cooling a sample on ice, and centrifugation of a sample all constitute steps.

In terms of technical ease, the best methods are this mercury saturation assay (6 steps), and the cadmium saturation assay described by Hamilton et al. (1987) (7 steps). The remaining six methods require between 11 and 20 steps. The cadmium saturation assay of Klein et al. (1990) requires the greatest number of steps of any assay examined (20 steps). It is relevant that their assay is specifically designed to determine MT with high copper content. The large number of steps reflects the need for the chelation of copper away from MT prior to cadmium saturation. By using mercury as a displacing metal, these considerations can be bypassed, resulting in a much simpler assay.

In terms of time requirements, the mercury saturation assay requires three minutes to complete per replicate (Table 2), while the other cadmium and silver saturation assays require between 18 and 51 minutes per analysis. The incubation period of the mercury-displacement assay is very rapid; no differences in MT estimates were observed between samples which were incubated for between 0.5 and 10 min, so 1 min incubation is

Table 2. A comparison of technical requirements for 8 metal saturation assays. Values in all columns were extracted from the materials and methods sections of the referenced papers.

Reference	Sample Preparation Time (min)	Assay Time (min)	Number of Steps Required
Eaton and Toal (1982)	18	18	11
Onasaka and Cherian (1982)	20	23	13
Scheuhamer and Cherian (1986)	20	24.5	12
Nolan and Shaikh (1986)	20	25.5	13
Hamilton et al. (1987)	83	31	7
Spry and Wood (1989)	12	44	15
Klein et al. (1990)	60	51	20
This paper	10	3	6

sufficient. This method comparison demonstrates that the mercury saturation assay is more rapid and easier to perform than seven conventional cadmium and silver saturation assays.

One aspect of MT metal saturation assays which has not been thoroughly addressed is the importance of sample preparation procedures used to isolate MT prior to analysis. Sample preparation may be the most time consuming aspect of a metal saturation assay, and the speed of a rapid metal saturation method may be seriously diminished by a slow sample preparation procedure (Table 2). Furthermore, if the prepared samples are affected in such a way as to limit accuracy of detection, the sample preparation procedure must be reconsidered.

Heat treatment is often used during sample preparation to selectively denature proteins other than MT, which is heat stable (Eaton and Toal 1982, Spry and Wood 1989). Most methods use a centrifugation step prior to heat treatment, but this is slow, so Lauren and McDonald (1987) heat-treated homogenate without prior centrifugation, speeding up sample preparation. Unfortunately, heat treatment alters zinc and copper distributions in rainbow trout liver cytosols (Figs. 9 and 10), releasing large quantities of copper into the supernatant after only 2 minutes. The presence of large quantities of copper is undesirable, especially for cadmium displacement assays, which are known to be adversely affected by copper (Eaton 1985, Cherian 1988).

Clearly, the choice of sample preparation procedure can

greatly affect the estimation of MT (Table 1). High, variable MT estimates produced from direct analysis of whole homogenate indicates that some treatment to isolate MT is necessary with the mercury saturation assay. Centrifugation (10 min at 10,000g; S10) was the simplest procedure tested and it produced results in agreement with the metal summation, S30, HDS½, and HDS1 estimates of MT. Short heat treatment steps (less than 2 min) produced consistent MT estimates. Lengthy heat treatments may more thoroughly remove proteins from the samples, but this appears to be offset by an elevation of metal binding in heat-treated supernatants, as indicated by high MT estimates in HDS2, HDS6, and HDS10 samples. Therefore, sample preparation procedures (centrifugation or short heat treatments) which do not alter in situ cytosolic metal distributions are recommended. This is particularly important if cytosolic metal distributions are to be examined.

The recovery of a spike or internal standard of MT can be used to verify the accuracy of a metal saturation assay (Zelazowski and Piotrowski 1977, Dieter et al. 1987). With the mercury saturation assay, the addition of spikes of rabbit liver MT-II to homogenates prior to sample preparation is routinely used to check sample preparation losses and assay accuracy. Recovery of MT spikes from 53 liver, kidney, gill, and intestine homogenates from white sucker (Catostomus commersoni) and lake charr (Salvelinus namaycush) was 98.1% ± 2.1 (SEM), indicating that neither sample preparation pro-

cedures or the mercury saturation assay cause the loss or degradation of MT (J.F. Klaverkamp, unpublished data).

Research on the role of MT in the toxicology of cadmium, copper, zinc, and mercury, and the use of this protein as a biochemical indicator of metal exposure stress and acclimation to metal toxicity, has been hindered by the lack of a consistent, validated method for measuring MT (Haux and Forlin 1988). Using metal binding characteristics as a means of measuring MT has been criticized because it is an indirect measure of MT (Dieter et al. 1987). However, the metal-binding characteristic is a hallmark of MT (Hamer 1986) and is the only reliable detection method for MT other than immunological methods (Kikuchi et al. 1990). Therefore, saturating MT with mercury under acidic conditions provides MT metal-binding data that are reliable and highly specific. This specificity is ensured by dilution replication.

In summary, the mercury saturation method presented here overcomes weaknesses commonly cited for metal saturation assays for metallothionein. The method is simple, specific, rapid, and can be standardized with commercially available MT. The mercury saturation assay therefore should serve as a reliable and standardized method for metallothionein measurement in fish.

CHAPTER III

HEPATIC AND RENAL METALLOTHIONEIN IN WHITE SUCKER (*Catostomus commersoni*) AND NORTHERN PIKE (*Esox lucius*) IN RELATION TO COPPER, ZINC, AND CADMIUM IN LIVER, KIDNEY, AND SEDIMENT FROM LAKES NEAR THE SMELTER AT FLIN FLON, MANITOBA, CANADA

INTRODUCTION

The copper/zinc smelter at Flin Flon, Manitoba, has been in production since 1930 (Franzin 1984). Until 1974, off-gases from ore roasting and smelting were released directly to the atmosphere from two stacks of 58 and 69 meters in height. Since 1974, the off-gases have been released from a 251 meter stack which was installed to reduce deposition of sulfur dioxide, particulates, and metals on the Flin Flon townsite (Klaverkamp et al. 1991). Electrostatic precipitators were installed in 1982 in an effort to reduce particulate emissions (Zoltai 1988, Phillips et al. 1986).

Over the time period covering these changes at the Flin Flon smelter, several studies have documented the status of aquatic ecosystems in the vicinity of the smelter. Van Loon and Beamish (1977) found that fish populations collected in 1973-1974 from eight lakes near Flin Flon were surviving in lakes with high zinc concentrations, and were more tolerant of elevated zinc concentrations than would be expected from extrapolations from laboratory toxicity tests. If major changes in recruitment, growth, and survival of the fish populations were occurring, they were long-term changes (Van Loon and Beamish 1977). Based on sampling conducted in 1976 and 1977, McFarlane and Franzin (1978) concluded that the white sucker (Catostomus commersoni Lacepede) population from Hamell Lake (located approximately 5 km west of the smelter)

was stressed by the elevated levels of heavy metals in the lake. In toxicity testing conducted in 1981, white suckers from Hamell Lake were 1.9 and 2.3 times more tolerant to 10 and 30 mg Cd/L (respectively) than white suckers from the relatively unpolluted Thompson Lake (Klaverkamp et al. 1991). This increased tolerance was accompanied by corresponding increases of 2.7 and 2.0-fold (respectively) in hepatic and renal MT in Hamell Lake suckers relative to the Thompson Lake fish. The findings from these studies near Flin Flon, coupled with the known ability of fish to acclimate to heavy metal exposure (Klaverkamp et al. 1984) suggested the possibility that metallothionein could be used as an indicator of metal contamination in freshwater ecosystems.

The research presented in this chapter follows from the previous research near Flin Flon by testing the hypothesis that metallothionein is a more sensitive biomarker of heavy metal pollution than whole-tissue metal concentrations. Rather than comparing just two lakes (Klaverkamp et al. 1991), eight lakes were studied along a presumed gradient of metal deposition near the Flin Flon smelter. This in turn permitted testing for a dose-response relationship between MT and metal exposure (metal exposure was indicated by metal concentrations in lake sediments). Such a relationship is an important property of a successful biomarker (Haux and Forlin 1988, Henderson et al. 1989, Phillips 1980).

MATERIALS AND METHODS

Study Area

Eight lakes between 5 and 31 km from the Flin Flon smelter were selected to provide a gradient in metal deposition among the study lakes. These lakes were located approximately on the major (northwest-southeast) axis of the broadly elliptical deposition area around the smelter (Zoltai 1988), and were within the zone of significant deposition (Harrison and Klaverkamp 1990). The locations and morphometry of the study lakes are presented in Figure 11 and Table 3. Distances from the smelter to the sites of sediment core sampling in the lakes were measured from 1:50,000 topographical maps.

The study area is near the southwest boundary of the Precambrian Shield. The majority of consolidated rock in the area is Precambrian, although evidence of the transitional nature of the region is seen in outliers of dolomite and Pleistocene glacial debris (Heywood 1966, Zoltai 1988).

Water Sampling

A single water sample was collected from each lake at arm's length below the water surface in 1 L acid-washed

Figure 11. Map of the study area at Flin Flon, Manitoba.

MAP OF THE STUDY AREA.

LAKE	SURFACE AREA	MAXIMUM DEPTH	DISTANCE FROM SMELTER
JOHNSON	677 ha	3.0 m	25.7 km
TYRELL	441	7.9	21.1
NESOOTAO	40	10.0	12.4
HAMELL	233	6.1	5.8
HOOK	90	18.3	9.8
WHITE	110	13.4	11.4
NESO	338	13.4	25.1
TWIN	185	24.4	31.0

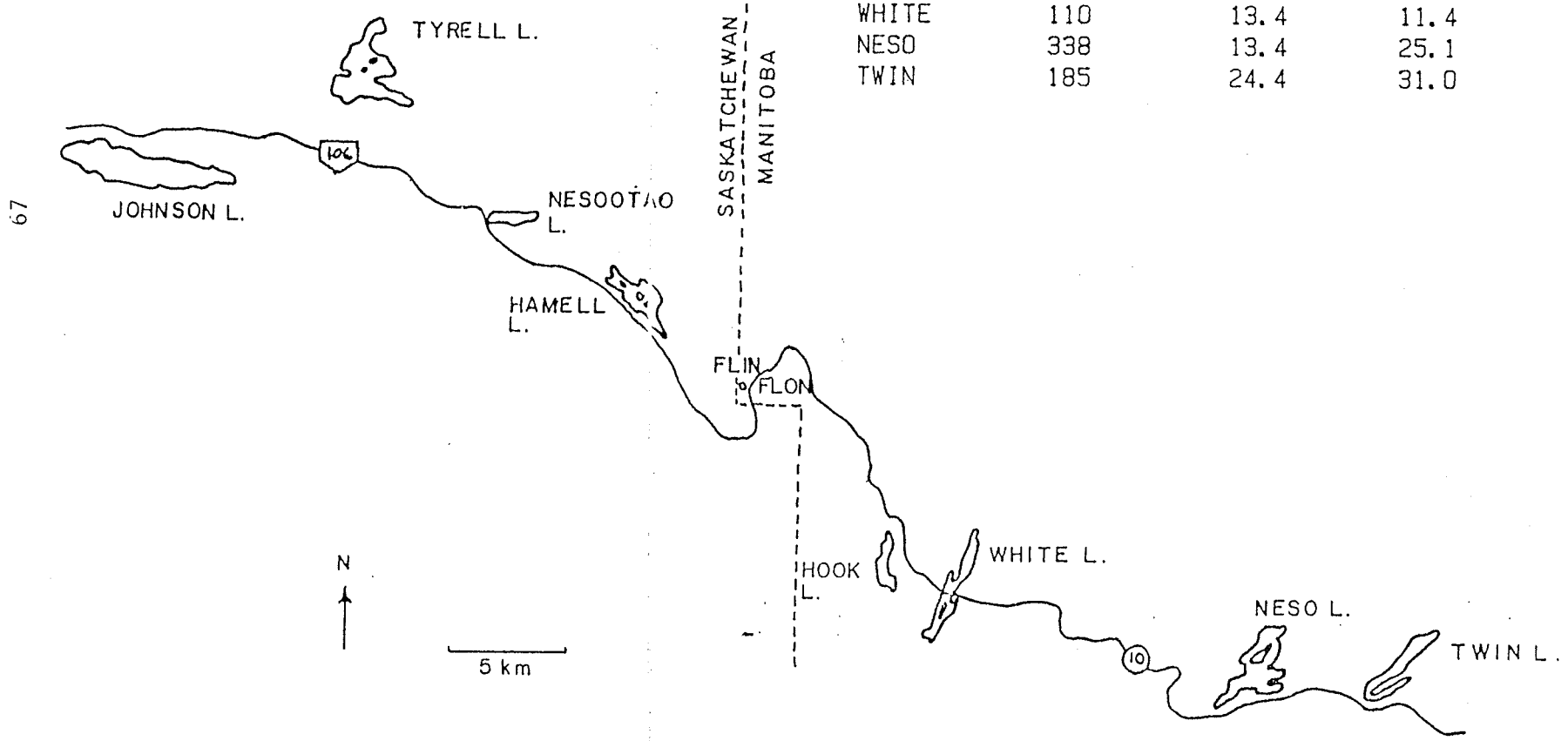


Table 3. Location, morphometry, and selected water chemistry for the study lakes.

Lake	Dist (km)	Area (ha)	pH	Ca (umol/L)	Mg (umol/L)	Na (umol/L)	K (umol/L)	Cl (umol/L)	SO_4 (umol/L)	Alkalinity (ueq/L)	Cond (uS/cm)	DIC (umol/L)	DOC (umol/L)
Hamell	5.8	233	7.6	311.9	108.6	87.0	34.0	124.1	123.9	590.1	98	310	1030
Hook	9.8	90	8.3	890.7	310.2	92.2	32.0	73.3	129.1	2194.4	229	1960	830
White	11.4	110	8.0	406.7	238.2	87.0	26.9	36.7	94.7	1167.6	126	730	1220
Nesootao	12.4	40	7.5	217.6	81.3	69.2	27.9	28.2	104.1	464.1	70	190	1290
Tyrell	21.1	441	7.4	141.0	71.0	64.8	24.9	19.7	60.4	389.0	50	170	1260
Neso	25.1	338	7.6	205.6	95.5	59.6	18.2	19.7	34.4	599.6	65	120	1210
Johnson	25.7	677	7.6	197.1	93.8	82.6	27.4	39.5	65.6	521.1	68	210	1660
Twin (small basin)	31.1	79	7.8	286.9	140.7	52.6	17.7	14.1	39.6	832.3	83	460	1050
Reference Lakes[a]													
ELA L. 240	-	44	6.5	44.9	32.9	39.1	7.7	22.6	43.7	92.4	21	120	606
ELA L. 313	-	114	6.6	54.9	28.8	43.5	10.2	22.6	45.8	106.9	19	79	904

a). Data from Beamish et al. (1976).

polyethylene bottles. Analyses for pH, SO_{42-} , Ca^{2+} , Mg^{2+} , Na^{+} , Cl^{-} , dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), and conductivity were conducted by the methods of Stainton et al. (1977).

Sediment Sampling and Metal Analysis

Four sediment cores were collected by SCUBA divers at the maximum depth in each lake, with the exception of Twin Lake, where cores were collected at the maximum depth of the smaller basin of the lake, at 8 m depth. Ten core sections of 1 cm thickness were sliced from the cores starting immediately below the sediment-water interface. Below 10 cm, 3 cm sections were removed, to a maximum core depth of 25 cm.

The sediment core sections were dried to constant weight, powdered, and digested in a nitric, perchloric, and hydroflouric acid digestion procedure (Sturgeon et al. 1982). Total copper, zinc, and cadmium were analyzed by atomic absorption on a Varian AA-5, using an air-acetylene flame.

Fish Sampling and Metal Analysis

White sucker (Catostomus commersoni Lacepede) and northern pike (Esox lucius Linnaeus) were collected by gill net in August and September of 1986. Nets were emptied

approximately every 30 minutes and the fish were transferred to a holding pen prior to processing. Fish were anaesthetized with buffered tricaine methanesulfonate (MS-222). Livers and kidneys were removed, immediately frozen in liquid nitrogen, and stored on dry ice in the field. Samples were stored at -120°C upon return to the laboratory. Left pectoral fins were removed for ageing according to the method of Chalanchuk (1983).

White sucker and pike were not collected from all eight of the study lakes. White suckers could not be caught in either Hamell Lake or Johnson Lake, so only six lakes are represented in analyses of white sucker data. Pike were collected only from the four study lakes northwest of the smelter in Saskatchewan, as a preliminary investigation of MT in this species, for which little information exists. In addition to the fish collected from the Flin Flon lakes, white suckers were collected from Lake 240 and Lake 313 of the Experimental Lakes Area (ELA) of Northwestern Ontario (approximately 800 km southeast of Flin Flon), in September 1988, to provide comparative reference material for aspects of the research. Reference pike were collected only from Lake 240.

Individual samples of liver and kidney (approximately 0.5 g wet weight) were digested in batches with a nitric, sulfuric, hydrogen peroxide method. Samples were made up to 25 mL with distilled, deionized water. Copper and zinc were measured by flame atomic absorption spectrophotometry on a Varian AA-5

with background correction, using an air-acetylene flame. Where necessary because of low concentrations, cadmium was analyzed after extraction by 5% diethyl dithioaminocarbamate (DDDC) in butyl acetate. National Bureau of Standards bovine liver, or National Research Council TORT-1 (lobster hepatopancreas) reference materials were determined with each batch of tissue. These determinations were within the specifications of the reference material (92.4 ± 6.8% of reference value (mean ± SD) (Zn), 97.4 ± 6.5% (Cu), 100.5 ± 31.2% (Cd); n=13).

MT Analysis by Gel Filtration Chromatography and Metal Summation

Samples of liver and kidney from each species from each lake were pooled and homogenized in 2 volumes of 10 mM phosphate buffer pH 7.6, with 5 mM 2-mercaptoethanol, 0.02% sodium azide, and 0.15 M KCl (distilled, deionized water was used for all aqueous solutions). Phenyl methyl sulfonyl flouride (PMSF) was added to the mixture (0.1 M) as a protease inhibitor. In the pooling of tissues for this method, no attempt was made to use equal quantities from each fish, so the resultant MT estimates were weighted estimates. The homogenates were centrifuged at 30,000g for 30 min at 4°C. The 30,000g supernatants were then recentrifuged at 100,000g for 120 min at 4°C. The surface lipid layer was removed from the surface of the supernatant. The final supernatant (cytosol)

was further fractionated by gel filtration chromatography. The cytosol was filtered through a 0.45 um cellulose acetate filter and chromatographed on a 1.9 x 100 cm column of Sephadex G-75 at a flow rate of 0.5 mL/min. Fractions of 10 mL were collected and analyzed for copper, zinc, and cadmium. The chromatography column was calibrated with blue dextran 2000, bovine serum albumin, ovalbumin, chymotrypsinogen A, ribonuclease A, and MT (Sigma rabbit liver MT-II, Sigma Corp., St. Louis Mo.). MT in the chromatographed cytosols was determined by co-elution of cadmium, copper, and zinc in chromatographic fractions which corresponded to the elution volume of MT (as determined from column calibration). MT concentration in the cytosols (nmol MT/g) was calculated under the assumption that MT binds 7 moles of zinc or cadmium (Kagi and Schaffer 1988), and 12 moles copper per mole of MT (Neilson et al. 1985).

MT Analysis by Mercury Saturation

MT was also estimated from liver and kidney of individual fish using the mercury saturation assay described in Chapter 2. These samples were analyzed before it was known that 10 min heat treatments result in somewhat elevated MT estimates (Chapter II). The tissues were prepared for the assay by homogenization in 4 volumes of 0.9% NaCl and a heat treatment at 100°C for 10 minutes in 1.5 mL polypropylene microcentri-

fuge tubes. The heat-treated homogenates were cooled on ice for 5 min and centrifuged for 10 min at 10,000g at room temperature in a benchtop microcentrifuge (Eppendorf 5412). The resulting supernatants were stored at -120°C until analysis. Liver samples were incubated with 10 µg of mercury as described in chapter II, but kidney samples were incubated with 5 µg of mercury, in an attempt to increase the sensitivity of the assay. This was done because it was assumed that kidney MT concentrations were much lower than liver MT concentrations.

Data Analyses

Metal deposition equations were calculated from surficial sediment metal concentrations using the power function $[Metal] = a(Distance)^{-b}$ (Franzin et al. 1979).

The mean hepatic and renal copper, zinc, cadmium, and MT concentrations from each fish species were regressed against the respective mean metal concentrations in surficial sediments (0-1.0 cm core section) to test for the presence of a dose-response relationship between the tissue variables and the sediment metal concentrations.

In an attempt to establish the relative importance of the three metals to MT induction, hepatic and renal MT concentrations (expressed as (nmol Hg bound)/g) were regressed against the respective metal concentrations (nmol/g) for both fish

species from each lake. This approach is similar to those of Olsson and Haux (1985) and Scheuhamer and Templeton (1990).

The metal summation and Hg saturation estimates of MT were used to estimate the degree of saturation of metal-binding sites of MT *in situ*, which may be of value as an indirect estimate of rates of MT synthesis. This index of MT saturation was calculated as the ratio of metals bound to MT before and after saturation with mercury. To do this, the MT concentrations in liver and kidney of individual fish (as determined by Hg-displacement assay) were multiplied by the appropriate weighting values to generate a weighted mean corresponding to that of the pooled MT estimates obtained by gel filtration chromatography.

Procedure GLM of the Statistical Analysis System (SAS) (SAS Institute, Cary, N.C., U.S.A.) was used for regression analysis. Where appropriate, differences among variables between lakes were tested for significance with Tukey's hsd multiple comparison in Procedure GLM of SAS. Procedure CORR of SAS was used to examine (1) the relationship between age of fish and copper, zinc, cadmium, and MT concentrations in liver and kidney, and (2) the relationship between water chemistry variables and distance from the smelter.

RESULTS

Water Chemistry

The water chemistry parameters of the study lakes are presented in Table 3. The study area is located in a geological transition zone between Precambrian Shield and sedimentary rocks of the Great Plains (Heywood 1966); much of the region is Precambrian Shield, with some areas being overlain with sedimentary rock (Gale et al. 1982, Syme et al. 1982) and calcareous glacial till (Heywood 1966). The influence of the sedimentary rocks on water chemistry can be seen from the elevated concentrations of cations, especially Ca^{2+} , in the study lakes (Golterman and Kouwe 1980). All of the lakes have Ca^{2+} concentrations in excess of 197 µmol/L, and alkalinity values in excess of 389 µeq/L. Hook Lake is distinct, having a Ca^{2+} concentration of 891 µmol/L, which is attributable to the presence of a lens of clastic sedimentary rock northwest of the lake (Heywood 1966). The water chemistry parameters (particularly alkalinity) of the reference lakes from ELA clearly demonstrate the differences between softwater Precambrian shield lakes and those near Flin Flon.

Sulfate concentration in water was negatively correlated with distance from the smelter ($r = -0.937$, $p < 0.001$). However, in spite of elevated sulfate concentrations in lakes nearest the smelter, none of the lakes were acidic (Table 3).

Metals in Sediment

Deposition equations for copper, zinc, and cadmium indicate that the study lakes northwest of the smelter (Saskatchewan lakes) received patterns of metal deposition which were different from those of the lakes southeast of the smelter (Manitoba lakes) (Table 4). The Manitoba lakes had a smaller radius of deposition for zinc than the Saskatchewan lakes, whereas the converse was true for copper and cadmium. The metal deposition equations establish the smelter as the source of elevated sediment metals in the study lakes, in agreement with earlier research (Franzin et al. 1980, Harrison and Klaverkamp 1990).

Metal concentrations in surficial sediment reflected both distance from the smelter, and the differences in deposition between lakes northwest and southeast of the smelter (Table 5). The highest sediment zinc concentrations were found in Hook Lake, 9.8 km south east of the smelter, while copper and cadmium concentrations were highest in Hamell Lake, 5.8 km northwest of the smelter. The surficial sediment metal concentrations in White Lake and Nesootao Lake (11.4 and 12.4 km from the smelter, respectively) were intermediate, while the more distant lakes (those more than 21 km from the smelter) had much lower sediment metal concentrations. In all lakes, the molar ratio of (Cu+Zn):Cd in the surficial sediment of the study lakes varied between 300:1 and 1100:1 (Table 6),

Table 4. Parameters for metal deposition equations from the Flin FLon study lakes. Equations are of the form [Sediment metal] = a(distance from smelter)$^{-b}$. The radius of deposition increases as b becomes smaller. The magnitude of deposition ioncreases as a increases. All equations are highly significant (the probability of a greater F value for the equation is less than 0.01).

	All Eight Lakes			Manitoba Lakes			Saskatchewan Lakes		
Metal	(b)	(a)	r^2	(b)	(a)	r^2	(b)	(a)	r^2
Cu	1.948	904.01	0.887	1.397	190.59	0.766	2.360	2653	0.994
Cd	1.783	10.02	0.868	1.645	6.31	0.719	1.846	12.63	0.986
Zn	1.936	5291	0.865	2.335	24396	0.931	1.871	4470	0.988

Table 5. Cu, Zn, and Cd concentrations (umol/g dry weight) in surficial sediment (0-1 cm sediment core section) from the eight Flin Flon study lakes. For each metal, lakes with the same letter are not significantly different.

Lake	Dist (km)	Cu (umol/g)	Zn (umol/g)	Cd (umol/g)
Hamell	5.8	43.6 a	116.1 ab	0.495 a
Hook	9.8	12.5 b	150.2 a	0.265 ab
White	11.4	4.1 b	68.8 bc	0.065 b
Nesootao	12.4	6.5 b	37.2 c	0.125 ab
Tyrell	21.1	2.0 b	13.8 c	0.040 bc
Neso	25.1	1.7 b	10.4 c	0.023 c
Johnson	25.7	1.3 b	8.2 c	0.035 bc
Twin	31.0	2.0 b	10.5 c	0.033 bc

Table 6. Molar ratios of Cu, Zn, and Cd in surficial sediment, liver, and kidney from the study lakes.

Lake	Dist (km)	Sediment		Fish Species	Liver		Kidney	
		Cu:Zn:Cd	(Cu+Zn):Cd		Cu:Zn:Cd	(Cu+Zn):Cd	Cu:Zn:Cd	(Cu+Zn):Cd
Hamell	5.8	88:234:1	322:1	Pike	12:32:1	44:1	9:302:1	311:1
Hook	9.8	47:563:1	610:1	Sucker	33:53:1	86:1	-	-
White	11.4	61:1031:1	1092:1	Sucker	53:78:1	130:1	-	-
Nesootao	12.4	51:293:1	344:1	Pike	37:113:1	150:1	102:2178:1	2280:1
				Sucker	20:35:1	55:1	1.3:17:1	18.3:1
Tyrell	21.1	58:387:1	445:1	Pike	83:222:1	305:1	205:9798:1	2000:1
				Sucker	45:110:1	155:1	6.5:78:1	84.5:1
Neso	25.1	83:518:1	601:1	Sucker	44:55:1	99:1	1.9:19:1	20.9:1
Johnson	25.7	41:262:1	303:1	Pike	70:153:1	223:1	40:1606:1	1646:1
Twin	31.0	71:364:1	435:1	Sucker	63:98:1	161:1	2.6:34:1	36.6:1
Reference Lakes								
ELA L. 240	-	-	-	Pike	159:785:1	944:1	7:862:1	869:1
				Sucker	101:147:1	248:1	1.6:20:1	21.6:1
ELA L. 313	-	-	-	Pike	228:276:1	504:1	5:822:1	827:1
				Sucker	63:78:1	141:1	0.8:9:1	9.8:1

indicating that the metal pollution problem near Flin Flon is largely one of copper and zinc.

Profiles of copper (Fig. 12), zinc (Fig. 13), and cadmium (Fig. 14) in sediment cores verified the relative importance of copper and zinc inputs to the Flin Flon study lakes. Although metal concentrations in surficial sediments were elevated above concentrations in the deepest core sections, the peak metal concentrations in sediments generally occurred at approximately 5 cm depth in the core sections.

The deposition equations from Table 4 were used to predict the radius at which no enrichment of metals would occur in the surficial sediment. The predicted deposition radii were 74, 74, and 33 km, respectively, for copper, zinc, and cadmium southeast of the smelter in Manitoba. The corresponding estimates were 39, 88, and 33 km for copper, zinc, and cadmium in the region northwest of the smelter, or 49, 90, and 33 km, when deposition curves for all eight lakes were used. Using these latter estimates, the area impacted by metal deposition is approximately 7543 km^2 for copper, 25447 km^2 for zinc, and 3421 km^2 for cadmium.

Metals in Fish Tissues

Linear regressions of copper, zinc, and cadmium concentrations in liver and kidney against the respective surficial sediment metal concentrations produced only two significant

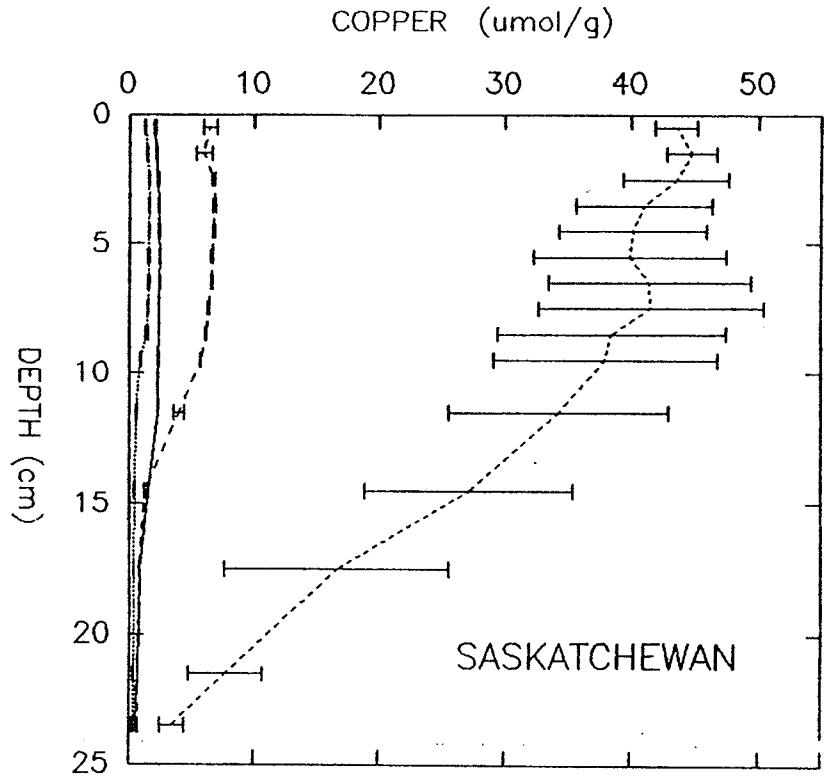
Figure 12. Profiles of copper concentration (µmol/g dry weight; mean ± SEM; n=4) in sediment cores from the Flin Flon study lakes.

Upper panel - Saskatchewan lakes.

Legend:
- dotted line - Johnson Lake
- solid line - Tyrell Lake
- coarse broken line - Nesootao Lake
- fine broken line - Hamell Lake

Lower panel - Manitoba Lakes.

Legend:
- dotted line - Twin Lake
- solid line - Neso Lake
- coarse broken line - White Lake
- fine broken line - Hook Lake


COPPER (umol/g)
0
10
20
30
40
50
0
5
10
15
20
25
DEPTH (cm)
SASKATCHEWAN

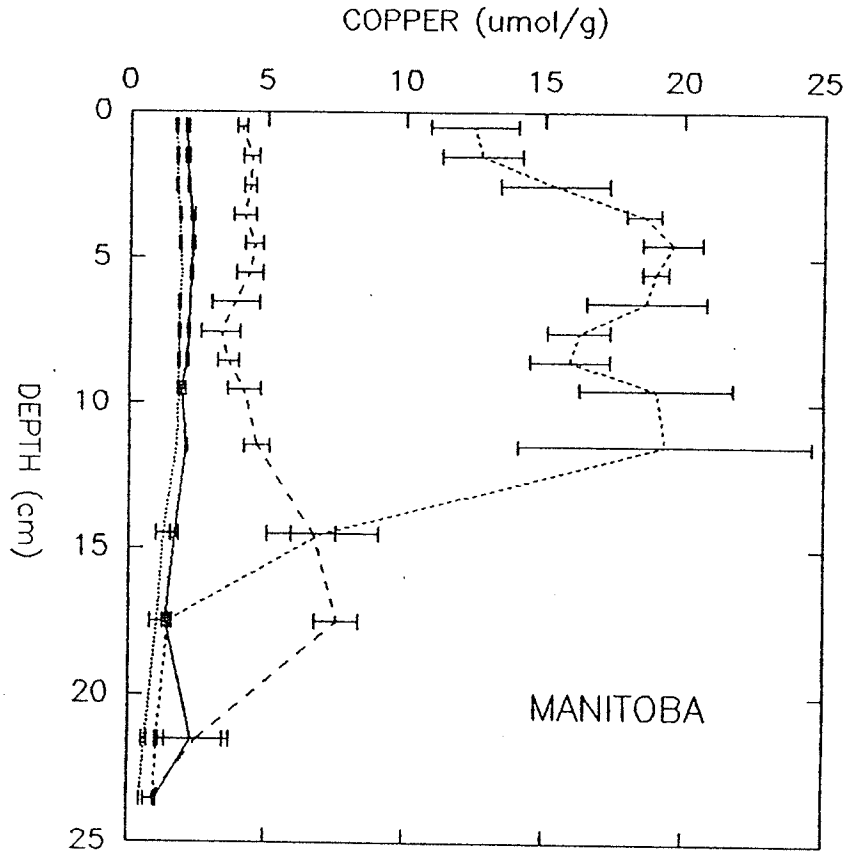

COPPER (umol/g)
0
5
10
15
20
25
0
5
10
15
20
25
DEPTH (cm)
MANITOBA

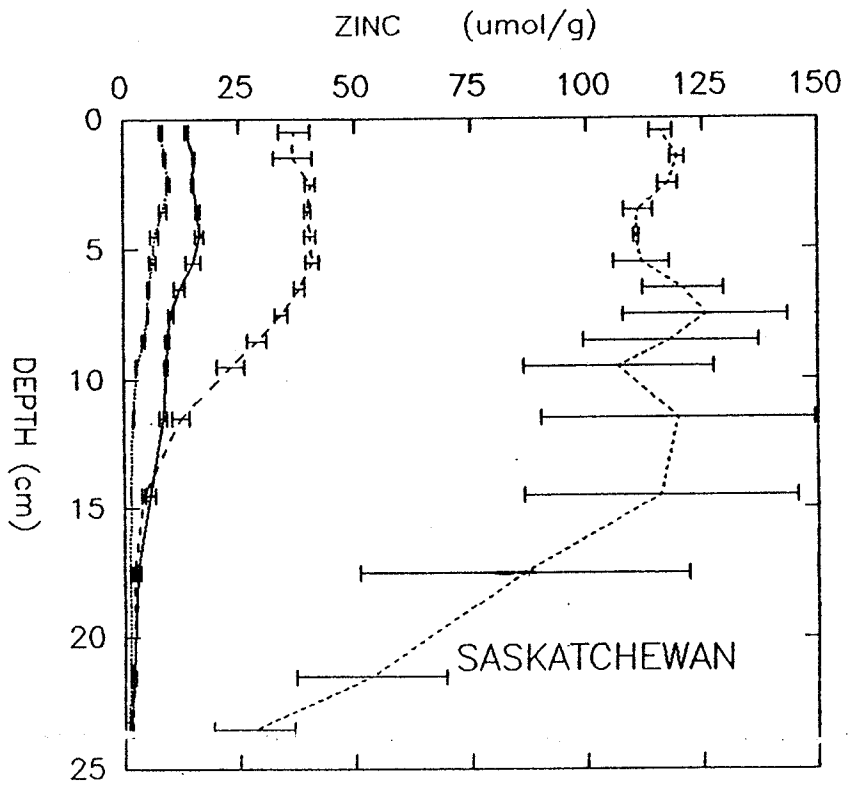
Figure 13. Profiles of zinc concentration (µmol/g dry weight; mean ± SEM; n=4) in sediment cores from the Flin Flon study lakes.

Upper panel - Saskatchewan lakes.

Legend:
- dotted line - Johnson Lake
- solid line - Tyrell Lake
- coarse broken line - Nesootao Lake
- fine broken line - Hamell Lake

Lower panel - Manitoba Lakes.

Legend:
- dotted line - Twin Lake
- solid line - Neso Lake
- coarse broken line - White Lake
- fine broken line - Hook Lake


ZINC (umol/g)
0
25
50
75
100
125
150
0
5
10
15
20
25
DEPTH (cm)
SASKATCHEWAN

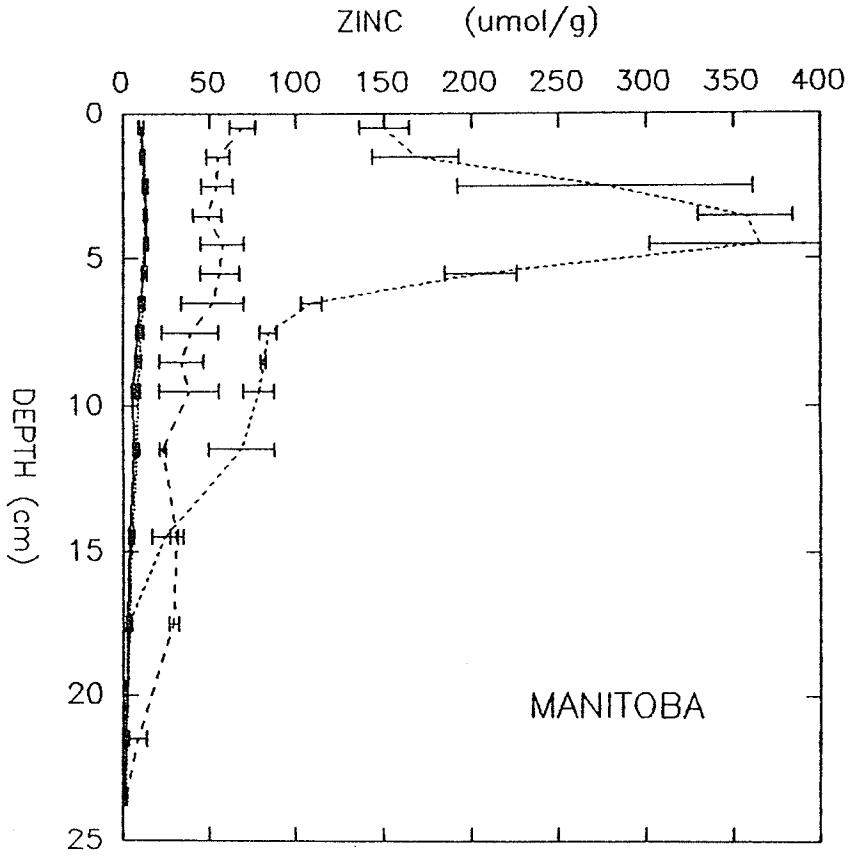

ZINC (umol/g)
0
50
100
150
200
250
300
350
400
0
5
10
15
20
25
DEPTH (cm)
MANITOBA

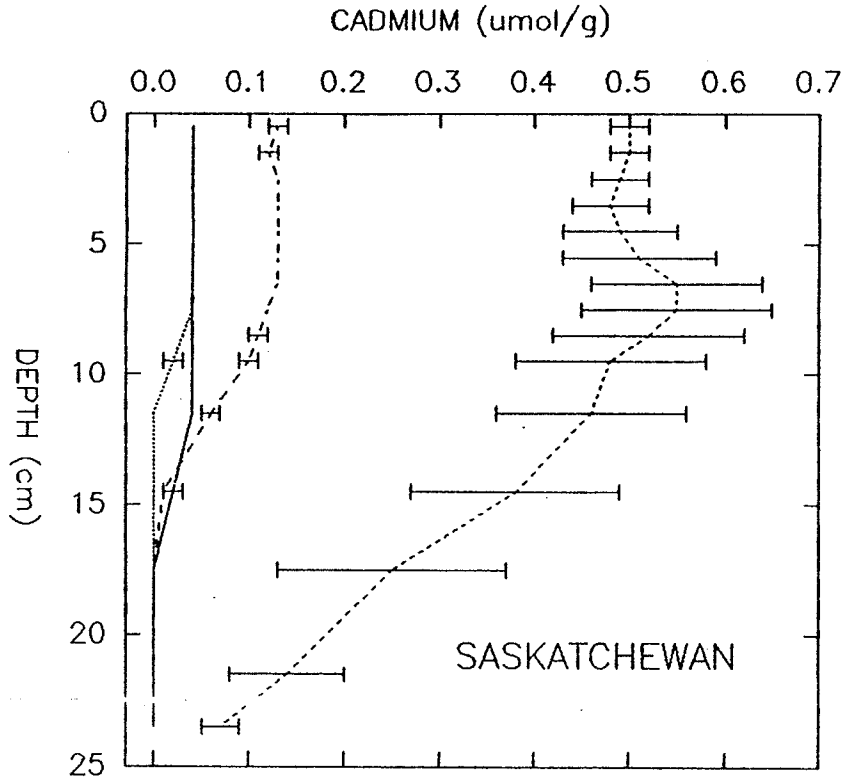
Figure 14. Profiles of cadmium concentration (µmol/g dry weight; mean ± SEM; n=4) in sediment cores from the Flin Flon study lakes.

Upper panel - Saskatchewan lakes.

Legend:
- dotted line - Johnson Lake
- solid line - Tyrell Lake
- coarse broken line - Nesootao Lake
- fine broken line - Hamell Lake

Lower panel - Manitoba Lakes.

Legend:
- dotted line - Twin Lake
- solid line - Neso Lake
- coarse broken line - White Lake
- fine broken line - Hook Lake


CADMIUM (umol/g)
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0.1
0.2
0.3
0.4
0.5
0.6
0.7
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5
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15
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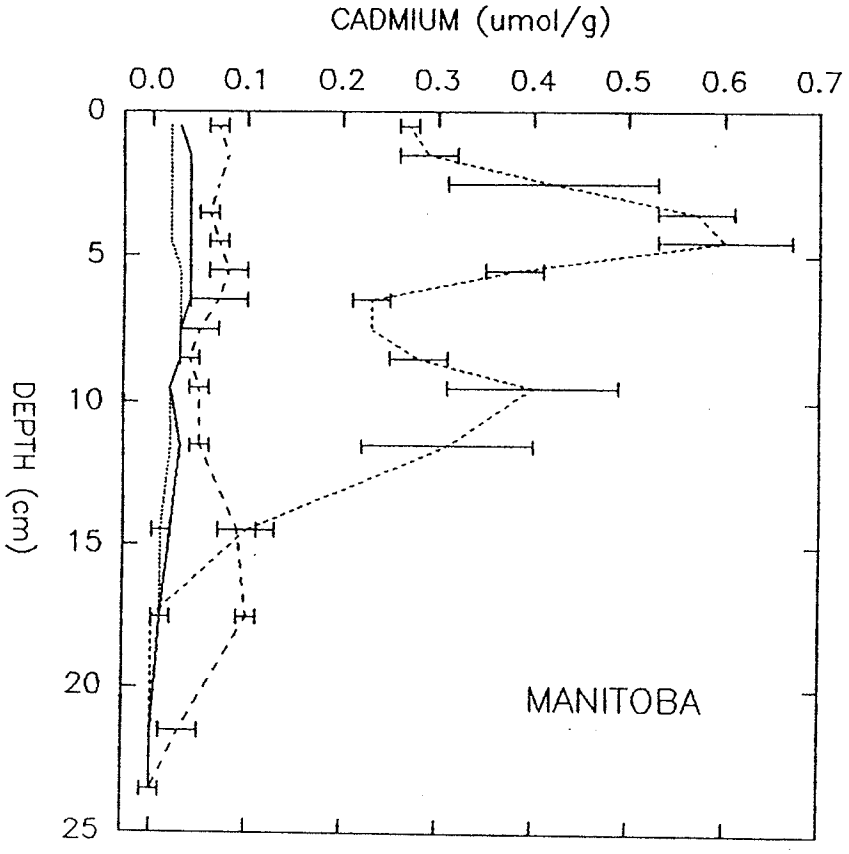
25
DEPTH (cm)
SASKATCHEWAN


CADMIUM (umol/g)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
5
10
15
20
25
DEPTH (cm)
MANITOBA

equations, both for cadmium concentrations in pike tissues:

Liver Cd = 35.370(Sediment Cd) + 1.656 ($p=0.029$ $r^2=0.943$)

Kidney Cd = 13.474(Sediment Cd) - 0.192 ($p=0.021$ $r^2=0.958$)

Significant differences in hepatic metal concentrations existed between Flin Flon lakes for both pike (Table 7) and sucker (Table 8), but the ranges encompassed those of pike and sucker from the reference lakes, as did renal cadmium concentrations. However, the copper and zinc concentrations in sucker kidney were higher in the Flin Flon lakes than in the ELA reference lakes (Table 8), as were copper concentrations in pike kidney (Table 7).

No consistent pattern of bioaccumulation (age-dependent accumulation) of copper, zinc, or cadmium was observed in pike from the Flin Flon study lakes, with the exception of copper and zinc in Hamell Lake pike liver ($r = 0.733$, $p = 0.025$ (copper); $r = 0.679$, $p = 0.044$ (zinc)). In contrast to fish from the Flin Flon study lakes, cadmium concentrations in kidney of pike from ELA Lake 240 were correlated with age ($r = 0.997$, $p = 0.003$).

In white sucker, only one significant correlation was observed between tissue metal concentrations and age (for zinc concentrations in livers of Nesootao Lake sucker), and it was a negative correlation ($r = -0.809$. $p = 0.015$). Again, cadmium concentrations in liver and kidney of suckers from one

Table 7. Mean metal and MT concentrations (± SEM) in liver and kidney of northern pike. Values followed by the same letter are not significantly different.

Lake	n	Liver				Kidney			
		Cu	Zn	Cd	MT	Cu	Zn	Cd	MT
			(nmol/g)				(nmol/g)		
Hamell	9	231.5 ab (35.3)	627.0 a (71.2)	19.7 a (6.2)	54.7 a (7.7)	59.6 a (3.6)	2021.7 a (97.5)	6.7 a (1.6)	13.3 a (2.9)
Nesootao	6	129.8 b (20.3)	391.8 b (41.7)	3.5 b (0.5)	47.9 a (6.6)	81.5 a (25.6)	1742.5 ab (268.9)	0.8 b (0.1)	13.3 a (4.7)
Tyrell	4	257.7 ab (91.8)	689.9 a (88.7)	3.1 b (0.9)	54.1 a (18.1)	41.0 a (15.5)	1959.0 ab (136.6)	0.2 b (0.1)	11.2 a (1.6)
Johnson	10	231.8 a (38.4)	708.9 a (80.6)	4.7 b (0.6)	61.0 a (7.2)	36.2 a (5.5)	1445.8 b (91.8)	0.9 b (0.1)	26.4 a (4.1)
Reference Lakes									
ELA L.240	4	132.9 (24.3)	653.5 (79.3)	0.8 (0.2)	73.8 (4.8)	15.4 (1.6)	1982.4 (79.9)	2.3 (0.4)	-
ELA L. 313	1	495.8	598.7	2.2	75.6	13.4	2300.2	2.8	-

Table 8. Mean metal and MT concentrations (± SEM) in liver and kidney of white sucker. Values followed by the same letter are not significantly different.

		Liver				Kidney			
Lake	n	Cu	Zn (nmol/g)	Cd	MT	Cu	Zn (nmol/g)	Cd	MT
Hook	10	287.4 ab (48.6)	464.4 ab (41.3)	8.7 b (1.1)	66.7 ab (8.7)	-	-	-	11.8 ab (1.1)
White	8	343.5 ab (57.4)	503.7 ab (27.3)	6.5 b (1.0)	65.2 ab (7.4)	-	-	-	6.4 b (0.4)
Nesootao	8	346.0 a (34.3)	597.6 a (57.1)	16.9 a (3.3)	96.7 a (12.1)	41.6 a (4.5)	568.0 a (55.4)	33.1 a (3.3)	14.9 a (2.3)
Tyrell	9	143.5 b (34.5)	351.0 b (11.4)	3.2 b (0.6)	44.3 b (11.1)	31.7 a (2.0)	380.0 ab (19.2)	4.9 b (1.6)	8.0 b (1.2)
Neso	11	397.0 a (44.6)	494.9 a (23.9)	9.0 b (1.6)	81.1 ab (7.4)	35.1 a (3.6)	358.9 b (18.1)	19.0 ab (4.9)	6.9 b (1.0)
Twin	11	299.7 ab (45.2)	465.5 ab (34.5)	4.7 b (1.0)	32.4 b (4.5)	33.1 a (5.2)	437.6 ab (67.0)	12.7 b (3.5)	7.0 b (1.0)
Reference Lakes									
ELA L.240	5	274.7 (48.8)	401.8 (23.5)	2.73 (0.5)	44.9 (3.7)	23.5 (1.6)	296.7 (16.6)	15.0 (5.6)	-
ELA L. 313	8	296.9 (50.0)	368.7 (23.8)	4.7 (1.36)	52.0 (7.8)	21.3 (0.8)	236.3 (10.3)	26.2 (9.9)	-

reference lake (ELA Lake 313) were correlated with age ($r = 0.792$, $p = 0.019$; $r = 0.868$, $p = 0.005$, respectively).

Although ratios of (Cu + Zn):Cd in sediments of the Flin Flon lakes were all greater than 300:1, the metal ratios in liver and kidney indicated that cadmium was concentrated relative to copper and (or) zinc, with the exception of pike kidney (Table 6). Interestingly, the smallest (Cu + Zn):Cd ratios (ie. the greatest accumulation of cadmium relative to zinc and copper) occurred in kidney of suckers from the reference lakes which do not receive any known point source cadmium deposition.

MT in Fish Liver and Kidney

All regressions of hepatic and renal MT concentrations against copper, zinc, or cadmium in surficial sediment were statistically non-significant for both fish species examined. In spite of the lack of a statistical relationship between MT and sediment metals, there was a slight tendency for MT concentrations in white sucker to be higher in lakes with higher sediment metal concentrations. There were also no significant differences existed between lakes for either hepatic or renal MT concentrations in pike (Table 7). Differences between hepatic and renal MT concentrations were common to both fish species, with MT concentrations in liver

exceeding those in kidney by approximately 5-15 times (Tables 7 and 8).

The statistical relationships between MT and its inducing metals copper, zinc, and cadmium are presented in Table 9 (liver) and Table 10 (kidney). Three patterns are apparent among the MT-metal regressions in these two organs of the two fish species studied. First, in pike, all regressions of hepatic MT against hepatic copper were significant, but MT was not related to zinc, with the exception of Hamell Lake pike (Table 9). Second, MT in kidney of both fish species was not related to any of the three metals, with one exception (cadmium in Tyrell Lake pike) (Table 10). Third, in sucker liver, MT was most commonly related to zinc and copper (Table 9), but no consistent trend was present.

Metal summation estimates of MT from pooled liver samples were all lower than corresponding Hg saturation estimates of MT (Table 11). The ratio of these two estimates indicates the degree to which MT is saturated with metals. In liver, the index of saturation was less than 100%, indicating that not all metal-binding sites of MT were saturated <u>in situ</u>. In sucker kidney, the two estimates of MT were in close agreement (Table 11), indicating that the MT was fully saturated with metals. In contrast, the indices of saturation for pike kidney MT all exceeded 100%.

Table 9. Regressions of hepatic MT against hepatic Cd, Cu, or Zn, and total hepatic metals.

White Sucker

	Cd			Cu			Zn			Total Metals (Cd+Cu+Zn)		
Lake	Slope	Inter-cept	Signif-icance	Slope	Inter-cept	Signif-icance	Slope	Inter-cept	Signif-icance	Slope	Inter-cept	Signif-icance
Hook	44.00	83.30	**	1.06	162.09	**	1.19	-87.25	**	0.58	21.83	**
White	-20.75	590.80	ns	0.72	208.89	*	1.73	-413.80	**	0.54	-8.00	**
Nesootao	-6.38	784.74	ns	1.91	17.14	*	1.30	-102.25	**	0.86	-151.87	**
Tyrell	-102.43	640.29	*	1.35	115.89	ns	0.40	-1108.29	**	1.25	-310.35	ns
Nesc	-9.64	654.66	ns	0.27	459.93	*	1.60	-225.70	ns	0.39	219.00	ns
Twin	2.21	217.55	ns	0.48	75.53	ns	0.48	-2.37	ns	0.26	18.00	*
ELA 240	42.07	199.31	ns	0.45	191.35	ns	0.74	17.68	ns	0.32	94.23	ns
ELA 313	0.08	363.60	ns	1.03	59.11	**	2.02	0.44	**	0.73	-123.05	**

Northern Pike

Hamell	3.17	320.25	ns	1.13	120.69	*	0.55	37.96	*	0.38	53.66	*
Nesootao	70.09	89.15	ns	2.01	74.05	*	0.90	-162.36	ns	0.65	-8.13	*
Tyrell	137.04	-46.55	*	1.36	28.54	*	1.30	-516.29	ns	0.69	-277.89	*
Johnson	60.69	143.40	*	1.03	96.04	**	-0.04	456.21	ns	0.12	298.89	ns
ELA 240	212.96	59.07	*	1.36	55.78	*	-0.03	254.16	ns	0.11	153.24	ns

Table 10. Regressions of renal MT against renal Cd, Cu, or Zn, and total renal metals.

White Sucker

Lake	Cd Slope	Cd Intercept	Cd Significance	Cu Slope	Cu Intercept	Cu Significance	Zn Slope	Zn Intercept	Zn Significance	Total Metals (Cd+Cu+Zn) Slope	Total Metals (Cd+Cu+Zn) Intercept	Total Metals (Cd+Cu+Zn) Significance
Hook	-	-	-	-	-	-	-	-	-	-	-	-
White	-	-	-	-	-	-	-	-	-	-	-	-
Nesootao	3.12	0.94	ns	0.99	63.09	ns	-0.07	145.97	ns	-0.05	139.35	ns
Tyrell	-1.22	61.74	ns	1.46	9.50	ns	0.07	30.08	ns	0.07	26.09	ns
Neso	0.31	44.84	ns	0.83	21.69	ns	-0.16	108.04	ns	-0.09	87.46	ns
Twin	0.26	45.35	ns	-0.40	61.95	ns	-0.02	57.30	ns	-0.02	57.28	ns

Northern Pike

Lake	Cd Slope	Cd Intercept	Cd Significance	Cu Slope	Cu Intercept	Cu Significance	Zn Slope	Zn Intercept	Zn Significance	Total Metals (Cd+Cu+Zn) Slope	Total Metals (Cd+Cu+Zn) Intercept	Total Metals (Cd+Cu+Zn) Significance
Hamell	3.74	72.57	ns	-1.53	188.42	ns	0.02	53.10	ns	0.02	54.11	ns
Nesootao	128.46	-3.54	ns	-0.94	169.55	ns	-0.04	168.00	ns	-0.05	182.11	ns
Tyrell	160.43	-48.55	*	0.67	50.92	ns	0.02	31.74	ns	0.03	20.31	ns
Johnson	-68.96	249.80	ns	-0.97	219.87	ns	-0.06	266.04	ns	-0.06	269.74	ns

Table 11. MT estimates and index of saturation for white sucker and northern pike from Flin Flon lakes.

Lake	Liver			Kidney		
	Metal Summation Estimate[a]	Mercury Saturation Estimate[b]	Index of Saturation[c] (%)	Metal Summation Estimate[a]	Mercury Saturation Estimate[b]	Index of Saturation (%)
Sucker						
Hook	16.4	62.7	26.2	11.8	12.4	95.5
White	27.1	68.1	39.8	-	-	-
Nesootao	38.9	89.4	43.5	17.1	15.4	111.1
Tyrell	11.6	44.5	26.1	9.2	7.9	83.2
Neso	41.1	81.2	50.6	7.5	6.9	108.9
Twin	24.7	30.5	81.1	5.5	6.6	83.2
Pike						
Hamell	23.7	53.2	44.5	29.3	14.2	206.9
Nesootao	13.1	48.2	27.2	18.7	11.1	168.3
Tyrell	20.0	45.6	43.9	52.0	9.3	561.6
Johnson	19.2	59.1	32.5	42.9	24.9	172.0

a). nmol MT = nmol Zn/7 + nmol Cd/7 + nmol Cu/12
b). nmol MT = nmol Hg/7
c). a/b x 100

DISCUSSION

Metals in Sediments

The metal deposition area around the Flin Flon smelter is broadly elliptical, but elongated somewhat to the southeast (Zoltai 1988). Based on the metal deposition equations for copper, zinc, and cadmium in surficial sediment (Table 4), the predicted radius of deposition beyond which copper, zinc, and cadmium would not accumulate above background levels were 49, 90, and 33 km, respectively. The resulting areas of deposition would be approximately 7543 km^2 (copper), 25447 km^2 (zinc), and 3421 km^2 (cadmium). However, because the zone of metal deposition is not a circle, but is broadly elliptical, these predicted areas of deposition probably overestimate the area impacted by fallout from the smelter. These estimates of deposition radii are partially in agreement with the earlier estimates of Franzin et al. (1979) (33-60 km for copper, 131-264 km for zinc, and 113-284 km for cadmium) and Zoltai (1988) (110 km for copper and 77 km for zinc). The high values for zinc and cadmium deposition radii of Franzin et al. (1979) are not readily explainable, but their estimates for copper deposition is very similar to the estimate produced by this study.

Metals in Fish Tissues

Dose-response relationships between sediment metal concentrations and metal concentrations in fish have not been consistently demonstrated in studies conducted near smelters (McFarlane and Franzin 1980, Bradley and Morris 1985, Harrison and Klaverkamp 1990), although metal concentrations in fish near smelters tend to be higher than in more distant lakes. In this study, only liver and kidney cadmium concentrations in northern pike were related to sediment metal concentrations. In liver and kidney, copper and zinc (the major pollutants released by the smelter) were not related to their respective concentrations in sediment.

The installation of the tall stack in 1974 and the addition of electrostatic precipitators in 1982 resulted in reduced metal deposition to lakes near the smelter. These cahnges in metal release from the smelter may account for the reduction of elevated hepatic metal concentrations in fish from Flin Flon lakes to near-background levels over the period from 1976 to 1982 (Harrison and Klaverkamp 1990). In this study, hepatic metal concentrations of fish sampled in 1986 were not elevated relative to concentrations in reference lakes (Tables 7 and 8), consistent with the findings of Harrison and Klaverkamp (1990).

Metal concentrations in kidney were not reported in earlier research at Flin Flon (McFarlane and Franzin 1980,

Harrison and Klaverkamp 1990). This precludes comparisons with the present findings of elevated copper and zinc concentrations in sucker kidney, and elevated copper in pike kidney, relative to fish from reference lakes (Tables 7 and 8). Unlike the liver and intestine, which are the primary organs responsible for copper and zinc excretion (Cousins 1985), the kidney does not have the same capacity to regulate metals, and is therefore very susceptible to heavy metal toxicity (Elinder 1985, Foulkes 1986, Miller 1987). The presence of high copper and zinc concentrations in kidney of Flin Flon fish (even though metal concentrations in liver appear to be normal) suggests that metal concentrations in kidney may be more sensitive biomarkers of metal pollution than liver metal concentrations. Since kidney is the target organ of cadmium toxicity in mammals (Elinder 1985, Foulkes 1986), further emphasis on the kidney as a target organ in fish is warranted.

The bioaccumulation of cadmium is of particular interest. cadmium is a toxic metal with no known physiological function, so its bioaccumulation in animals is widely used as a biomarker of environmental cadmium exposure (Bendell-Young et al. 1986, Jeffery et al. 1989, Frank et al. 1989, Evtushenko et al. 1990). In several lakes near Flin Flon, cadmium was found to be bioaccumulating in livers of the Hamell and Nesootao pike populations in 1976 (McFarlane and Franzin 1980). Harrison and Klaverkamp (1990) resampled Hamell Lake in 1982 and did not observe hepatic cadmium bioaccumulation. In 1986,

cadmium bioaccumulation was not observed in any of the Flin Flon lakes (although copper and zinc were bioaccumulating in Hamell Lake pike). Further complicating the interpretation of these findings is the fact that cadmium bioaccumulation was occurring in white sucker and northern pike from two relatively pristine softwater lakes in northwestern Ontario, which receive no known point source metal deposition.

Other research has shown that cadmium bioaccumulation can occur in aquatic organisms exposed to ambient cadmium concentrations much lower than those found near Flin Flon (Bendell-Young et al. 1986, Evtushenko et al. 1990), suggesting that cadmium bioaccumulation _per se_, may not be an adequate indicator of environmental cadmium pollution. The bioaccumulation of cadmium may simply represent its concentration in organs, at the expense of other metals. For example, the ratios of (Cu + Zn): Cd in fish tissues from Flin Flon lakes were decreased by up to 8-fold relative to those of sediment (Table 6), indicating that cadmium was concentrated in fish tissues (relative to copper and zinc). If cadmium ultimately becomes a large proportion of the metal (Cu + Zn) pool, it may approach a critical toxic level (Foulkes 1986). The smallest (Cu + Zn):Cd ratios found in this study were in kidney of sucker from ELA L. 313, where the Cu:Zn:Cd ratio was 0.8:9:1 (and where cadmium was bioaccumulating). Although the proportion of cadmium approached one tenth of the kidney metal pool in these fish, the mean cadmium concentration was 26.2 nmol/g

(2.95 µg/g), well below the 1779 nmol/g (200 µg/g) thought to be the critical level for cadmium in the mammalian renal cortex (Foulkes 1986). Clearly, the whole issue of the importance of cadmium bioaccumulation needs clarification.

MT in Fish Liver and Kidney

Metallothionein concentrations in liver and kidney of white sucker and northern pike were unrelated to sediment metal concentrations, indicating the absence of a dose-response relationship between MT and environmental metal loadings. Metallothionein is induced in liver and kidney in response to metal concentrations in the organs (Scheuhamer and Templeton 1990), so induced levels of MT should be associated with elevated metal concentrations in these organs. Given the lack of observable differences in hepatic metal concentrations between Flin Flon fish and ELA fish, it is not surprising that there are no differences in hepatic MT concentrations either (Tables 7 and 8).

The relationships between MT, copper, zinc, and cadmium in liver (Table 9) and kidney (Table 10) demonstrate that there are distinct differences in MT gene expression in these two organs. In kidney of white sucker and northern pike there were no significant regressions between MT and metal concentrations, with one exception (in Tyrell Lake pike, MT was

linearly related to cadmium). This general lack of relationship between MT and metals would seem to indicate that MT synthesis is not highly inducible in kidney. On the other hand, significant regressions between MT and copper (in both fish species) and between MT and zinc in pike liver, support the general interpretation that MT is involved in the metabolism of copper and (or) zinc (Bremner 1987a, Bremner 1987b, Hamer 1986). The general lack of statistical relationship between MT and cadmium in liver and kidney probably reflects the relatively low cadmium concentrations in the fish collected for this study.

This research follows from the findings that metallothionein concentrations and metal tolerance in suckers from a heavily-polluted lake (Hamell Lake) were significantly greater than in a relatively unpolluted lake (Thompson Lake) (Klaverkamp et al. 1991). Their two-lake comparison was suggestive of a dose-response relationship between MT and metal exposure.

A similar comparison between the Nesootao and the Tyrell or Twin Lake sucker populations sampled in this follow-up study would also have pointed toward the existance of a gradient of MT concentrations in lakes near Flin Flon. However, the dose-response relationship seen when two lakes are compared, is not apparent when six lakes are considered. The reasons for this lack of a dose-response relationship are uncertain, although there are several possible explanations.

Many chemical characteristics of natural waters can moderate metal availability and toxicity, including chemical speciation, water hardness and dissolved organic carbon (Buffle 1988, Moore and Ramamorthy 1984). Therefore, the bioavailable metal concentration may be quite different from the total metal concentration in a lake. The chemical characteristics of the Flin Flon study lakes are such that two of the lakes with the highest sediment metal concentrations (Hook and White) have very high Ca concentrations, 891 and 407 µmol/L (Table 3), which may reduce metal bioavailability and prevent bioaccumulation in these lakes. Therefore, the lack of a dose-response relationship between MT concentrations and sediment metal loading may be a function of bioavailability, and not because MT induction is unresponsive.

A more fruitful approach might be to resample the same lakes over a period of years. Assuming that water chemistry characteristics of lakes remain relatively constant over time, the question of bioavailability can be avoided by such a sampling scheme. Deniseger et al. (1990) used such an approach to demonstrate that MT in rainbow trout from Buttle Lake, British Columbia, responded in a dose-dependent manner to reductions in waterborne zinc, copper, and cadmium concentrations. Over a four year period, rainbow trout hepatic MT concentrations dropped from 213 nmol MT/g to 64 nmol/g, while waterborne zinc concentrations dropped from 370 µg/L to 50 µg/L.

Because MT is an inducible protein, a simple measurement of MT synthesis would be useful for measuring induction in field samples (McCarter and Roch 1983). The index of MT saturation is proposed here as a prospective simple and indirect measure of MT synthesis rates. An index of saturation near 100% indicates that the metal-binding sites of MT are saturated in situ, and that MT synthesis and degradation are at equilibrium. An index of saturation that is much less than 100% indicates that a large percentage of the metal-binding sites of MT are unoccupied by metals, signifying high rates of thionein synthesis.

There are several lines of evidence which support the view that unsaturated MT exists in vivo. First, newly synthesized MT is thought to be the metal-deficient apometallothionein (Cousins 1985). Second, metal-deficient MT can be generated in vitro (Neilson et al. 1985, Hamer 1986). Third, the overproduction of MT after metal exposure has been documented (McCarter and Roch 1983, Petering et al. 1987). By quantifying the the proportion of unoccupied binding sites of MT with the index of saturation, it can be determined whether such overproduction of MT is occuring. Hobson and Birge (1989) used this approach, and found MT to be 75% saturated after 7 and 14 days exposure to 1.8 mg Zn/L, and 97% saturated after 21 days of exposure, while MT concentration increased 2.5-fold. These independent findings are in agreement with Cousins (1983), who has proposed that an appreciable amount of metal-

free thionein may be present during induction of MT synthesis.

In five of the Flin Flon lakes from which sucker was sampled, the index of saturation was inversely related to metal deposition, with hepatic metallothionein being most-saturated (81%) in Twin Lake, the most distant lake from the smelter. In Tyrell Lake sucker, the index of saturation was only 26%, suggesting relatively high rates of MT synthesis in spite of only moderate metal deposition to Tyrell Lake. In sucker kidney, MT was near saturation in all lakes, but in pike kidney, the index of saturation varied between 170 and 560 % (Table 11). These extreme values in pike are probably related to the high renal zinc concentrations (ca. 2000 nmol/g), which can interfere with metal saturation assays for MT estimation (Eaton 1985). Sample preparation for MT analysis involved a 10 min heat denaturation step to remove non-MT proteins from solution. In Chapter II it was shown that such a heat denaturation step mobilized large quantities of copper into rainbow trout cytosols. It is conceivable that the high zinc concentrations in pike kidney may also have been mobilized into the cytosol after heat treatment, resulting in an underestimation of MT by the Hg saturation assay.

The index of saturation remains to be verified as a simple measurement of MT synthesis. In this study, there was no apparent relationship between MT saturation and sediment metal concentration, so the utility of this approach is uncertain. If it can be verified by the methods of molecular

biology, the value of this method is its relative simplicity, which would make estimates of MT synthesis readily available to ecotoxicologists.

CHAPTER IV

TESTING THE IMPORTANCE OF GROWTH RATE IN HEPATIC CADMIUM BIOACCUMULATION IN FISH

INTRODUCTION

Cadmium is a rare element, with an average crustal concentration of 0.1-0.2 mg/kg (0.89-1.78 µmol/kg), 1/350 as common as zinc (Nriagu 1980a). Cadmium and zinc are closely related chemically, and cadmium is typically found in zinc ores, the smelting of which has released cadmium to the atmosphere for thousands of years (Elinder 1985). However, it is only within this century that atmospheric emissons of cadmium have dramatically increased (Elinder 1985), so that anthropogenic sources of atmospheric cadmium may now exceed natural sources by approximately 9 times (Nriagu 1980a). Even in remote regions of earth, cadmium concentrations have increased between 15- and 60-fold in the last century (Nriagu 1980b). In North American freshwaters, cadmium concentrations have increased over the past two decades (Smith et al. 1987), indicating that cadmium pollution is a serious problem on this continent.

Non-ferrous metal smelting contributes approximately 76% of the anthropogenic cadmium emissions, while fossil fuel combustion accounts for the remaining 24% (Nriagu 1980b). Point source emissions of cadmium are of obvious importance near smelters (Franzin et al. 1979, Franzin 1984, Harrison and Klaverkamp 1990), but elevated cadmium concentrations in the water and sediments of remote lakes in eastern North America indicate that long-range atmospheric transport of cadmium is

also significant (Johnson et al. 1987, Smith et al. 1987). In either case, the emission of cadmium by human activity is usually associated with the release of nitrogen and sulfur oxides, and the subsequent acidification of poorly buffered receiving waters of the Precambrian Shield. Because cadmium concentration in freshwater is typically inversely related to pH (Breder 1988, Stephenson and Mackie 1988), cadmium may be retained for longer periods in the water column of acidified lakes relative to non-acidified lakes, in which cadmium is normally partitioned to the particulate phase and rapidly deposited to sediments (Breder 1988). The longer retention of cadmium in the water column of acidified lakes may, in turn, increase cadmium accumulation both through the food web and through exposure via water.

An important biochemical characteristic of cadmium is its tendency to bioaccumulate (increase in concentration with the age of an organism) in liver and kidney of terrestrial and aquatic biota, including humans (Kjellstrom and Nordberg 1985), horses (Jeffery et al. 1989), cattle (Frank et al. 1989), and fish (Sprenger et al. 1988, Harrison and Klaverkamp 1990, Mcfarlane and Franzin 1980, Bendell-Young et al. 1986). Among fish, cadmium bioaccumulation is not common to all lakes near smelters (McFarlane and Franzin 1980, Harrison and Klaverkamp 1990), nor is it common to all acidified lakes (Sprenger et al. 1988). Of great interest is the finding that fish from northern Ontario lakes (far removed from any point

sources of cadmium deposition) were bioaccumulating cadmium, while in lakes near the Flin Flon smelter (which receive elevated inputs of cadmium) fish were not bioaccumulating cadmium (Chapter III). If these inconsistencies can be understood, cadmium accumulation in fish may be a useful biomarker of continued environmental cadmium pollution and its relation to acidification.

Hepatic cadmium accumulation in fish is affected by several factors, including; route of uptake, concentration in the environment, excretory capacity of the organism, and bioavailability. Bendell-Young et al. (1986) added fish growth rate to this list, in a model which assumes that bioavailability and growth rate are the determinants of hepatic cadmium accumulation in fish. Based on their model, they concluded that hepatic cadmium accumulation will be favored in rapidly-growing fish.

Their cadmium accumulation model requires validation, because the relationship between cadmium accumulation and growth may simply reveal an autocorrelation between growth and cadmium dose. Fish growth is largely determined by the rate of food intake. Therefore, for food of a given cadmium concentration, a doubling of ration will also double the dietary cadmium dose received by each fish. The interdependency between ration and cadmium dose could therefore account for different rates of cadmium accumulation between fish of different growth rates.

This experiment is a test of the Bendell-Young hepatic cadmium bioaccumulation model. By using the same Cd-labelled food for all treatments, bioavailability could be assumed to be constant in the experiment, permitting the isolation and manipulation of growth rate (the second component of the Bendell-Young model), and enabling the testing of the hypothesis that growth rate influences the hepatic bioaccumulation of dietary cadmium.

MATERIALS AND METHODS

Experimental Design

The effect of growth rate on cadmium bioaccumulation in rainbow trout (Oncorhynchus mykiss) was tested in a 3X3 factorial experiment. The two experimental factors were dietary cadmium concentration and ration, each with three treatment levels (4.5, 45, and 450 nmol Cd/g; and 0.5, 1.0, and 1.5% of body weight/day, respectively).Each treatment combination of these two factors was randomly assigned to one of nine tanks.

Rainbow trout (Tagwerker disease resistant strain, Spring Valley Trout Farms, New Dundee, Ontario) were anesthetized with buffered MS-222 (0.067 mg/L) in water containing 150 mM NaCl (to minimize osmotic shock during handling; S.B. Brown, pers. comm.). The length and weight of the fish were recorded, and, to permit the growth of individual fish to be measured,

each fish was tagged with a visual implant tag. The fish were not fed for 3 days prior to tagging, to ensure that initial weights did not include weight of gut contents. The fish were then randomly distributed to the 9 160 L tanks, where they were held for 13 days (without feeding) prior to the start of the experiment. The fish (254 in total) were maintained in 10°C dechlorinated City of Winnipeg water at a flow rate of 1.8 L/min/tank for the entire experiment.

Preparation of Cadmium-labelled Food and Feeding Procedures

The diets containing nominal cadmium concentrations of 4.5, 45, and 450 nmol/g were prepared by sprinkling aqueous $CdCl_2$ onto Martin Trout Chow #5 (Martin Feed Mills, Elmira, Ontario) and drying the food in a fume hood.

Fish were fed daily, with the exception described below. The cadmium-labelled food was sprinkled over the entire surface of the tank in small portions, so that food did not sit on the tank bottom for extended periods. This feeding procedure prevented the formation of social feeding heirarchies and presumably minimized the leaching of cadmium from food into the water.

Sampling

Fish were sampled from each tank on day 3 (n=3), day 9 (n=3), day 20 (n=4), day 31 (n=4), day 61 (n=6), and day 94 (n= all fish remaining in tank). Except for day 3 samples, feeding was stopped 3 days before sampling, to allow for gut clearance by sampling date.

Fish were killed by overdose of buffered MS-222 (0.2 g/L). At sampling, weight, length, tag number, and weight of gut contents (if any) were recorded for each fish. Livers were removed and immediately frozen at -120 C. Growth was calculated as:

$$[(W_s - W_g) - W_i]/W_i \times 100$$

Where W_s = Weight of fish at sampling

W_g = Weight of gut contents at sampling

W_i = Initial weight of fish

Metal Analyses

Food and liver samples were prepared for metal analysis by digestion in concentrated HNO_3 and oxidation of residual organic matter with 30% H_2O_2. Quality assurance (QA) samples were digested with each batch of samples. For analysis of cadmium concentrations in food, low cadmium (NBS bovine liver) and high cadmium (NRCC TORT-1 (lobster hepatopancreas)) QA

samples were digested and analyzed. Results of these analyses were 4.18 ± 0.27 nmol/g (mean ± SEM) (n=3), and 213.77 ± 1.42 nmol/g, respectively, while the specifications for these QA samples were 3.91 ± 0.53 nmol/g, and 234.00 ± 18.68 nmol/g.

NRCC DOLT-1 (dogfish liver) was used as a QA sample for liver digestions. Metal concentrations in DOLT-1 (n=12) were determined to be: 36.92 ± 0.44 nmol/g (mean ± SEM) (Cd), 1339.86 ± 19.88 nmol/g (Zn), and 297.42 ± 5.35 nmol/g (Cu). Product specifications for these three metals were:37.19 ± 2.49 nmol/g (Cd), 1414.81 ± 35.18 nmol/g (Zn), and 327.32 ± 18.88 nmol/g (Cu).

Statistical Analyses

For each sampling day, analysis of variance (ANOVA) was undertaken using Procedure GLM of SAS (Statistical Analysis Systems, Cary, S.C.), using a factorial model which tested for main effects due to dietary cadmium concentration and ration and interactions between main effects.

RESULTS

Cadmium, Zinc, and Copper Concentrations in Food

The measured cadmium concentrations (mean ± SEM) in the

4.5, 45, and 450 nmol Cd/g diets were 4.3 ± 0.1 nmol/g (n=4), 42.4 ± 5.9 nmol/g (n=4), and 384.8 ± 43.55 nmol/g (n=4), respectively. For clarity, the following discussion will refer to nominal concentrations. Zinc and copper concentrations in the Martin Trout Chow were 2590.0 ± 82.6 nmol/g and 401.0 ± 23.7 nmol/g (n=12). Therefore, ratios of (Cu + Zn):Cd in the 4.5, 45, and 450 nmol Cd/g treatments were 665, 66.5, and 6.65, respectively.

Growth of Fish

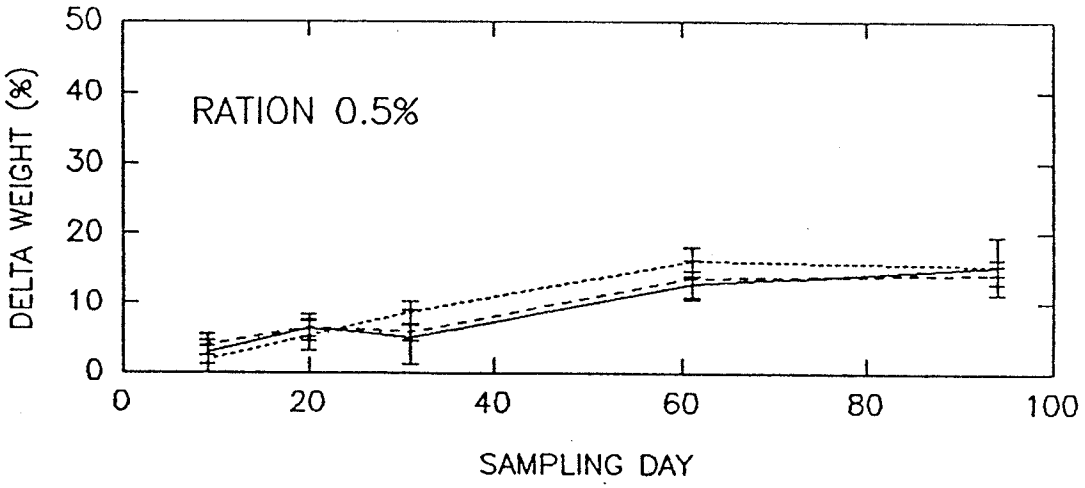
Growth of individual fish could be monitored because all fish were tagged. Only 32 of the 254 fish in the experiment had no tag at sampling (12.6% tag loss). However, it was possible to establish the correct identity of all fish which had lost tags.

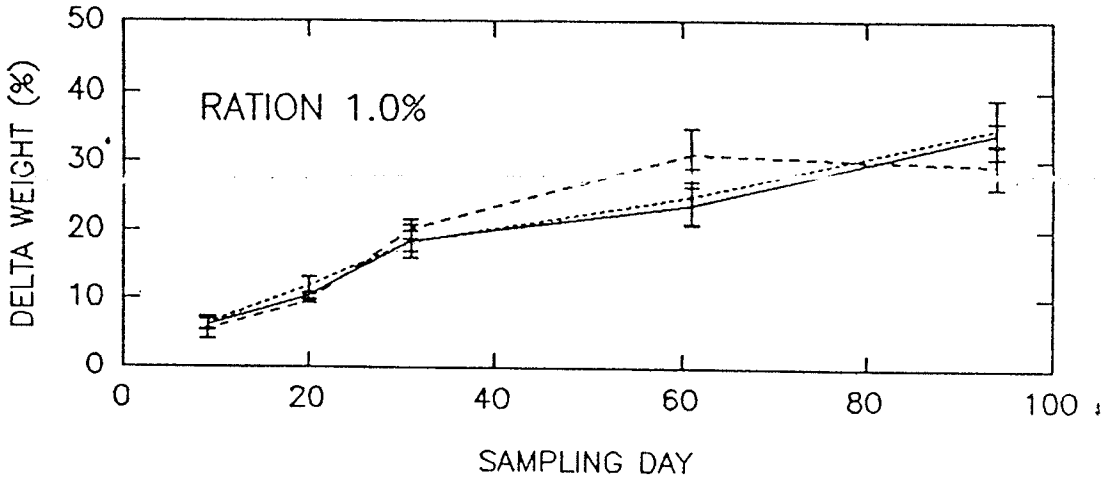
Trends in weight change over the duration of the experiment indicate that the greatest change in growth occurred between the 0.5% and the 1.0% ration levels (15 and 33% weight changes, respectively, after 94 days) (Fig. 15). The relatively small differences between the 1.0 and 1.5% ration treatment levels indicates a loss of efficiency in conversion of food to body mass at the highest level of ration.

On all five sampling days considered (day 3 samples were excluded from growth analysis), the factorial ANOVA model for growth was significant (Table 12). Partioning the model into

Figure 15. Growth of individual cadmium-fed rainbow trout (MEAN ± SEM) (expressed as per cent increase in weight) over the 94 days of the experiment.

Legend: Solid line - 450 nmol Cd/g food; broken line - 45.0 nmol Cd/g food; dotted line - 4.50 nmol Cd/g food.


RATION 0.5%
DELTA WEIGHT (%)
SAMPLING DAY
0
10
20
30
40
50
0
20
40
60
80
100


RATION 1.0%
DELTA WEIGHT (%)
SAMPLING DAY
0
10
20
30
40
50
0
20
40
60
80
100

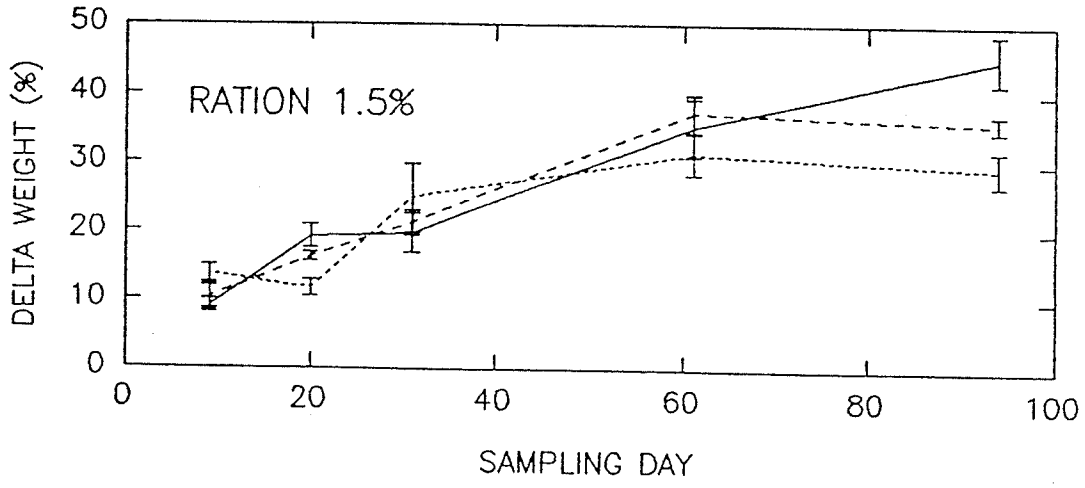

RATION 1.5%
DELTA WEIGHT (%)
SAMPLING DAY
0
10
20
30
40
50
0
20
40
60
80
100

Table 12. Analysis of Variance Summaries for Growth of Fish (expressed as % weight gain). The factorial model has been divided into sources due to the main effects (dietary Cd concentration and ration) and interaction between main effects.

Sampling Day	Source	F Value	Prob > F
9	Model	7.30	0.0003
	Cd	0.73	0.4960
	Ration	25.63	0.0001
	Cd*Ration	1.43	0.2680
20	Model	10.22	0.0001
	Cd	1.28	0.2956
	Ration	35.78	0.0001
	Cd*Ration	1.91	0.1382
31	Model	7.30	0.0001
	Cd	0.85	0.4380
	Ration	27.59	0.0001
	Cd*Ration	0.37	0.8255
61	Model	7.42	0.0001
	Cd	0.41	0.6661
	Ration	27.99	0.0001
	Cd*Ration	0.63	0.6413
94	Model	14.40	0.0001
	Cd	0.54	0.5837
	Ration	51.38	0.0001
	Cd*Ration	2.84	0.0316

treatment components indicated that the main effect, ration, was the sole determinant of growth on all sampling days except day 94. The significant cadmium-ration interaction component on day 94 indicated that the two factors were not independent at that sampling date. On no sampling day could the dietary cadmium concentration be considered a significant contributor to growth (Table 12). The significance of the factorial ANOVA model for fish growth over the entire experiment (Table 13) demonstrated that the growth rate component of the Bendell-Young cadmium accumulation model was successfully manipulated in this experiment, and that it was related to ration, not dietary cadmium concentration.

Cadmium, Copper, and Zinc in Liver

No consistent trend was apparent in hepatic copper and zinc concentrations during the experiment, with the exception of elevations of hepatic copper concentrations in all three tanks receiving the 0.5% ration (Fig. 16). The copper concentrations in liver were approximately 2000 nmol/g throughout the experiment (Fig. 16), while zinc concentrations were approximately 300 nmol/g (Fig. 17).

The hepatic cadmium concentration of trout fed dietary cadmium concentrations of 45 or 450 nmol/g increased in an approximately linear manner over the duration of the experiment, but in the 4.5 nmol/g treatments, hepatic cadmium

Table 13. Analysis of Variance Summary for Growth of Fish (expressed as % weight gain). All fish in the experiment are included in this analysis, with the exception of those sampled on day 3. The factorial model has been divided into sources due to the main effects (dietary Cd concentration and ration) and interaction between main effects.

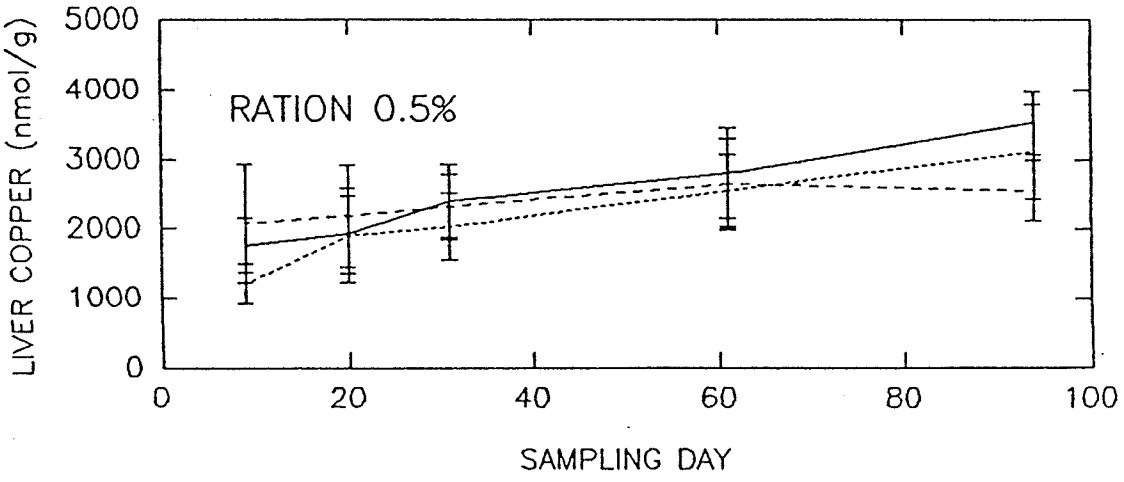
Sampling Day	Source	F Value	Prob > F
	Model	12.20	0.0001
	Cd	0.21	0.8108
	Ration	47.49	0.0001
	Cd*Ration	0.56	0.6939

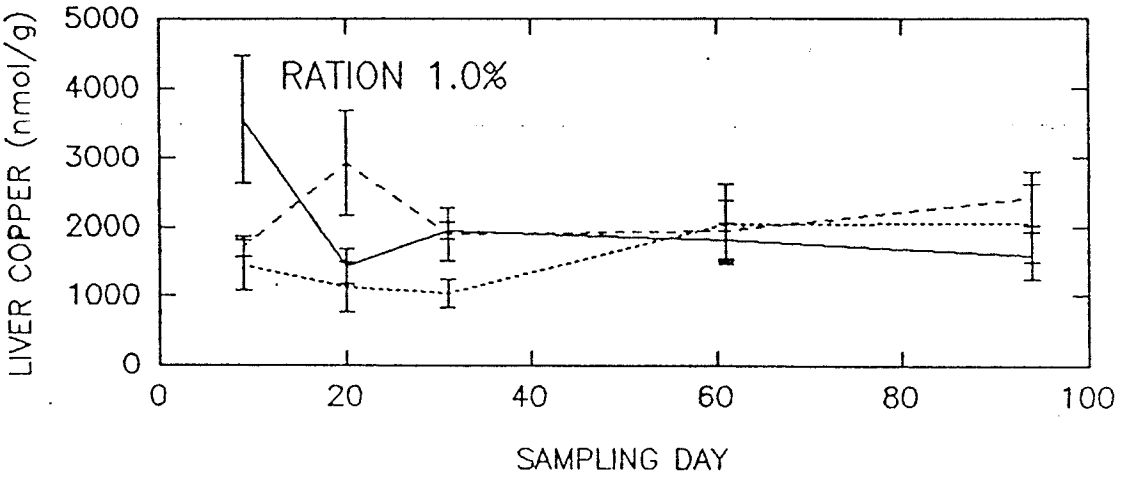
Results of Tukey's Studentized Range Test for Growth of Rainbow Trout in Relation to the Experimental Factors 'Ration' and 'Dietary Cd'.

Treatment	Level	Weight Increase (%)	Significance Grouping
Ration	1.5	27.3	A
	1.0	22.2	B
	0.5	10.6	C
Dietary Cd	450.0	19.5	A
	45.0	20.2	A
	4.5	20.5	A

Figure 16. Hepatic copper concentrations (MEAN ± SEM) in cadmium-fed rainbow trout over the 94 days of the experiment.

Legend: Solid line - 450 nmol Cd/g food; broken line - 45.0 nmol Cd/g food; dotted line - 4.50 nmol Cd/g food.


RATION 0.5%
LIVER COPPER (nmol/g)
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
SAMPLING DAY


RATION 1.0%
LIVER COPPER (nmol/g)
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
SAMPLING DAY

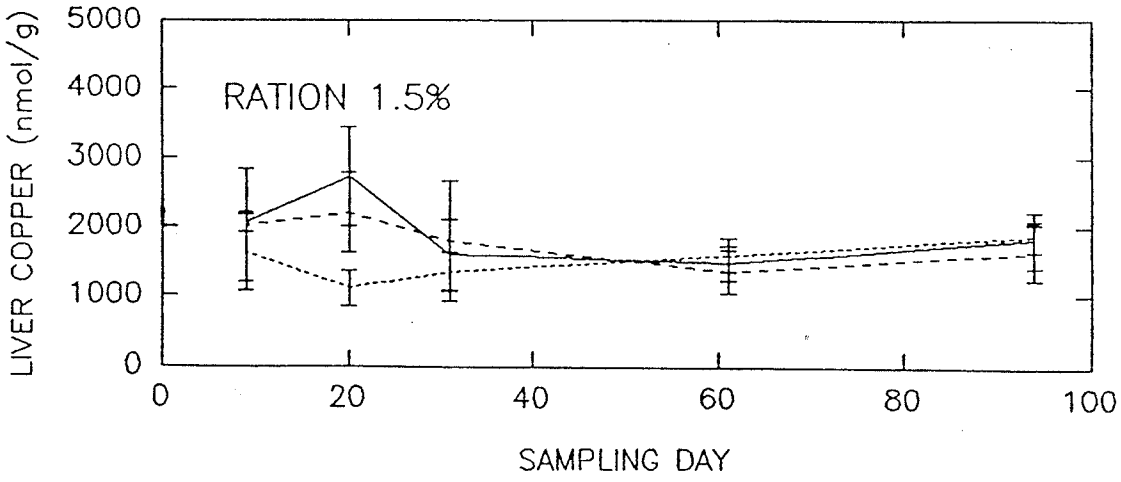
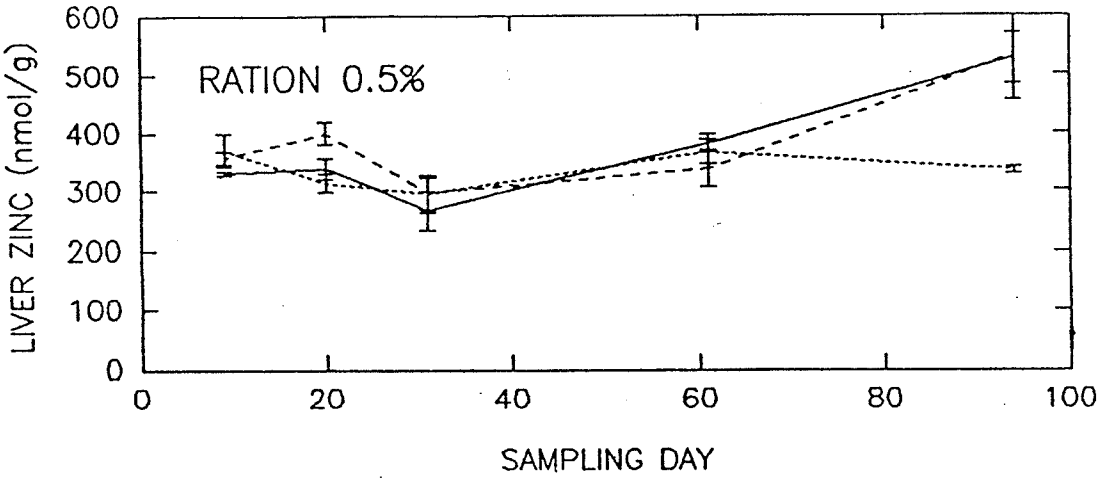
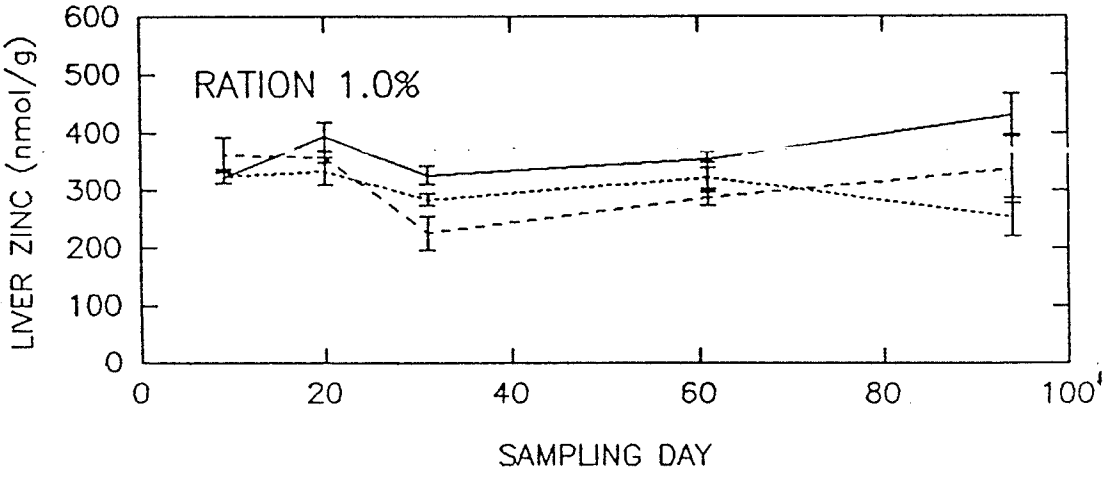
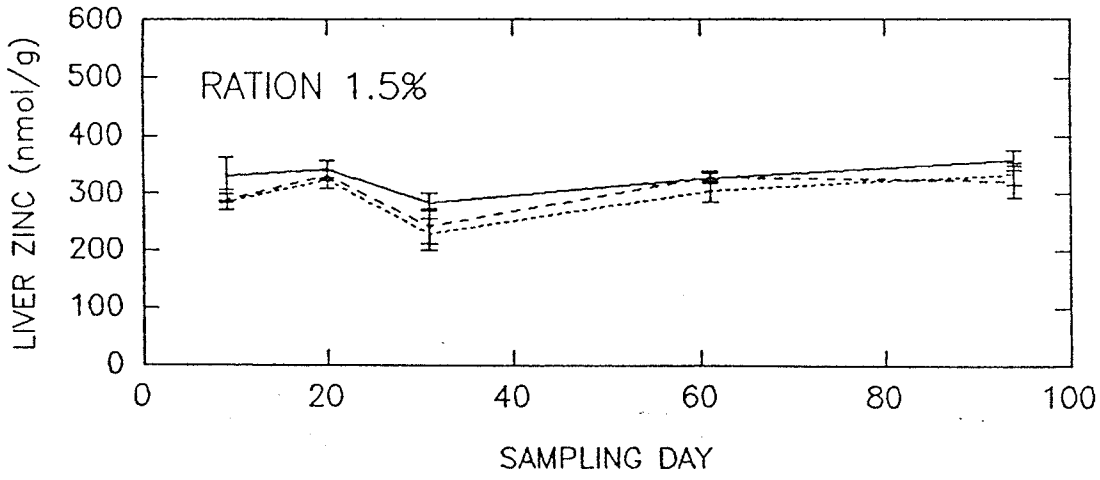

RATION 1.5%
LIVER COPPER (nmol/g)
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
SAMPLING DAY

Figure 17. Hepatic zinc concentrations (MEAN ± SEM) in Cd-fed rainbow tout over the 94 days of the experiment.

Legend: Solid line - 450 nmol Cd/g food; broken line - 45.0 nmol Cd/g food; dotted line - 4.50 nmol Cd/g food.


RATION 0.5%
LIVER ZINC (nmol/g)
600
500
400
300
200
100
0
0
20
40
60
80
100
SAMPLING DAY


RATION 1.0%
LIVER ZINC (nmol/g)
600
500
400
300
200
100
0
0
20
40
60
80
100
SAMPLING DAY


RATION 1.5%
LIVER ZINC (nmol/g)
600
500
400
300
200
100
0
0
20
40
60
80
100
SAMPLING DAY

concentrations were only slightly above detection limits, and did not exceed 0.08 nmol/g after 94 days of exposure (Fig. 18).

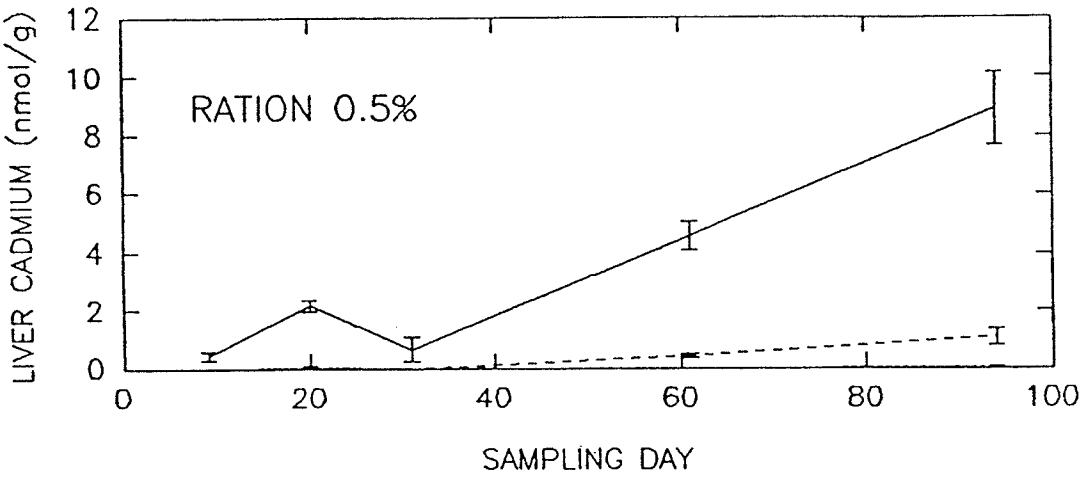
The factorial ANOVA model for hepatic cadmium concentration was significant on all six sampling days (Table 14). Partitioning the model into treatment components revealed that the main effect, dietary cadmium concentration, was the sole determinant of hepatic cadmium concentration on all sampling days. The absence of a significant main effect for ration on all sampling days demonstrated that ration did not contribute to the hepatic accumulation of cadmium from the diet (Table 14).

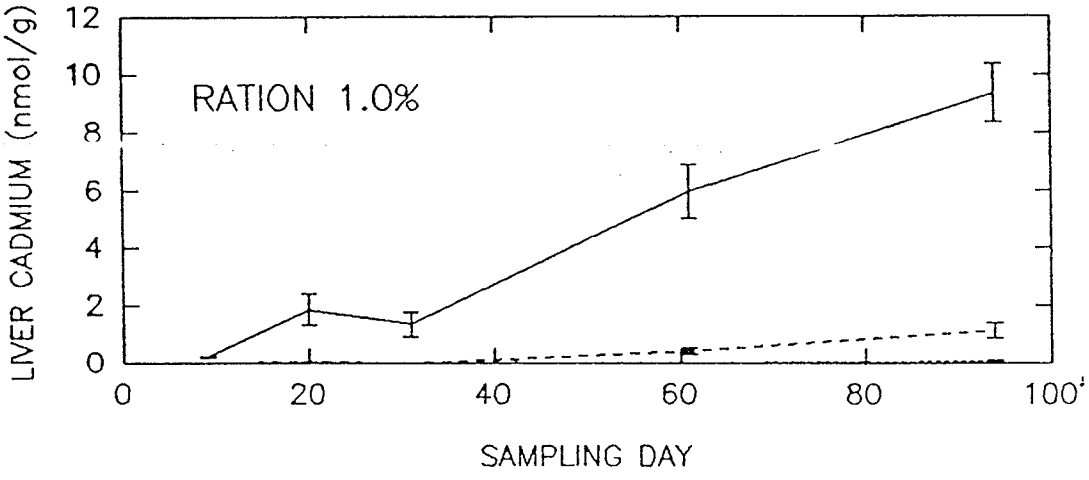
DISCUSSION

The cadmium accumulation model of Bendell-Young et al. (1986) assumes that the hepatic cadmium concentration in fish is due to cadmium bioavailability and growth rate. In this experiment, bioavailability was assumed to be constant among all treatments, because the cadmium-labelled food for all treatments was prepared in the same way (by adding aqueous $CdCl_2$ to fish food). By holding bioavailability constant, growth rate could then be manipulated to test the Bendell-Young model.

Figure 18. Hepatic cadmium concentrations (MEAN ± SEM) in Cd-fed rainbow tout over the 94 days of the experiment.

Legend: Solid line - 450 nmol Cd/g food; broken line - 45.0 nmol Cd/g food; dotted line - 4.50 nmol Cd/g food.


RATION 0.5%
LIVER CADMIUM (nmol/g)
SAMPLING DAY
0
2
4
6
8
10
12
0
20
40
60
80
100


RATION 1.0%
LIVER CADMIUM (nmol/g)
SAMPLING DAY
0
2
4
6
8
10
12
0
20
40
60
80
100

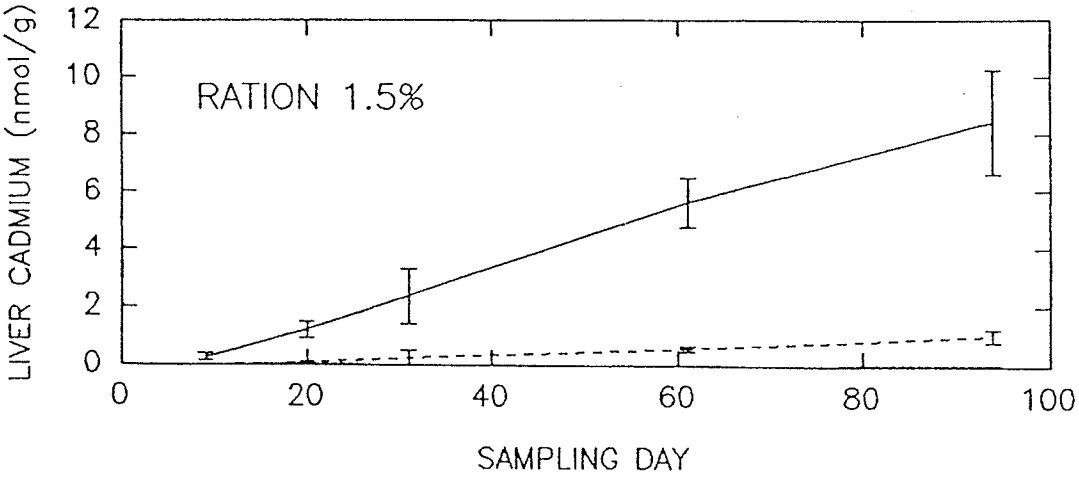

RATION 1.5%
LIVER CADMIUM (nmol/g)
SAMPLING DAY
0
2
4
6
8
10
12
0
20
40
60
80
100

Table 14. Analysis of Variance Summaries for Hepatic Cadmium Concentrations. The factorial model has been divided into sources due to the main effects (dietary Cd concentration and ration) and interaction between main effects.

Sampling Day	Source	F Value	Prob > F
3	Model	2.86	0.0307
	Cd	6.33	0.0083
	Ration	1.72	0.2078
	Cd*Ration	1.69	0.1961
9	Model	5.42	0.0014
	Cd	19.29	0.0001
	Ration	0.79	0.4579
	Cd*Ration	0.79	0.5452
20	Model	18.02	0.0001
	Cd	66.88	0.0001
	Ration	1.75	0.1935
	Cd*Ration	1.73	0.1735
31	Model	4.84	0.0009
	Cd	14.12	0.0001
	Ration	2.27	0.1229
	Cd*Ration	1.49	0.2338
61	Model	20.44	0.0001
	Cd	80.35	0.0001
	Ration	0.54	0.5881
	Cd*Ration	0.44	0.7790
94	Model	33.30	0.0001
	Cd	132.85	0.0001
	Ration	0.15	0.8625
	Cd*Ration	0.11	0.9793

For each treatment level of dietary cadmium concentration, growth rates were altered by feeding at three different rates (ie. rations of 0.5, 1.0, and 1.5% of body weight per day), which resulted in significantly different growth rates among rainbow trout fed for up to 94 days (Table 13) (a necessary prerequisite for testing whether growth rate influences cadmium accumulation). In spite of the altered growth rates, cadmium was not observed to accumulate in rainbow trout liver as a function of growth rate. Rather, cadmium accumulated only as a function of the dietary cadmium concentration (Table 14).

These two statistical inferences, although mathematically sound, are physically unreasonable. The two experimental factors, dietary cadmium concentration and ration, appear to be unrelated at first inspection, but in fact, they are related in terms of dose (the quantifiable amount of material introduced into an organism (Rand and Petrocelli 1985)). Consequently, the three levels of dietary cadmium represent three cadmium doses, as do the three levels of ration.

The conclusion that ration does not affect hepatic cadmium bioaccumulation implies that dose <u>does not</u> affect hepatic cadmium bioaccumulation, whereas the conclusion that dietary cadmium concentration does affect hepatic cadmium bioaccumulation implies that dose <u>does</u> affect hepatic cadmium bioaccumulation. This contradiction regarding dose underlines the fact that statistical inferences must be based upon

physical reality, not just mathematical correctness; for without physical reality, the inferences are arbitrary and unintelligable (Vollenweider 1990). In order to make the statistical inferences of this experiment physically meaningful, the contradiction regarding dose must be overcome. In other words one experimental factor must be redefined into terms which are independent of dose.

Such a redefinition could involve dietary metal ratios, because cadmium is known to interact with other metals in the diet (reviewed by Neathery 1980 and Spivey-Fox 1988). Because dietary copper and zinc concentrations were constant among all treatments (401 and 2590 nmol/g respectively), as the cadmium concentration in the food increased from 4.5 to 45 and 450 nmol/g, the ratio of copper and zinc to cadmium in the food decreased from 665 to 66.5 and 6.65. Therefore, the factor defined as dietary cadmium concentration could also be meaningfully defined as the ratio of copper and zinc to cadmium in the diet. This definition appears to be appropriate because it is independent of ration (ie. for each level of dietary cadmium concentration, the ratio of copper and zinc to cadmium is not altered by a change in ration), thereby avoiding the contradiction (regarding dose) which results when the factor is defined as dietary cadmium concentration.

Therefore, it is the ratio of copper and zinc to cadmium in food which determines cadmium accumulation in rainbow trout liver, not just the dietary cadmium concentration. This is

plausible, because dietary copper and zinc supplementation alleviates the uptake and toxicity of dietary cadmium (Bremner 1979, Webb 1979, Neathery 1980, Spivey-Fox 1988). In the natural environment, this effect would be attenuated by complexation reactions which limit the bioavailability of these three metals, but in this experiment the bioavailability of the metals was held constant between treatments, and so was not a factor.

Typically, cadmium bioaccumulation is examined without considering other metals even though they are known to interact with cadmium (Bendell-Young et al. 1986, Jeffery et al. 1988, Frank et al. 1990), in metal exposure situations which may be complex. The results of this experiment indicate that metals such as copper and zinc are important factors determining cadmium accumulation in fish, so they should be measured in addition to the cadmium concentrations to which fish are exposed.

Furthermore, this experiment could not validate the cadmium accumulation model of Bendell-Young et al. (1986); growth rate was found to not affect dietary cadmium bio-accumulation in rainbow trout. Instead, hepatic cadmium accumulation from the diet could only be explained as a function of metal ratios in the diet, specifically, the ratio of (Cu + Zn):Cd. However, this conclusion is based upon cadmium exposure via the diet only. Further research is required to: (1) determine whether similar results hold

waterborne cadmium exposure, and (2) verify that metal:cadmium ratios determine cadmium accumulation. The latter experiment could be acheived by manipulating dietary metal concentrations simultaneously.

If present trends hold, cadmium concentrations will continue to increase in North American waters (Smith et al. 1987), so trends in cadmium bioaccumulation may be of some significance to the overall understanding of environmental cadmium pollution. However, the mechanisms underlying cadmium bioaccumulation must be understood in greater detail before cadmium concentration in fish tissues can be utilized as a biomarker of environmental cadmium exposure.

CHAPTER V

GENERAL DISCUSSION

GENERAL DISCUSSION

The research reported in this thesis was conducted over a five year period. The differences among chapters II, III, and IV demonstrate how my research developed over this period. One problem which results from presenting these separate research projects together as one body of research, is the occurrence of several apparent methodological inconsistancies.

The first of these inconsistencies concerns the use of heat treatment for sample preparation prior to analyzing MT by the mercury saturation assay. In chapter II it was recommended that heat treatment be restricted to less than 2 minutes, yet in the Flin Flon research in chapter III, 10 minute heat treatment was used. This inconsistency could not be avoided because the Flin Flon samples were analyzed 2 years before it was realized that long heat treatments can elevate MT estimates.

A second apparent inconsistency concerns the failure (in chapter IV) to follow my recommendation (from chapter III) that future emphasis on the effects of heavy metals on the kidney is warranted. This inconsistency reflects the fact that the experiment in chapter IV was specifically conducted to test the hepatic cadmium bioaccumulation model of Bendell-Young et al. (1986). Although the behavior of cadmium in the kidney is ultimatley of great interest and in need of study,

it was simply beyond the scope of the experiment.

Finally, the use of laboratory and field experimental approaches in this thesis is (arguably) inconsistent. Ecotoxicology is defined as the study of the fate and effects of chemical pollutants on ecosystems (Brouwer et al. 1990, Moriarty 1988). Useful ecotoxicological assessments (eg. validating biomarkers) require the integration of analytical, toxicological, and ecological information. Ecological and analytical information are usually provided by field studies, while toxicological information is generally obtained from laboratory studies involving single species of organism (Brouwer et al. 1990). The ability of laboratory toxicity testing methods to predict the ecosystem effects of chemical pollutants may be inadequate (Kimball and Levin 1985), largely because of differences in biotic and abiotic complexity between the laboratory and the field.

However, if greater emphasis is placed upon mechanisms determining the fate and effects of chemical pollutants on ecosystems, the gaps between laboratory and field research are minimal. Mechanism-oriented ecotoxicological research utilizes laboratory toxicity tests as sources of baseline data, from which the mechanisms are then studied in laboratory, semi-field (ie. microcosm or mesocosm), and field experiments (Brouwer et al. 1990).

Experiments are planned inquiries to obtain new facts, or to confirm or deny the results of previous experiments (Steel

and Torrie 1980). The power of experimentation lies in the ability of the experimenter to control and manipulate experimental variables. The inability of the experimenter to control variables in field experiments can limit the statistical power of field experiments.

For example, the field survey at Flin Flon (Chapter III) was an experiment consisting of eight treatments (ie. Cu, Zn, and Cd concentrations in surficial sediments of eight lakes). The inability of the survey to establish a dose-response relationship between MT and sediment metal concentrations probably reflects a lack of experimental control, as several important factors could not be manipulated. First, total metal concentrations in surficial sediment were used to estimate metal exposure, even though it was not known what proportion of sediment metals were actually bioavailable. Second, the contribution of waterborne metals to the overall exposure situation was not established. Third, the water chemistry parameters which were measured indicated that inter-lake variability in water chemistry was large, which may have affected metal bioavailability. Fourth, fish were exposed to metal mixtures, the composition of which varied between lakes. Clearly, the Flin Flon survey was not a conclusive test of MT as a biomarker of metal exposure.

A second example which demonstrates the lack of experimental control which exists in field survey experiments

concerns the research of Bendell-Young et al. (1986). After surveying six lakes in southern Ontario, the authors produced a cadmium accumulation model for fish. The model used several assumptions which were required due to a lack of experimental control (water chemistry and cadmium loadings varied among lakes). These assumptions (and the model) were found to be in error when the model was tested in a simple laboratory experiment (Chapter IV).

In order for field surveys to provide useful ecotoxicological assessments, the experimental designs employed must accomodate the lack of experimental control which is available in such studies. For example, multi-year studies of the same lakes can overcome problems of inter-lake variability among water chemistry parameters to some extent. However, in order to determine the fate and effects of chemical pollutants in aquatic ecosystems, preliminary laboratory experimentation can reveal potential mechanisms at considerably less expense and with considerably more certainty than field surveys.

Ultimately, the two experimental approaches are complementary, not opposite. The current conflict concerning laboratory and field ecotoxicology will have been a useful excercise if, as a result, the complementary nature of these two approaches becomes accepted.

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