Accumulation, Distribution, and Toxicology of Nickel in Lake Whitefish (*Coregonus clupeaformis*) and Lake Trout (*Salvelinus namaycush*) Exposed through the Dietary Route of Uptake

By

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A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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GENERAL ABSTRACT

Metal mining, milling, and smelting, metal processing, fuel combustion, and waste incineration activities release significant amounts of nickel (Ni) into freshwater systems. Benthic-feeding fish residing in Ni-contaminated systems are exposed to Ni through ingestion of contaminated food items and sediments. Laboratory-based research is needed to provide insight into the potential impacts of the chronic exposure of freshwater fish to dietary Ni. A short-term trial was conducted to investigate the uptake and toxicity of dietary Ni in adult lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*) fed diets containing 0, 1000, and 10000 μg Ni/g, prepared with and without brine shrimp, over a period of 18 days. Results from this study were used to determine which diet concentrations, diet type, and fish species to use in the long-term experiment. In the long-term experiment, adult lake whitefish were fed diets containing 0, 10, 100, and, 1000 μg Ni/g for 10, 31, and 104 days. Ni concentrations were highest in intestine and pyloric caeca of whitefish fed 1000 μg Ni/g on day 10, but decreased on subsequent sampling days, possibly due to protective mechanisms. Ni accumulation in stomach, kidney, liver, gill, skin, and scales was dose- and duration-dependent. The tissues that best assess dietary Ni bioavailability are kidney and scales. Concentrations of Cu and Zn were measured in 6 tissues but results were inconsistent. The toxicity of Ni was assessed through the measurement of responses, through a range of levels of biological organization. In livers and kidneys of treated fish lesions were observed. The frequency and severity of renal histopathologies increased with the dose and duration of exposure. Increases in intestinal metallothionein and plasma lipid peroxide concentrations were

observed in whitefish fed 1000 μg Ni/g, but did not persist. Results indicate that metallothionein and lipid peroxides may be important biochemical indicators of Ni exposure in fish. Hematological, organ, and whole organism parameters were not affected by Ni exposure. The measurement of tissue residues in kidney and scales and the histopathological assessment kidney and liver are recommended for use in field bio-monitoring programs, to evaluate exposure of natural populations of fish to Ni.

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ABBREVIATIONS

BW	body weight
cc	cubic centimeter
CEPA	Canadian Environmental Protection Act
cm	centimeter
°C	degrees celcius
d	day
D	distal tubule
DL	detection limit
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EEM	environmental effects monitoring
dw	dry weight
F	female
g	gram
H&E	hematoxylin and eosin stain
L	litre
LPO	lipid peroxides
LSI	liver somatic index
M	male
mg	milligram
min	minute
ml	milliliter
MMLER	metal mining liquid effluent regulations
MS-222	tricaine methane sulfonate
MT	metallothionein
NS	no shrimp
P1	first segment of the proximal tubule
P2	second segment of the proximal tubule
PUFA	polyunsaturated fatty acids
rpm	revolutions per minute
R^2	coefficient of determination
SE	standard error of the mean
t	tonne
µg	microgram
µm	micron
µl	microliter
ww	wet weight
ww calc.	wet weight calculated
WS	with shrimp

GENERAL INTRODUCTION

Nickel (Ni) is present in Canadian freshwater environments because of mining, milling, and smelting operations, and other human activities, including refining, fuel combustion, alloy production, and waste incineration activities. Ni is also released into the environment from natural processes including the weathering and leaching of rock and in soil dust (Environment Canada, 1994). Ni occurs in aquatic systems as dissolved species or as soluble salts adsorbed onto or associated with clay minerals, iron and manganese oxides, or organic matter (WHO, 1991; AQUAMIN, 1996).

Ni released from metal mining, milling, and smelting operations enters aquatic systems through atmospheric deposition, liquid effluents, and leachates (Chau and Kulikovsky-Cordeiro, 1995). Although, there has been a significant reduction in the amount of Ni released from these operations during the last 30 years, annual releases of Ni from these operations are still substantial. For example, in 1998, a total of 312 t of Ni was released into the environment, with 307 t of Ni released as air emissions and 5.1 t released as effluents, from Canadian metal mining, milling, and smelting operations (MAC, 1999b).

The release of Ni into aquatic systems, through air emissions and liquid effluents, is currently regulated in Canada through metal mining liquid effluent regulations (MMLER) and the Canadian Environmental Protection Act (CEPA). The MMLER address water pollution issues related to the mining industry, including the reduction and control of dissolved Ni in mine waters and tailings pond discharges (AQUAMIN, 1996). In the fall of 2000, a revised MMLER will be implemented that requires mining companies to conduct an environmental effects monitoring (EEM) program to effectively

monitor and evaluate the effects of mine effluent on the aquatic environment. The key components of the proposed EEM program include a review of the existing information, site characterization, initial monitoring, data assessment and interpretation, periodic monitoring, focused monitoring, and the investigation of cause of effects. Under the proposed EEM program, water, sediment, air, and biota will be routinely monitored to identify the effects of contaminant exposure on ecosystems. When the cause of an effect cannot be identified, additional research will be conducted to provide information that can be utilized to improve the monitoring programs (Environment Canada, 1998).

The CEPA regulates the quality of water and sediments in aquatic systems (CEC, 1995; NRCAN 1996). The Canadian water quality guidelines recommend that Ni concentrations in freshwater should be below 25 μg/L to protect aquatic life. The Canadian sediment quality guidelines define a probable effect level (PEL) for Ni of 36 μg Ni/g (dry weight, dw) in freshwater sediments. Ni concentrations in sediments that exceed this concentration are predicted to cause frequent adverse effects in aquatic organisms (Environment Canada, 1994).

In freshwater systems located near Canadian base-metal, gold, and uranium mining, milling, and smelting operations, elevated concentrations of Ni greatly exceeding the PEL concentration have been observed in surficial sediments (Environment Canada, 1994). For example, surficial sediments collected downstream from 2 gold-mining operations located near Red Lake, Ontario accumulated concentrations up to 1100 μg Ni/g (dw) (J.F. Klaverkamp, unpublished data). Concentrations of nickel as high as 12000 μg Ni/g (dw) have been observed in sediments near Canadian base metal smelting operations near Sudbury, Ontario (Nriagu *et al.*, 1982).

The elevated concentrations of Ni observed in the surficial sediments are often associated with the presence of elevated Ni concentrations in aquatic organisms (Hutchinson et al., 1976; Ney and Hassel, 1983; Dallinger and Kautzky, 1985; Bradley and Morris, 1986; Mastala *et al.*, 1992; Sharif *et al.*, 1993; Tariq *et al.*, 1993; Kashulin and Reshetnikov, 1995; Moiseenko *et al.*, 1995; Maletin *et al.*, 1996; Allen-Gil *et al.*, 1997: Brotheridge *et al.*, 1998; Klavins *et al.*, 1998a; Klavins *et al.*, 1998b; Klaverkamp *et al.*, 2000a). For example, zooplankton collected from the Wanapitei River, located near base-metal mining operations in Sudbury, Ontario, contained Ni concentrations that were 9x higher than those observed in zooplankton collected from a reference area (Hutchinson *et al.*, 1976). Elevated Ni concentrations, of 224 μg Ni/g (dw), were also observed in the surficial sediments of the Wanapitei River. In another example, lake whitefish (*Coregonus clupeaformis*) collected from Little McDonald Lake, near a uranium mine in Key Lake, Saskatchewan, accumulated Ni concentrations that were 9x higher in liver and 7x higher in kidney than concentrations observed in lake whitefish collected from a reference lake (Klaverkamp *et al.*, 2000a). Elevated concentrations of Ni, as high as 690 μg Ni/g (dw), were also observed in the surficial sediments of Little MacDonald Lake.

Freshwater fish inhabiting contaminated systems are exposed to Ni, primarily, through the ingestion of contaminated food and sediments (Dallinger and Kautzky, 1985). Despite the importance of the diet as a source of Ni uptake, laboratory studies conducted investigating the uptake and toxicity of Ni are based on the exposure of fish to contaminated water (Hughes *et al.*, 1979; Chaudhry and Nath, 1984; Tjalve *et al.*, 1988; Nath and Kumar, 1989; Nath and Kumar 1990; Ray *et al.*, 1990; Ghazaly, 1992; Sreedevi

et al., 1992; Jha *et al.*, 1994; Jha and Jha, 1994; Pyle, 1999). Laboratory based research investigating the exposure of fish to dietary Ni is needed to provide insight into the potential impacts of the chronic exposure of natural populations of freshwater fish to dietary Ni.

To address these research needs, two studies were conducted investigating the accumulation, distribution, and sublethal toxicity of dietary Ni exposure in two species of freshwater fish. The first study was a short-term, preliminary experiment that was conducted to investigate the uptake and sublethal toxicity of dietary Ni in adult lake whitefish and lake trout (*Salvelinus namaycush*) fed diets containing 0, 1000, and 10000 μg Ni/g, prepared with and without brine shrimp, over a period of 18 days (Chapter 1). The objectives of this experiment were: 1) to determine the concentrations of dietary Ni that would be readily consumed by lake trout and lake whitefish, 2) to determine if brine shrimp would increase the consumption of the Ni-contaminated diets, and 3) to determine which species of fish is most sensitive to Ni exposure, based on the extent of Ni uptake and toxic effects observed. The results of this research were used to guide the experimental design of the second experiment.

The second experiment was a long-term experiment that was conducted to investigate the uptake and sublethal toxicity of dietary Ni in adult lake whitefish fed 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days (Chapters 2 and 3). The objectives of this experiment were: 1) to examine the patterns of Ni accumulation and distribution in lake whitefish and determine which tissues provide the best indication of Ni bioavailability, 2) to examine the toxicological effects of dietary Ni exposure on biochemical, cellular, tissue, organ, and whole-organism parameters in lake whitefish and

to determine which responses can provide early warning signals of Ni exposure in fish, and 3) to determine if there is a link between Ni uptake and toxicological effects observed.

Information gained from this research can be used to provide valuable information and insight to other researchers and to individuals conducting field bio-monitoring programs in the mining industry.

Chapter One

Accumulation, distribution, and toxicology of dietary nickel

in lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*).

1) ABSTRACT

A preliminary experiment was conducted to investigate the uptake and sublethal toxicity of dietary Ni in adult lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*) fed diets containing 0, 1000, and 10000 μg Ni/g, prepared with and without brine shrimp, over a period of 18 days. The results of this experiment were used to determine which fish species, diet concentrations, and diet types to use in a long-term experiment, lasting 104 days (Chapters 2 and 3). Feed refusal was observed in lake trout and lake whitefish fed 10000 μg Ni/g, after 3 and 4-5 feedings, respectively. Lake trout fed high dose diets exhibited different patterns of accumulation than lake trout fed low dose diets. Similar accumulation patterns were observed in lake whitefish fed low and high dose diets. Increased Ni concentrations in all lake whitefish tissues, except intestine, were associated with increases in the dose of Ni in the diet. Cu and Zn concentrations in kidney and liver of lake whitefish were altered. The toxicology associated with the exposure was investigated at a numerous levels of biological organization. At the molecular level, metallothionein concentrations in kidneys of lake trout fed 1000 μg Ni/g and 10000 μg Ni/g and lake whitefish fed 10000 μg Ni/g and in livers of lake whitefish fed 10000 μg Ni/g (diet without shrimp only) increased significantly. Increased lipid peroxide production in the plasma of lake trout fed 10000 μg Ni/g was observed. Hematological parameters, including glucose and blood ions, were affected by Ni exposure. At the cellular level, histopathological alterations were sustained in kidneys of lake whitefish fed low and high dose diets, livers of whitefish fed high dose diets, and intestines of lake whitefish fed high dose diets and lake trout fed low and high dose diets. At the whole organism level, lake trout fed high dose diets exhibited significant decreases

in weight. Lake whitefish were selected for use in the long-term experiments. Diets selected for the longer-term experiments contained 0, 10, 100, and 1000 μg Ni/g, because diets containing 1000 μg Ni/g were readily consumed by lake trout and lake whitefish. Brine shrimp were not incorporated into the diets used in the long-term exposure because increased consumption of the high dose diets was not observed.

2) INTRODUCTION

Nickel (Ni) enters aquatic systems through atmospheric deposition and in liquid effluents released from mining, smelting, refining, metal processing, fuel combustion, and waste incineration operations (Chau and Kulikovsky-Cordeiro, 1995). In freshwater systems located near Canadian base-metal, gold, and uranium mining and milling operations, elevated concentrations of Ni have been observed in surficial sediments (Environment Canada, 1994). For example, surficial sediments collected from lakes located close to smelters in Sudbury, Ontario contained Ni concentrations as high as 12 000 μg Ni/g (dry weight, dw) (Nriagru *et al.*, 1982).

Increased concentrations of Ni have also been reported in invertebrates and fish residing in Ni-contaminated environments (Hutchinson et al., 1976; Ney and Hassel, 1983; Dallinger and Kautzky, 1985; Bradley and Morris, 1986; Mastala *et al.*, 1992; Sharif *et al.*, 1993; Tariq *et al.*, 1993; Kashulin and Reshetnikov, 1995; Moiseenko *et al.*, 1995; Maletin *et al.*, 1996; Allen-Gil *et al.*, 1997; Brotheridge *et al.*, 1998; Klavins *et al.*, 1998a; Klavins *et al.*, 1998b; Klaverkamp *et al.*, 2000a). For example, whitefish (*Coregonus lavaretus*) collected from a lake, with concentrations of 2227 μg Ni/g (dw) in the surficial sediments located near base-metal mining smelters in Russia, accumulated

28 μg Ni/g (d.w) in the kidney, 9.6 μg Ni/g (dw) in the gill, and 7.5, 2.7, and 1.3 μg Ni/g (dw) in the skeleton, liver, and muscle, respectively (Kashulin and Reshetnikov, 1995).

Freshwater fish residing in polluted systems are predominantly exposed to Ni through the ingestion of contaminated food and sediments (Dallinger and Kautzky, 1985). In spite of the importance of the dietary route of exposure, as a source of Ni uptake, laboratory studies conducted investigating the uptake and toxicity of Ni are based on the exposure of fish to waterborne Ni (Hughes *et al.*, 1979; Chaudhry and Nath, 1984; Tjalve *et al.*, 1988; Nath and Kumar, 1989; Nath and Kumar 1990; Ray *et al.*, 1990; Ghazaly, 1992; Sreedevi *et al.*, 1992; Jha *et al.*, 1994; Jha and Jha, 1994; Pyle, 1999). Research investigating the exposure of fish to dietary Ni is needed to elucidate the potential impacts of chronic dietary Ni exposure on natural populations of freshwater fish.

To address these research needs, a preliminary study was conducted assessing the accumulation, distribution, and toxicity of dietary Ni in two species of freshwater fish. Lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush)* were fed control and Ni-contaminated (1000 and 10 000 μg Ni/g) diets, prepared with and without brine shrimp for 18 days. The patterns of Ni accumulation and distribution were determined by measuring Ni concentrations in twelve tissues. The toxicology associated with the exposure was assessed by measuring molecular (metallothionein induction, lipid peroxide production, hematological parameters (hemoglobin, blood ions, and glucose)), cellular (histopathology) and tissue (hematological parameter (hematocrit), and organ and whole organism (condition factor, growth, and LSI) responses. Lake trout and lake whitefish were selected for use in the experiments because of their sensitivity to

environmental contaminants, economic importance, and distribution (Bodaly, 1986; DFO, 1997; Cooley and Klaverkamp, 2000; Pedlar and Klaverkamp, 2000). The concentrations and form of Ni incorporated into the diets were environmentally relevant, as they were based on concentrations and forms of Ni reported to occur in oxic sediments near base-metal, gold, and uranium mining and milling operations in Canada (Chau and Kulikovsky-Cordeiro, 1995; AQUAMIN, 1996).

The primary objectives of this experiment were, first, to determine the concentrations of Ni that lake trout and lake whitefish would consume and tolerate; second, to determine the effectiveness of brine shrimp in increasing consumption of the contaminated diet; and third, to determine which species of fish is most sensitive to Ni exposure, based on the extent of Ni uptake and toxic effects observed. The results from this study were used to guide the experimental design of a long-term exposure, lasting 104 days (presented in Chapters 2 and 3).

3) MATERIALS AND METHODS

3.1) Fish

Adult lake whitefish and lake trout were obtained from stocks at the Freshwater Institute, Winnipeg, Manitoba. Twenty-four lake whitefish, 3 years in age, with weights and fork lengths (mean ± SE) of 208 ± 5.71 g and 24.6 ± 0.205 cm, respectively, were randomly distributed into 6 tanks. Twenty-four lake trout, 2 years in age, with weights and fork lengths (mean ± SE) of 306 ± 8.43 g and 30.0 ± 0.268 cm, respectively, were randomly distributed into 6 additional tanks. The fish were acclimated for 4 weeks.

During the acclimation period fish were fed a ration of a commercially prepared control diet equal to 0.5% of the total body weight per tank every Monday, Wednesday and Friday.

3.2) Tanks

Twelve 200 L fiberglass tanks, with transparent, plexi-glass lids were used. Aerated, de-chlorinated, treated municipal water was supplied to the tanks (Wagemann *et al.*, 1987). Photoperiod was controlled by a timed artificial lighting system; with 11.5 hours of light and 11.5 hours of darkness, separated by two 30-minute periods of intermediate light.

3.3) Diets

Diets were prepared at the Freshwater Institute, Winnipeg, Manitoba. Six diets were prepared: Diets 1-3 contained nominal concentrations of 0, 1000, and 10000 μg Ni/g and diets 4-6 contained nominal concentrations of 0, 1000 and 10000 μg Ni/g with added brine shrimp. Diets 4-6 were prepared with brine shrimp because it was anticipated that fish might refuse to eat Ni-contaminated diets. The form of Ni used in the diets was nickel sulphate hexahydrate, $NiSO_4 \cdot 6H_2O$ (Fisher Scientific, Fairlawn, NJ). Commercial sinking pellets (Martin Feed Mills, Elmira, ON), containing 42% crude protein, 16% crude fat, 2% crude fiber, 5% ash, and 0.9% calcium, were ground in a feed mill to produce flour. Frozen brine shrimp were ground using a food processor. Control diet mixture #1 was prepared by mixing 2 parts flour with 1 part deionized, distilled water. Contaminated diets mixtures 2 and 3 were prepared by adding $NiSO_4 \cdot 6H_2O$ to

flour, to produce mixtures with nominal concentrations of 1000 and 10000 μg Ni/g. Following that, one part deionized, distilled water was added to 2 parts of the contaminated diet mixture. Diet mixtures 4-6 were prepared in a similar manner, by adding 1 part brine shrimp and 1 part deionized, distilled water, to 3 parts flour or 3 parts of a flour and $NiSO_4 \cdot 6H_2O$ mixture, to produce mixtures with nominal concentrations of 0, 1000, and 10000 μg Ni/g mixture. Mixtures were homogenized using a large stainless steel food mixer and processed using an extruder to produce noodles. The noodles were dried to remove water, cut into No.3 size pellets, and stored at -20° C. To prevent contamination between diet mixtures, diets were produced in ascending order of Ni concentrations and all equipment used to prepare the diets was thoroughly cleaned.

Diets were analyzed in triplicate to verify Ni concentrations, with a detection limit of 0.05 μg Ni/g (ww). Diets 1-3, with nominal concentrations of 0, 1000, and 10000 μg Ni/g (no shrimp) contained < 0.05, 1100 ± 33, and 11000 ± 700 μg Ni/g (ww), respectively, expressed as mean ± SE. Diets 4-6, with nominal concentrations of 0, 1000, and 10000 μg Ni/g (shrimp) contained 1.5 ± 0.93, 1500 ± 110, and 14000 ± 270 μg Ni/g (ww), respectively. Diets 4-6 contained more water than diets 1-3, due to the addition of brine shrimp. As a result, these diets contained higher Ni concentrations after they were dried.

In subsequent sections, when diet types need to be differentiated, diets 1-3, containing 0, 1000, and 10000 μg Ni/g, will be denoted as "(NS)" and diets 4-6, containing 0, 1000, and 10000 μg Ni/g with added shrimp, will be denoted as "(WS)".

3.4) Water Quality

Water quality was monitored daily. Dissolved oxygen (99 ± 3.0 % saturation), pH (7.9 ± 0.08), and temperature (10.6 ± 0.316° C) were measured, expressed as mean ± SE for all tanks. Concentrations of major ions, total anion and cations, organic acids, total dissolved inorganic and organic carbon, total suspended solids, conductivities, and alkalinities of water supplied to the tanks are presented in Cooley and Klaverkamp (2000).

To minimize exposure of fish to Ni-contaminated waste products, solid waste was siphoned from tanks daily, tanks were flushed weekly, and tanks received water at a flow rate of 1 L every 54 seconds. This flow rate resulted in 90 % replacement of tank water in 7 hours and 99% replacement of tank water in 15 hours (Sprague, 1973).

3.5) Experimental Design and Procedures:

Each of 6 tanks of lake whitefish and 6 tanks of lake trout were exposed to a combination of two treatments. Treatment 1 was the concentration of Ni in the diet (0, 1000, or 10000 μg Ni/g) and treatment 2 was the diet type (WS or NS). The sample size was 4 individuals per treatment group.

Lake trout and lake whitefish were fed control and Ni-contaminated diets at a ration equal to 0.5% total body weight per tank every Monday, Wednesday and Friday. Fish were administered this ration 7 times, over a period of 18 days. Feeding activity was monitored visually throughout experiment.

3.6) Sampling:

On August 28, 1997, the 18th day of the experiment, after 2 days of fasting, all fish were sampled. Fish were anesthetized with a pH-neutralized solution of tricaine methane sulfate (MS-222) (Sigma Chemical Co., St. Louis, MO), at a concentration of 338 mg MS-222/L. Fish were removed from the anesthetic after 1-2 minutes, when there was no response to a tail pinch. Fork lengths and weights were measured.

Blood was removed from the caudal artery and vein, using a 20-gauge needle and 3 cc syringe pre-treated with ammonium heparin. A drop of whole blood was transferred to a blood glucose electrode and glucose concentrations were measured using a blood glucose sensor (Medisense, Inc., UK). Hematocrit content for the whole blood was determined by transferring a small amount of whole blood to a capillary tube, sealing the tube, centrifuging for 5 min at 8000 rpm using a Damon/IEC Division Clinical Centrifuge (Needham, MA), and measuring hematocrit using a Damon/IEC Division microcapillary hematocrit reader (Needham, MA). A 20 µl sample of blood was transferred to a tube containing Drabkin's solution for hemoglobin measurements. The remaining quantity of the whole blood sample was transferred into a vacutainer, pre-treated with ammonium salt heparin (Sigma Chemical Co., St. Louis, MO), and centrifuged for 3 minutes at 10000 rpm. The resultant plasma was pipetted into labeled microcentrifuge tubes and frozen at –90 °C for lipid peroxide and blood ion analyses.

Tissues were dissected using the methods described in Cooley and Klaverkamp (2000). Kidney and liver were stored at –90 °C for metals and biochemical analyses. Bile, bone, gall bladder, gills, gonads, heart, intestine, muscle, pyloric caeca, and stomach were stored - 40 °C for metals analyses. Anterior and posterior kidney, liver, cardiac

stomach, pyloric caeca, intestine, rectum, and thyroid samples were collected and immersed in Bouin's fixative for histological analyses.

3.7) Metals Analyses

Tissues were prepared for metals analyses as follows. Gill filaments were removed from the gill arch. Muscle was separated from the skin. Opercular bones were scraped to remove overlying tissue. Gall bladders were cut longitudinally. Opecular bones and gall bladders were rinsed with deionized, distilled water and blotted. Stomach and intestine (small and large pooled) were separated, cut longitudinally, lightly scraped using a spatula, rinsed with deionized, distilled water and blotted dry.

All analytical work was conducted in the Environmental Chemistry Lab at the Freshwater Institute, Winnipeg, MB. Twelve tissues, including bile, bone, gall bladder, gills, gonads, heart, intestine, kidney, liver, muscle, pyloric caeca, and stomach, of lake trout and lake whitefish were analyzed to determine Ni content. Samples were digested in 4 ml of trace metal, analytical- grade nitric acid and heated at 135 ° C until dried. The digestion process was repeated twice, using 2.5 ml and 1.0 ml of nitric acid, respectively. Charred samples were treated with 2 drops of 30% hydrogen peroxide, following the addition of nitric acid. Tubes were cooled, 150 µl of nitric acid was added, and volumes were diluted to 12.5 ml with deionized, distilled water, and heated for 1 hour at 80 ° C. After cooling, volumes were diluted to 25 ml with deionized, distilled water and mixed with a vortex. Tubes holding gonad, heart, intestine, kidney, liver, muscle, pyloric caeca, stomach, or feed samples were cooled, 150 µl of nitric acid was added, and volumes were diluted to 12.5 ml with deionized, distilled water, and heated for 1 hour at 80 ° C. After

cooling, volumes were diluted to 25 ml with deionized, distilled water and mixed with a vortex. It was anticipated that Ni concentrations would be considerably lower in the bile, bone, and gall bladder samples because of low tissue weights. Accordingly, these samples were diluted to a lesser extent. Tubes were cooled, 100 µl of nitric acid was added, and volumes were diluted to approximately 6 ml with deionized, distilled water, and heated for 1 hour at 65 ° C. After cooling, volumes were diluted to 12.5 ml with deionized, distilled water and mixed with a vortex.

Samples were analyzed using flame atomic absorption spectroscopy (Varian SpectrAA-20 Atomic Absorption Spectrometer) to detect the higher range of Ni concentrations (25 - 2000 µg/L). Lower concentrations of Ni (1.0 - 50 µg/L) were measured using graphite furnace atomic absorption spectroscopy (Hitachi Polarized Zeeman Atomic Absorption Spectrometer Model 8200). Samples were diluted if necessary. Detection limits were calculated using the following formula: ((analytical detection limit of equipment X the volume of sample)/tissue weight). The weights of tissue samples ranged from 9 mg for bile samples to 1.532 g for muscle samples. The calculated detection limits, expressed as mean ● SE, for Ni in lake trout bile, bone, gall bladder, gills, gonads, heart, intestine, kidney, liver, muscle, pyloric caeca, and stomach were 0.65 ± 0.08, 0.07 ± 0.00, 1.1 ± 0.13, 0.04 ± 0.00, 0.18 ● 0.03, 0.16 ± 0.01, 0.13 ± 0.01, 0.16 ± 0.01, 0.22 ± 0.01, 0.03 ± 0.00, 0.07 ± 0.00, and 0.10 ± 0.00 µg Ni/g (ww), respectively. The calculated detection limits, expressed as mean ± SE, for Ni in lake whitefish bile, bone, gall bladder, gills, gonads, heart, intestine, kidney, liver, muscle, pyloric caeca, and stomach were 0.73 ± 0.12, 0.07 ± 0.00, 0.63 ± 0.05, 0.05 ± 0.00, 0.39

± 0.06, 0.32 ± 0.01, 0.30 ± 0.02, 0.27 ± 0.01, 0.26 ± 0.01, 0.03 ± 0.00, 0.08 ± 0.01, and 0.16 ± 0.01 μg Ni/g (ww), respectively.

Zinc and copper concentrations in kidney and liver were measured using flame atomic absorption spectroscopy (Varian SpectrAA-20 Atomic Absorption Spectrometer). The calculated detection limits, expressed as mean ± SE, for Cu and Zn were 2.1 ± 0.10 μg/g (ww) in kidney and 2.8 ± 0.10 μg/g (ww) in liver of lake trout and 3.4 ± 0.12 μg/g (ww) in kidney and 3.2 ± 0.14 μg/g (ww) in liver of lake whitefish.

Analytical accuracy was verified using certified biological reference materials (National Research Council Canada) and reagent blanks as described in the materials and methods section in Chapter 2.

In the discussion section, where results regarding Ni accumulation in tissues from this study were compared to results from other studies, it was often necessary to convert concentrations given as dry weights to wet weights, to correct for the percentage moisture present in tissue samples. A calculated wet weight concentration, denoted as "μg Ni/g (ww calc)", was determined by multiplying the documented dry weight concentration by 0.2 (Jarvinen and Ankley, 1999). All other data for metal concentrations in tissues are expressed as μg Ni/g wet weight (ww).

3.8) Toxicological Analyses

3.81 Molecular Responses

i) Metallothionein Analysis

Metallothionein concentrations were measured in kidney and liver tissue, using the mercury displacement assay described in Klaverkamp *et al.*(2000b).

ii) Lipid Peroxide Analysis

Lipid peroxides in plasma were analyzed using the K-Assay LPO-CC kit (Kamiya Biomedical Company, Seattle, WA). Plasma samples were thawed on ice, combined with the reagent to produce a 30 µl sample, and analyzed in duplicate.

iii) Hematology

In addition to molecular and tissue level parameters measured in whole blood, that are described above, blood ion (Cl^-, K^+, and Na^+) concentrations in plasma were measured. Prior to analyzing plasma samples, to determine blood ion concentrations, samples were thawed, allowed to clot, and filtered to remove fibrinogen, using a syringe attached to a micropore filter containing 0.45 µm Millipore filter paper. Na^+ and K^+ concentrations were measured using an Instrumental Laboratory 943 flame photometer. Cl^- concentrations were measured using a Corning Chloride Analyzer 925.

Hematocrit is a tissue level parameter; however, in subsequent sections it will be included with molecular level hematological parameters.

3.82 Cellular Responses

Histopathology

After 48 hours, anterior and posterior kidney, liver, cardiac stomach, pyloric caeca, intestine, rectum, and thyroid samples immersed in Bouin's fixative were washed in 3 changes of 70% ethanol over a period of 3 days and stored in 70% ethanol. Tissues were processed using an ethanol/butanol series in an IL MVP automated tissue processor. Tissues were embedded in paraffin (Tissue Prep II). Paraffin blocks of the following lake trout tissues were sectioned into 7 μm thick sections: liver (all fish), posterior kidney (all fish), anterior kidney (all fish fed diets with shrimp and 1 fish from each treatment group fed the low and high dose diets without shrimp), cardiac stomachs (all fish fed diets without shrimp), pyloric caeca (1 fish from each treatment group fed diets with and without shrimp), rectum (1 fish from each treatment group fed diets with and without shrimp), thyroid (1 fish from each treatment group fed diets with and without shrimp), and intestine (all fish fed diets without shrimp). Paraffin blocks of the following lake whitefish tissues were sectioned into 7 μm thick sections: liver (all fish), posterior kidney (all fish), anterior kidney (all fish fed diet with shrimp and 1 fish from each treatment group fed low and high dose diets without shrimp), cardiac stomachs (all fish fed diets without shrimp), pyloric caeca (1 fish from each treatment group fed diets with and without shrimp), rectum (1 fish from each treatment group fed diets with and without shrimp), thyroid (1 fish from each treatment group fed diets with and without shrimp), intestine (all fish), and gills (all fish fed diets without shrimp). Sections were mounted on glass slides and stained using Harris' hematoxylin and eosin (Edwards, 1967). All

chemicals used to fix, process, and stain tissues were obtained from Fisher Scientific (Fair Lawn, NJ). A qualitative assessment of histological alterations was conducted using a Zeiss Photomicroscope III light microscope. Photomicrographs were taken with a Kodak DC-120 digital camera, using the Kodak Microscopy Documentation System (MDS120), edited using Adobe Photodeluxe 2.0, and printed.

3.83 Organ and Whole Organism Measurements

Measurements of liver somatic indices (LSI), growth, % change in length, and condition factor were made using conventional approaches. LSI were calculated using the formula: (liver weight x 100)/(final body weight-liver weight). Condition factors were calculated using the formula: (final body weight/final fork length^3) x 100. Percent change in weight was calculated using the formula: ((final body weight/initial body weight)-1) x 100. Percent change in length was determined using a similar calculation.

3.9) Statistics

All statistical analyses described below were performed using SPSS v. 9.0 software and were based on methods described in Neter *et al.* (1990), SPSS (1999), and Stevens (1992).

A one-way ANOVA and appropriate multiple comparison techniques were used to test for differences between fish fed control and Ni-contaminated diets. Separate ANOVAs were conducted for each species (lake trout or lake whitefish) and diet type (WS or NS) combination. First, the assumptions of the ANOVA were tested. The tests

for normality failed because of the small sample size of 4 fish per treatment group. Next, a non-parametric Kruskal-Wallis test was used to test for significant differences between control and treated fish. If results from the Kruskal-Wallis test were significant, data were ranked and analyzed using a one-way ANOVA. If results from the one-way ANOVA were significant, Dunnett's test ($p<0.05$) was used to identify significant differences between control and treated fish.

A two-way ANOVA was used to test for differences between species. Separate ANOVAs were conducted for each diet type (WS or NS), to test for differences between lake trout and lake whitefish fed control and Ni-contaminated diets. First, the assumptions of the ANOVA were tested. Because of the small sample size of 4 fish per treatment group tests for normality failed. Next, a non-parametric Kruskal-Wallis test was used to test for differences between treatment groups. If results from the Kruskal-Wallis test were significant, data were ranked, and tested for species effects using a two-way ANOVA, with $p<0.05$. The ANOVA was used to identify species-related differences.

Regression analyses could not be used to test for dose-dependent patterns of accumulation in tissues because of the small sample size.

4) RESULTS

4.1) Feeding Behavior

Feeding behavior was altered in lake trout and lake whitefish fed 10000 µg Ni/g. After 3 feedings, lake trout stopped eating the high dose diets. Lake whitefish fed high dose diets, with and without shrimp, stopped feeding after 4 and 5 feedings, respectively.

Visual observations confirmed that other treatment groups continued to consume the diets.

4.2) Organ and Whole Organism Measurements

Condition factor, % change in weight, % change in fork length, and liver somatic indices measured in lake whitefish and lake trout are presented in Table 1. Lake trout fed 10000 μg Ni/g (WS) exhibited significant decreases in weight compared to the control group. The remaining parameters were not significantly affected by dietary Ni exposure or feed refusal. Data on lake whitefish lengths and condition factors are not presented because fork lengths were not measured in a consistent manner.

4.3) Accumulation and Distribution of Ni

The concentrations of Ni in kidney, liver, gill, and muscle of lake trout and lake whitefish fed diets containing 0, 1000, and 10000 μg Ni/g, processed with and without shrimp, are presented in Figure 1. Concentrations of Ni measured in the remaining 8 tissues are presented in Table 2. Lake trout fed Ni-contaminated diets accumulated significant amounts of Ni in all tissues sampled, with the exceptions of bile in trout fed 1000 μg Ni/g (NS) and muscle in trout fed 10000 μg Ni/g (WS). Lake whitefish fed diets containing 10000 μg Ni/g accumulated significant amounts of Ni in a majority of tissues, with the exceptions of gonads, gill, and bile of whitefish fed the high dose diet (NS), and heart of whitefish fed the high dose diet (WS). Lake whitefish fed diets containing 1000 μg Ni/g accumulated significant amounts of Ni in stomach, pyloric caeca, muscle, liver, intestine (NS and WS), and in bone, kidney, heart, and gill (NS only).

In a small number of tissues lake trout accumulated higher concentrations of Ni compared to lake whitefish. Lake trout fed diets with shrimp accumulated higher concentrations of Ni in bile, gall bladder, and liver. Lake trout fed diets without shrimp accumulated higher concentrations of Ni in gall bladder, heart, kidney, and liver.

Lake trout fed 1000 μg Ni/g (NS) accumulated the highest mean concentrations of Ni (expressed as μg Ni/g (ww)) in intestine (80) > pyloric caeca (16) > stomach (10) > kidney (6.4) > gall bladder (3.1) > heart (2.1) > gonads (1.6), gill (1.5) > liver (1.1) > bile (0.88) > bone (0.77) > muscle (0.29). Similar patterns of accumulation were observed in lake trout fed 1000 μg Ni/g (WS). Lake trout fed 10000 μg Ni/g (NS) accumulated the highest mean concentrations of Ni (expressed as μg Ni/g (ww)) in bile (23) > kidney (17) > gall bladder (5.1) > intestine (4.5) > pyloric caeca (2.2) > stomach (1.8) = heart (1.8) > gonads (1.7) = gill (1.7) > bone (1.04), liver (0.96) > muscle (0.24). Similar patterns of accumulation were observed in lake trout fed 10000 μg Ni/g (WS).

Lake whitefish fed 1000 μg Ni/g accumulated the highest mean concentrations of Ni (expressed as μg Ni/g (ww)) in intestine (40) > kidney (4.0) > stomach (1.4) > bile (1.3) > gali bladder (1.2) > heart (1.1) > gonads (1.0) > bone (0.75) > gill (0.64) > liver (0.49) > pyloric caeca (0.47) > muscle (0.11). Similar patterns of accumulation were observed in lake whitefish fed 1000 μg Ni/g (WS). Lake whitefish fed 10000 μg Ni/g accumulated the highest concentrations of Ni (expressed as μg Ni/g (ww)) in intestine (9.8) > kidney (5.1) > pyloric caeca (3.7) > bile (2.9) > gall bladder (2.8) > stomach (1.8) = bone (1.8) > heart (1.6) > gonads (1.5) > gill (1.4) > liver (0.63) > muscle (0.21). Lake whitefish fed 10000 μg Ni/g (WS) exhibited similar patterns of accumulation.

An apparent association was observed between the dose of Ni administered in the diet and the accumulation of Ni in lake whitefish and lake trout tissues. In all lake whitefish tissues, except intestine, Ni concentrations appeared to increase with increased concentrations of Ni in the diet. In lake trout, Ni concentrations in bile, gall bladder, pyloric caeca, and kidney appeared to increase, as the concentration of Ni in the diet increased. However, in stomach, heart and intestine, Ni concentrations were most elevated in trout fed the low dose diets. In the remaining tissues, Ni concentrations in tissues of trout fed low and high dose diets appeared to be similar.

4.4) Copper and Zinc Accumulation

The concentrations of Cu and Zn measured in lake trout and lake whitefish kidney and liver are presented in Table 3. Cu concentrations in kidney were significantly reduced in lake whitefish fed 1000 μg Ni/g (WS) and 10000 μg Ni/g (NS). Significant increases in Cu and Zn concentrations were observed in liver of lake whitefish fed 10000 μg (NS). Cu and Zn concentrations were not altered in lake trout.

4.5) Toxicology

4.51 Molecular Responses

i) Metallothionein

Concentrations of metallothionein measured in kidney and liver of lake trout and lake whitefish are presented in Figure 2 and Table 4. Increased metallothionein concentrations were observed in the kidneys of lake trout fed 1000 μg Ni/g and 10000 μg

Ni/g, in kidneys of lake whitefish fed 10000 μg Ni/g, and in livers of lake whitefish fed 10000 μg Ni/g (NS).

ii) Lipid Peroxides

Concentrations of lipid peroxides measured in plasma of lake trout and lake whitefish are presented in Table 5. Increased lipid peroxide concentrations were observed in lake trout fed high dose diets.

iii) Hematological Parameters

Hemoglobin, glucose, hematocrit measured in whole blood and ions (Cl^-, K^+, Na^+) measured in plasma of lake trout and lake whitefish are presented in Table 6. Hemoglobin and hematocrit were unaffected. Significant decreases in glucose were observed in lake whitefish fed 1000 μg Ni/g (WS) and 10000 μg Ni/g (WS). Significant decreases in K^+, Na^+, and Cl^-, were observed in lake trout fed 10000 μg Ni/g (WS) and in K^+ in lake whitefish fed 10000 μg Ni/g (NS).

4.52 Cellular Responses

Histopathology

Lake whitefish fed Ni-contaminated diets exhibited histological alterations in the posterior kidney, liver, and intestine. Alterations were infrequent or minor in the kidneys, livers, and intestines of lake whitefish fed control diets (Figures 3a, 4a and 4c, 5a and 5d, respectively). In kidneys of lake whitefish fed low and high dose diets, alterations, including the presence of swollen, ruptured, and necrotic epithelial cells in tubules, desquamated epithelial cells, disintegrating tubules, dilated tubules, debris in the lumen of tubules and hematopoietic tissue, and depletion of hematopoietic tissue, were

observed (Figure 3b). In livers of lake whitefish fed high dose diets, minor areas of focal necrosis, most commonly characterized by swollen and ruptured hepatocytes, altered positioning of nuclei in hepatocytes, hepatocytes with pyknotic nuclei, the presence of debris in sinusoids, loss of cord structure, cellular dissolution, and altered staining, were observed (Figures 4b and d). Whitefish fed the high dose diet exhibited minor to moderate alterations in intestine, characterized by the desquamation of the lamina epithelialis (mucosal epithelium), presence of necrotic cells at the tips of the intestinal folds, and expansion of the lamina propria (Figures 5b,c, and e).

Lake trout fed low and high dose diets exhibited similar histological alterations in intestine to those observed in lake whitefish. No alterations were observed, however, in the other tissues examined.

5) DISCUSSION

Feed refusal was observed in lake whitefish fed 10000 µg Ni/g and was accompanied by decreased weight in lake trout fed 10000 µg Ni/g (WS). Lake trout stopped feeding on day 5 of the experiment, after consuming 3 rations of the high dose diets. Lake whitefish consumed the high dose diets for a slightly longer duration and stopped feeding on days 8 and 10 of the experiment, after consuming 4-5 rations of the high dose diets. This study demonstrates that fish can detect and avoid exposure to high concentrations of Ni in food. Similar avoidance behavior has been observed in rainbow trout (*Salmo gairdneri)* exposed to high concentrations of waterborne Ni (Giattina *et al.*, 1982). The addition of brine shrimp was not observed to increase the consumption of Ni-contaminated diets, as feed refusal was observed in fish fed both diet types.

Lake trout fed low and high dose diets exhibited different patterns of Ni accumulation and distribution. Lake trout fed the high dose diet exhibited feed refusal after consuming 43% of the total expected dose. Patterns of Ni accumulation and distribution in the high dose treatment groups reflect the reduced uptake and increased excretion of Ni resulting from a 2-week depuration period. In lake trout fed the high dose diet (NS) the highest Ni concentrations were observed in bile, kidney, and gall bladder. Conversely, in lake trout fed the low dose diet (NS), the highest Ni concentrations were observed in tissues of the digestive tract, including the intestine, pyloric caeca, and stomach.

Lake whitefish fed the low and high dose diets accumulated Ni in a similar manner. Although lake whitefish eventually refused to eat the high dose diets, a majority (71-86%) of the total expected dose was consumed. Lake whitefish fed the high dose diet (NS) exhibited the highest mean Ni concentrations in intestine, kidney, and pyloric caeca. Similarly, lake whitefish fed the low dose diet (NS) accumulated the highest mean Ni concentrations in intestine, kidney, and stomach. Furthermore, an apparent association was observed between the Ni concentrations in tissues of lake whitefish and the dose of Ni administered.

The highest concentrations of Ni were observed in intestines of lake trout and lake whitefish fed Ni-contaminated diets. The intestine is the major site of absorption of digested material, including metals (Luckey and Vengopal, 1977). The kidney is another important site of Ni accumulation. High concentrations of Ni observed in the kidney of lake trout and lake whitefish can be attributed to the principal role this organ plays in the accumulation, detoxification, and excretion of Ni (Sunderman, 1977; WHO, 1991; Eisler,

1998). The accumulation of Ni in liver, gall bladder, and bile observed in this study indicates that the liver may also play an important role in Ni detoxification and excretion in fish, particularly in lake trout.

The accumulation and distribution of Ni in natural populations of fish residing in Ni-contaminated areas have been investigated in numerous field studies. Patterns of Ni accumulation were similar in lake whitefish exposed to dietary Ni in the laboratory and freshwater fish collected in the field (Mastala *et al.*, 1992; Tariq *et al.*, 1993; Kashulin and Reshetnikov, 1995; Moiseenko *et al.*, 1995; Allen-Gil *et al.*, 1997; Brotheridge *et al.*, 1998; Klaverkamp *et al.*, 2000a). For example, whitefish (*Coregonus lavaretus*) collected from a lake, with concentrations of 2227 μg Ni/g (dw) in the surficial sediments, located near base-metal mining smelters in Kola Peninsula, Russia, accumulated 5.6 μg Ni/g (ww calc.) in kidney, 1.92 μg Ni/g (ww calc.) in gill, and 1.5, 0.5, and 0.3 μg Ni/g (ww calc.) in skeleton, liver, and muscle, respectively (Kashulin and Reshetnikov, 1995). In this study, comparable Ni concentrations were observed in kidney, gill, skeleton, liver, and muscle tissue of lake whitefish fed 10000 μg Ni/g (NS) for 14 days, at mean concentrations of 5.1, 1.4, 1.8, 0.63, and 0.21 μg Ni/g. In another study, Klaverkamp *et al.* (2000) collected lake whitefish (*Coregonus clupeaformis*) from Little Macdonald Lake in Key Lake, Saskatchewan. Little Macdonald Lake received treated mine water and had elevated concentrations of Ni in the surficial sediments, as high as 690 μg Ni/g (dw). Lake whitefish collected from this lake had elevated concentrations of Ni in kidney and liver, at mean concentrations of 4.7 μg Ni/g and 0.57 μg Ni/g, respectively. A similar range of concentrations was observed in lake whitefish

fed 1000 μg Ni/g (ns) and 10000 μg Ni/g (ns), with concentrations of 4.0 and 5.1 μg Ni/g in kidney and concentrations of 0.49 and 0.63 μg Ni/g in liver, respectively.

Conversely, lake trout in this study appear to accumulate Ni in different concentrations and patterns than those observed natural populations of fish collected from polluted environments (Hutchinson *et al.*, 1976; Ney and Hassel, 1983; Dallinger and Kautzky, 1985; Bradley and Morris, 1986; Mastala *et al.*, 1992; Kashulin and Reshetnikov, 1995; Moiseenko *et al.*, 1995; Maletin *et al.*, 1996; Allen-Gil *et al.*, 1997; Klaverkamp *et al.*, 2000a). For example, lake trout collected from Lake Nelson, located near mining operations in Sudbury, Ontario, accumulated mean concentrations of 1.0 μg Ni/g (ww calc.) in kidney and < 0.4 μg Ni/g (ww calc) in liver. Concentrations of Ni in the surficial sediments of Lake Nelson were 444 μg Ni/g (dw). The concentrations observed in this study in kidney and liver of lake trout fed low dose diets were much higher, with mean concentrations in the kidney and liver ranging from 6.4-7.6 and 1.1-1.2 μg Ni/g, respectively.

Significant alterations in Cu and Zn concentrations related to Ni exposures were observed in lake whitefish. In lake whitefish fed the high dose diet (NS), significant decreases in Cu concentrations were observed in the kidney, whereas, significant increases in Cu and Zn concentrations were observed in liver. Increases in endogenous concentrations of essential metals, like Cu and Zn, can induce metallothionein production in aquatic organisms (Roesijadi, 1992). The observed increases in Cu and Zn concentrations in the livers of lake whitefish fed the high dose diet (NS) may have contributed to the increased hepatic metallothionein concentrations observed.

The sublethal toxicity associated with exposure to dietary Ni was assessed through the measurement of responses at various levels of biological organization. One molecular response evaluated in this study was metallothionein production. Metallothionein (MT) is a low-molecular weight, cysteine-rich protein, which functions in the regulation of essential metals and the detoxification of non-essential metals (Hamilton and Mehle, 1986; Roesijadi, 1992). Harmful intracellular reactions, such as free radical formation, are restricted by MT, which sequesters or donates essential metals to biochemical reactions and sequesters non-essential metals into nonavailable intracellular compartments (Roesijadi, 1992). Environmental and physiological factors, including exposure to free radicals, starvation, temperature changes, stress, and inflammation, can also induce MT production (Sunderman and Fraser, 1983; Robertson *et al.*, 1988; Fleet *et al.*, 1990; Hildalgo *et al.*, 1990; Petering *et al.*, 1990; Hylland *et al.*, 1994; McNamara and Buckley, 1994; Srivastava *et al.*, 1995). In fish, naturally occurring fluctuations in basal MT concentrations can occur due to gender, reproductive state, developmental stage, and water temperature (Ollson *et al.*, 1998), but were not apparent in this study. Much of what is known about the ability of Ni to induce MT production has been discovered through mammalian studies (Sunderman and Fraser, 1983; Nation *et al.*, 1985; Khandelwal *et al.*, 1989; Fleet *et al.*, 1990; Arizono *et al.*, 1991; Srivastava *et al.*, 1995). However, because of conflicting results reported in these studies, the status of Ni as an MT inducer in fish is open to question.

Only one laboratory-based study has been conducted investigating the effects of Ni exposure on MT production in fish (Pyle, 1999). Pyle (1999) observed increases in MT concentrations in the gills of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed

for 7 days to water containing 6.6 mg Ni/L, as nickel chloride. No significant changes were observed in hepatic MT concentrations of exposed rainbow trout.

Results from this study demonstrate that exposure to dietary Ni can induce MT production in fish. Lake trout fed diets without shrimp contained higher concentrations of hepatic MT and lake trout fed diets with shrimp contained higher levels of renal and hepatic MT, compared to lake whitefish. This trend was observed in all treatment groups, including the control groups. The higher concentrations of MT observed in lake trout may offer increased protection against Ni toxicity, as fewer toxicological effects were observed in lake trout compared to lake whitefish in the current study. MT induction observed in liver of lake whitefish may be attributed to increases in the endogenous concentrations of Zn. Increased renal MT concentrations observed in lake trout fed high dose diets, however, may be due to effects of starvation and lipid peroxide production.

Another molecular response evaluated in this study was the production of lipid peroxides. Lipid peroxides are formed from a self-propagating process, in which free radicals react with polyunsaturated fatty acids (PUFA) found in the lipids of cell membranes, endoplasmic reticulum, and mitochondria (Muriel, 1997). Lipid peroxidation is recognized as an important molecular mechanism for cellular injury, as it results in the disruption of integral cellular components including cell membranes (Muriel, 1997). Numerous mammalian studies have shown that lipid peroxidation can be induced by Ni (Sunderman *et al.*, 1989; Knight and Voorhees, 1990; Misra *et al.*, 1990; Sole *et al.*, 1990; Srivastava *et al.*, 1990; Misra *et al.*, 1991; Rodriguez *et al.*, 1991; Iscan *et al.*, 1992; Stinson *et al.*, 1992; Novelli *et al.*, 1995; Srivastava *et al.*, 1995; Chen *et al.*,

1998; Chakrabarti and Bai, 1999)). The specific mechanisms involved in the generation of lipid peroxides in mammals exposed to Ni and the resultant cellular damage have not been identified.

This is the first study to investigate the induction of lipid peroxides in fish due to Ni exposure. Significant increases in the production of lipid peroxides were observed only in lake trout fed the high dose diets. The incidence of feed refusal in this treatment group confounds the interpretation of these findings, because starvation has been observed to enhance lipid peroxidation in mammals (Hildalgo *et al.*, 1988).

Previous studies that have examined the histological effects of Ni on fish have been limited to aqueous exposures (Department of Environment, 1971; Hughes *et al.*, 1979; Nath and Kumar, 1989; Nath and Kumar 1990; Jha *et al.*, 1994). The most marked histological alterations in this study were observed in posterior kidneys of whitefish fed high dose diets, indicating that the kidney is a target organ for Ni toxicity in lake whitefish. Renal histological alterations observed in whitefish fed the low and high dose diets were consistent with those reported in mammalian studies (Gitlitz et al., 1975; Pereira et al, 1997; USPHS, 1997). For example, tubular necrosis and altered epithelial cells have been observed in rats and mice exposed to Ni (Gitlitz et al., 1975; Pereira et al. 1997). The results of this study provide further evidence that renal histopathologies are useful indicators of exposure of fish to contaminants, as suggested by other researchers (Hinton *et al.*, 1992; Cooley *et al.*, 2000).

The presence of hepatic lesions in lake whitefish and the accumulation of elevated concentrations of Ni in the bile of lake whitefish and lake trout fed the high dose diets indicate that biliary excretion may be an important route of Ni excretion in fish. Similar

hepatic lesions have been reported in studies that exposed fish to waterborne Ni (Department of Environment, 1971; Jha *et al.*, 1994). For example, climbing perch (*Anabas testudineus*) exposed to waterborne Ni exhibited similar areas of focal necrosis, characterized by hepatocytes with altered nuclear positioning and pyknotic nuclei, ruptured hepatocytes, and loss of cord structure (Jha *et al.*, 1994). The role of liver in the detoxification and excretion of Ni needs to be investigated further in freshwater fish.

In intestine of lake whitefish fed high dose diets and lake trout fed the low and high dose diets, desquamation of the mucosal epithelium was observed. Mammalian studies indicate that increased production of mucus and desquamation of epithelial layers in the intestine result in decreased absorption of Ni (Tallkvist and Tjalve, 1997). Accordingly, the desquamation of the intestinal epithelium observed in this study may offer fish increased protection against Ni exposure.

The cellular injuries observed in kidneys and livers of lake whitefish were not accompanied by significant increases in lipid peroxide concentrations. These results contrast with the findings of Cooley *et al.* (2000), who observed an association between lipid peroxide production and histological alterations in the kidney and liver of lake whitefish exposed to dietary uranium. Another explanation for the increase in lipid peroxides observed in lake trout fed the high dose Ni diets is that cellular injury and damage may have occurred in an organ that was not included in the histological assessment. Additionally, a stronger association might have been seen between lipid peroxide production and cellular injury in lake whitefish, if lipid peroxides were measured directly in kidney and liver homogenate instead of in plasma. More research is

needed to elucidate the relationship between histopathology and lipid peroxidation in fish.

At the tissue level, a number of hematological responses were investigated. Generally, the use of hematological responses as indicators of exposure of fish to contaminants can be confounded by stress related to capture, anesthesia, and bleeding (Heath, 1995). Other environmental and physiological events can also result in transient alterations of these parameters. Hematological alterations, however, have been reported in fish exposed to waterborne Ni in the laboratory (Chaudhry and Nath, 1984; Ghazaly, 1992; Jha and Jha, 1994). Ghazaly (1992) exposed *Tilapia nilotica* to 19.2, 32, and 51.2 mg/L of nickel sulfate for 96 hours and observed increases in blood glucose, hematocrit, and hemoglobin. The results from this study contrast with these findings, as significant decreases in glucose were observed in lake whitefish fed 1000 μg Ni/g (WS) and 10000 μg Ni/g (NS) and hematocrit and hemoglobin concentrations were unaltered. Significant decreases in K^+, Na^+, and Cl^-, were observed in lake trout fed 10000 μg Ni/g (WS) and in K^+ in lake whitefish fed 10000 μg Ni/g (NS). Comparisons cannot be made with these results as no other laboratory studies evaluating the effect of Ni on plasma electrolytes have been conducted.

Organ and whole-organism responses usually respond to contaminant exposure less rapidly than molecular responses (Adams *et al.*, 1990). However, due to their high ecological relevance and ease with which they are measured, these responses are advantageous for use in monitoring programs. Alterations were not observed at the organ and whole-organism levels, with the exception of the decreased weight gain observed in lake trout, which also exhibited feed refusal behavior.

A comprehensive discussion of the uptake and toxicology associated with long-term exposure of lake whitefish to dietary Ni is presented in Chapters 2 and 3.

6) CONCLUSIONS AND RECOMMENDATIONS

The results from this study were used to guide the experimental design of a long-term exposure. Lake whitefish were selected for use in the long-term experiments for the following reasons: 1) lake whitefish consumed the high dose diet for a longer period of time, 2) increased Ni concentrations in all lake whitefish tissues, except intestine, were apparently associated with increases in the dose of Ni in the diet, 3) lake whitefish fed Ni-contaminated diets accumulated Ni at concentrations similar to those observed in natural populations of whitefish residing in Ni-contaminated environments, 4) lake whitefish fed 10000 μg Ni/g (prepared without shrimp) exhibited alterations in the endogenous concentrations of Cu and Zn in kidney and liver, and 5) lake whitefish fed Ni-contaminated diets sustained renal and hepatic lesions. Diets selected for the longer-term experiments will contain 0, 10, 100, and 1000 μg Ni/g, because diets containing 1000 μg Ni/g were readily consumed by lake trout and lake whitefish. Brine shrimp will not be incorporated into the diets used in the long-term exposure because increased consumption of the high dose diets was not observed.

Table 1. Organ and whole organism measurements of lake trout and lake whitefish fed diets containing 0, 1000, and 10000 µg Ni/g processed with and without brine shrimp.

Fish Species	Dose	Diet Type	Mean Percent Change in Weight (±SE)	Mean Percent Change in Length (±SE)	Mean Condition factor (±SE)	Mean Liver Somatic Index (±SE)
lake trout	0 µg Ni/g	shrimp	-0.443 (0.919)	3.49 (0.586)	0.948 (0.007)	0.56 (0.06)
		no shrimp	-3.82 (1.87)	4.19 (0.141)	0.987 (0.014)	0.76 (0.08)
	1000 µg Ni/g	shrimp	-1.71 (0.916)	2.26 (0.731)	1.03 (0.022)	0.69 (0.03)
		no shrimp	-2.40 (1.61)	4.55 (0.583)	0.831 (0.128)	0.64 (0.03)
	10000 µg Ni/g	shrimp	-9.83 (1.11)*	2.97 (0.635)	0.978 (0.021)	0.60 (0.03)
		no shrimp	-9.41 (1.37)	2.06 (1.33)	0.949 (0.027)	0.62 (0.05)
lake whitefish	0 µg Ni/g	shrimp	-0.684 (3.59)	-	-	0.32 (0.09)
		no shrimp	5.59 (3.56)	-	-	0.46 (0.08)
	1000 µg Ni/g	shrimp	2.90 (4.16)	-	-	0.38 (0.13)
		no shrimp	4.71 (3.74)	-	-	0.46 (0.07)
	10000 µg Ni/g	shrimp	-0.129 (1.17)	-	-	0.44 (0.07)
		no shrimp	-1.79 (1.58)	-	-	0.37 (0.11)

"*": represents significant differences observed between control and treatment groups for each diet type ($p<0.05$)
"-": lake whitefish lengths and condition factors were not presented due to an inconsistency in measurement technique
Calculations used to determine condition factors and LSIs are described in the materials and methods section.

Table 2. Ni concentrations in the stomach, pyloric caeca, intestine, gall bladder, bile, gonads, heart, and bone of lake trout and lake whitefish fed diets containing 0, 1000, and 10000 µg Ni/g, processed with and without shrimp.

Fish Species	Dose	Diet Type	Mean Ni Concentration (± SE) (µg Ni/g)							
			Stomach	Pyloric Caeca	Intestine	Gall Bladder	Bile	Gonads	Heart	Bone
lake trout	0 µg Ni/g	shrimp	0.21 (0.03)	0.10 (0.02)	0.51 (0.11)	0.41 (0.21)	2.3 (2.1)	0.07 (0.02)	0.18 (0.03)	0.06 (0.01)
		no shrimp	0.21 (0.09)	0.23 (0.17)	1.6 (1.3)	0.48 (0.16)	0.40 (0.24)[a]	0.14 (0.02)	0.12 (0.02)	0.04 (0.01)
	1000 µg Ni/g	shrimp	5.3 (0.81)*	39 (12)*	62 (6.0)*	2.8 (0.33)*	1.8 (0.37)*	2.3 (0.41)*	3.7 (1.2)*	1.1 (0.09)*
		no shrimp	10 (2.2)*	16 (2.9)*	80 (18)*	3.1 (0.81)*	0.88 (0.18)[b]	1.6 (0.22)*	2.1 (0.05)*	0.77 (0.05)*
	10000 µg Ni/	shrimp	1.9 (0.04)*	3.5 (0.75)*	9.3 (1.8)*	3.8 (0.58)*	30 (8.4)*	2.0 (0.31)*	1.8 (0.28)*	0.97 (0.14)*
		no shrimp	1.8 (0.21)*	2.2 (0.27)*	4.5 (1.4)*	5.1 (0.78)*	23 (8.6)*[a]	1.7 (0.22)*	1.8 (0.15)*	1.0 (0.27)*
lake whitefish	0 µg Ni/g	shrimp	0.07 (0.01)	0.04 (0.01)	1.5 (1.0)	0.25 (0.09)	0.14 (0.08)	0.78 (0.57)	0.53 (0.31)	0.13 (0.06)
		no shrimp	0.17 (0.02)	0.04 (0.01)	0.29 (0.06)	0.38 (0.10)	0.20 (0.12)[b]	0.36 (0.23)	0.14 (0.07)	0.05 (0.02)
	1000 µg Ni/g	shrimp	1.2 (0.43)*	0.79 (0.37)*	29 (15)*	7.9 (6.8)	0.39 (0.07)[a]	1.5 (0.54)	1.3 (0.34)	0.89 (0.29)
		no shrimp	1.4 (0.21)*	0.47 (0.05)*	40 (13)*	1.2 (0.50)	1.3 (0.94)[a]	1.0 (0.27)	1.1 (0.22)*	0.75 (0.18)*
	10000 µg Ni/	shrimp	6.0 (2.1)*	4.8 (1.6)*	18 (2.0)*	2.3 (0.25)*	14 (5.6)*[a]	2.1 (0.89)	2.3 (0.70)	1.5 (0.29)*
		no shrimp	1.8 (0.21)*	3.7 (0.72)*	9.8 (1.6)*	2.8 (0.51)*	2.9 (1.3)[a]	1.5 (0.32)	1.6 (0.31)*	1.8 (0.42)*

"*" represents significant differences ($p<0.05$) observed between control and treatment groups for each diet type.

n=4 for all treatment groups except those denoted by the following:
"a" represents treatment groups with n=3
"b" represents treatment groups with n=2

Table 3. Cu and Zn concentrations in the kidney and liver of lake trout and lake whitefish fed diets containing 0, 1000, and 10000 µg Ni/g, processed with and without shrimp.

Fish Species	Dose	Diet Type	Mean [Zn] (± SE) (µg Zn/g)		Mean [Cu] (± SE) (µg Cu/g)	
			Kidney	Liver	Kidney	Liver
lake trout	0 µg Ni/g	shrimp	63 (25)	49 (0.89)	1.7 (0.10)	38 (5.7)
		no shrimp	43 (8.6)	45 (1.8)	1.6 (0.07)	31 (2.9)
	1000 µg Ni/g	shrimp	53 (20)	51 (3.5)	1.8 (0.28)	59 (11)
		no shrimp	35 (1.6)	45 (1.6)	2.2 (0.28)	36 (4.2)
	10000 µg Ni/g	shrimp	59 (6.6)	49(1.7)	2.0 (0.33)	40 (7.7)
		no shrimp	42 (4.1)	50 (1.7)	2.2 (0.19)	42 (9.5)
lake whitefish	0 µg Ni/g	shrimp	28 (1.3)	30 (1.9)	2.6 (0.22)	14 (3.6)
		no shrimp	33 (1.7)	28 (1.0)	2.4 (0.22)	17 (1.5)
	1000 µg Ni/g	shrimp	29 (2.2)	32 (2.8)	1.8 (0.19)*	23 (3.9)
		no shrimp	30 (1.6)	28 (0.8)	2.3 (0.38)	13 (2.7)
	10000 µg Ni/g	shrimp	31 (1.0)	31 (0.6)	2.5 (0.29)	15 (2.4)
		no shrimp	28 (1.8)	36 (1.4)*	1.8 (0.11)*	32 (3.3)*

"*" represents significant differences ($p<0.05$) observed between control and treatment groups for each diet type.

Table 4. Metallothionein concentrations in the liver of lake trout and lake whitefish fed diets containing 0, 1000, and 10000 µg Ni/g processed with and without brine shrimp.

Fish Species	Dose	Diet Type	Mean Metallothionein Concentration (± SE) (µg/g)
lake trout	0 µg Ni/g	shrimp	653 (111)
		no shrimp	491 (11.7)
	1000 µg Ni/g	shrimp	727 (118)
		no shrimp	524 (76.0)
	10000 µg Ni/g	shrimp	649 (135)
		no shrimp	616 (156)
lake whitefish	0 µg Ni/g	shrimp	156 (27.3)
		no shrimp	199 (30.8)
	1000 µg Ni/g	shrimp	277 (59.0)
		no shrimp	146 (32.1)
	10000 µg Ni/g	shrimp	207 (35.7)
		no shrimp	417 (91.4)*

"*" represent significant differences ($p<0.05$) observed between control and treatment groups for each diet type.

Table 5. Lipid peroxide concentrations in the plasma of lake trout and lake whitefish fed diets containing 0, 1000, and 10000 µg Ni/g processed with and without shrimp.

Fish Species	Dose	Diet Type	Mean LPO Concentration (± SE) (nmol/ml)
lake trout	0 µg Ni/g	shrimp	2.0 (0.67)
		no shrimp	1.6 (0.56)
	1000 µg Ni/g	shrimp	1.4 (0.37)
		no shrimp	3.2 (0.38)
	10000 µg Ni/g	shrimp	6.5 (1.6)*
		no shrimp	5.2 (1.4)*
lake whitefish	0 µg Ni/g	shrimp	4.1 (3.1)
		no shrimp	3.3 (0.79)
	1000 µg Ni/g	shrimp	6.5 (0.96)
		no shrimp	6.4 (1.6)
	10000 µg Ni/g	shrimp	7.4 (1.0)
		no shrimp	6.2 (2.3)

"*" represent significant differences ($p<0.05$) observed between control and treatment groups for each diet type.

Table 6. Hematological parameters in lake trout and lake whitefish fed diets containing 0, 1000, and 10000 µg Ni/g processed with and without brine shrimp.

Fish Species	Dose	Diet Type	Mean Hemoglobin Concentration (± SE) (g/dl)	Mean Glucose Concentration (± SE)(mmol/L)	Mean Percent Hematocrit (± SE)	Mean Ion Concentration (± SE)		
						Cl^- (meq/L)	K^+ (mmol/L)	Na^+ (mmol/L)
lake trout	0 µg Ni/g	shrimp	9.5 (0.40)	5.0 (0.25)	33.6 (1.43)	151 (2.14)	2.6 (0.12)	156 (0.557)
		no shrimp	9.3 (0.28)	5.2 (0.09)	35.1 (0.819)	146 (1.48)	2.3 (0.08)	151 (0.588)
	1000 µg Ni/g	shrimp	11 (0.84)	5.9 (0.60)	38.8 (2.20)	150 (1.81)	2.3 (0.10)	155 (1.28)
		no shrimp	8.6 (0.86)	5.3 (0.04)	30.7 (0.773)	149 (1.82)	2.5 (0.19)	156 (1.60)
	10000 µg Ni/g	shrimp	8.3 (0.53)	5.1 (0.26)	30.8 (1.51)	140 (2.92)*	1.8 (0.22)*	148 (1.90)*
		no shrimp	10 (1.2)	5.5 (0.60)	34.5 (2.56)	143 (1.62)	2.2 (0.17)	153 (2.33)
lake whitefish	0 µg Ni/g	shrimp	9.6 (1.2)	5.8 (0.63)	42.5 (2.59)	147 (2.05)	2.7 (0.20)	152 (2.52)
		no shrimp	11 (0.30)	4.7 (0.13)	43.8 (1.71)	140 (6.36)	3.8 (0.59)	150 (1.45)
	1000 µg Ni/g	shrimp	10 (0.40)	3.7 (0.09)*	43.9 (1.53)	144 (2.80)	2.7 (0.20)	151 (1.52)
		no shrimp	8.8 (0.46)	4.3 (0.40)	39.6 (2.90)	145 (1.73)	3.0 (0.22)	152 (0.769)
	10000 µg Ni/g	shrimp	10 (0.46)	4.1 (0.13)*	42.8 (1.44)	147 (1.77)	3.4 (0.49)	152 (0.928)
		no shrimp	11 (0.75)	4.9 (0.62)	46.1 (3.13)	142 (1.77)	2.4 (0.10)*	150 (0.928)

"*" represent significant differences ($p<0.05$) observed between control and treatment groups for each diet type.

Figure 1. Ni accumulation in kidney, liver, gill, and muscle of lake trout and lake whitefish fed diets containing 0, 1000, and 10000 μg Ni/g, processed with and without shrimp: a) kidney, b) liver, c) gill, and d) muscle. Data are expressed as mean (± SE). Asterisks represent significant differences ($p<0.05$) observed between control and treatment groups for each diet type.

Figure 1

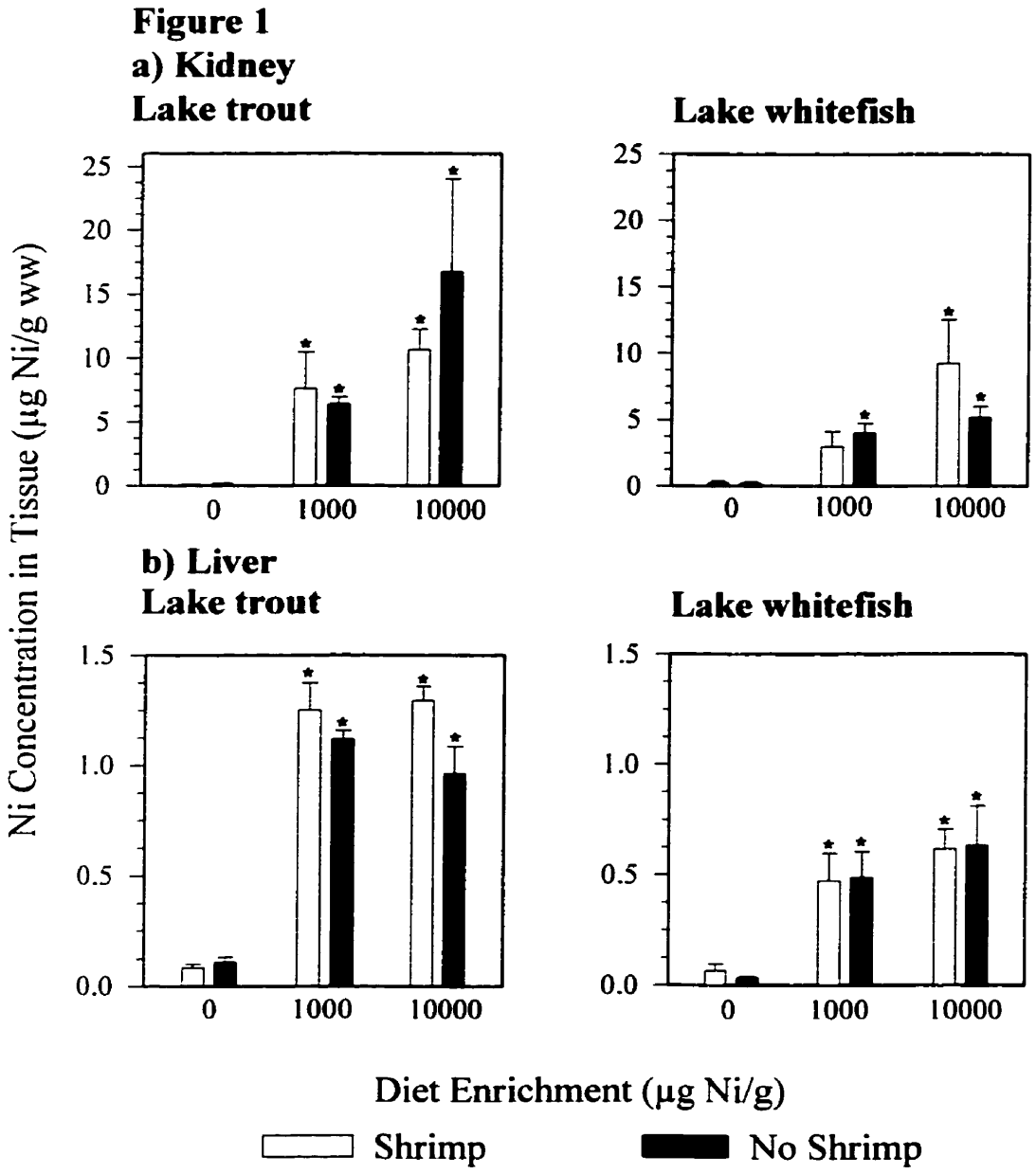

a) Kidney
Lake trout
Lake whitefish
b) Liver
Lake trout
Lake whitefish
Ni Concentration in Tissue (µg Ni/g ww)
25
20
15
10
5
0
1.5
1.0
0.5
0.0
0
1000
10000
Diet Enrichment (µg Ni/g)
Shrimp
No Shrimp

Figure 1

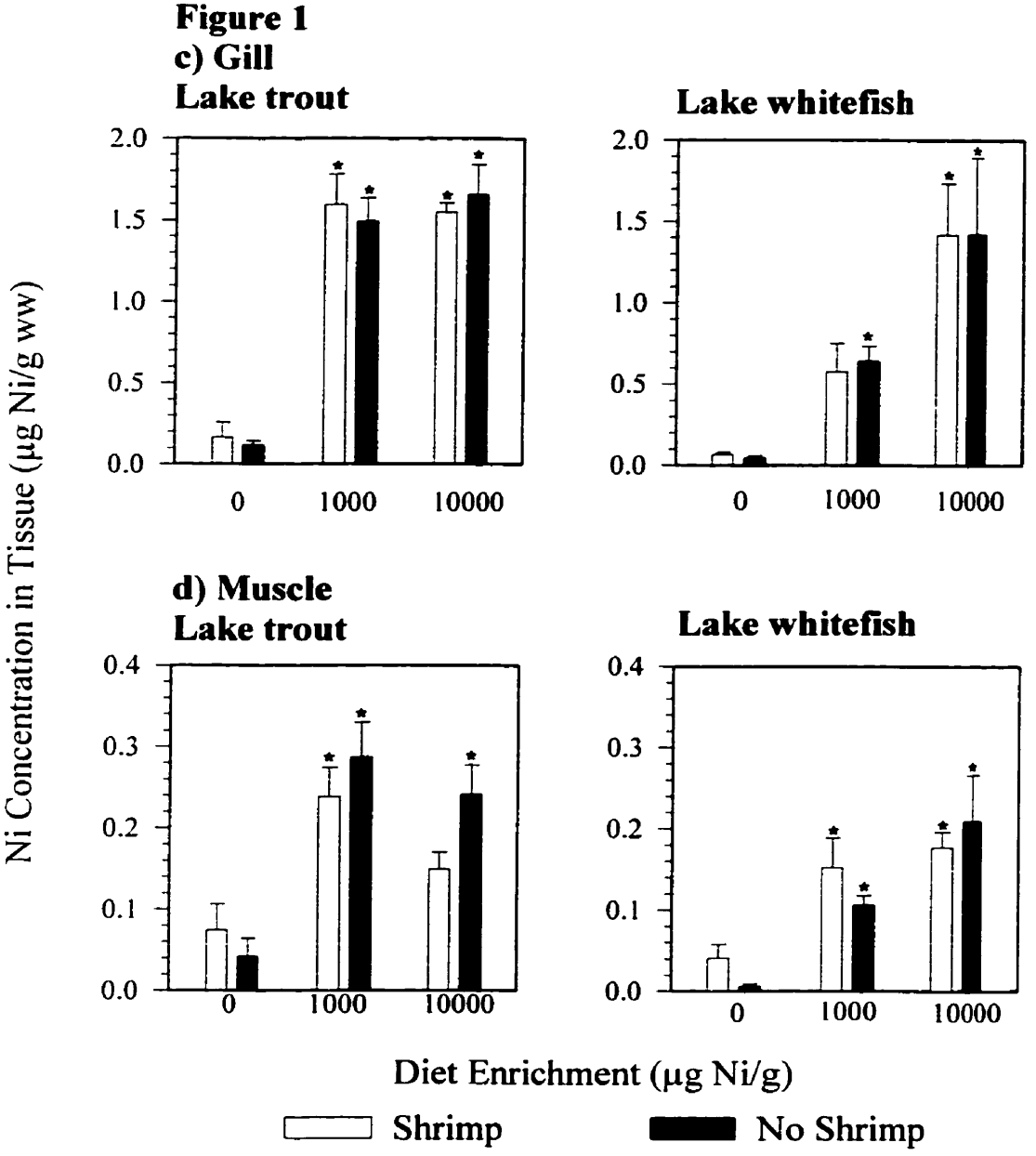
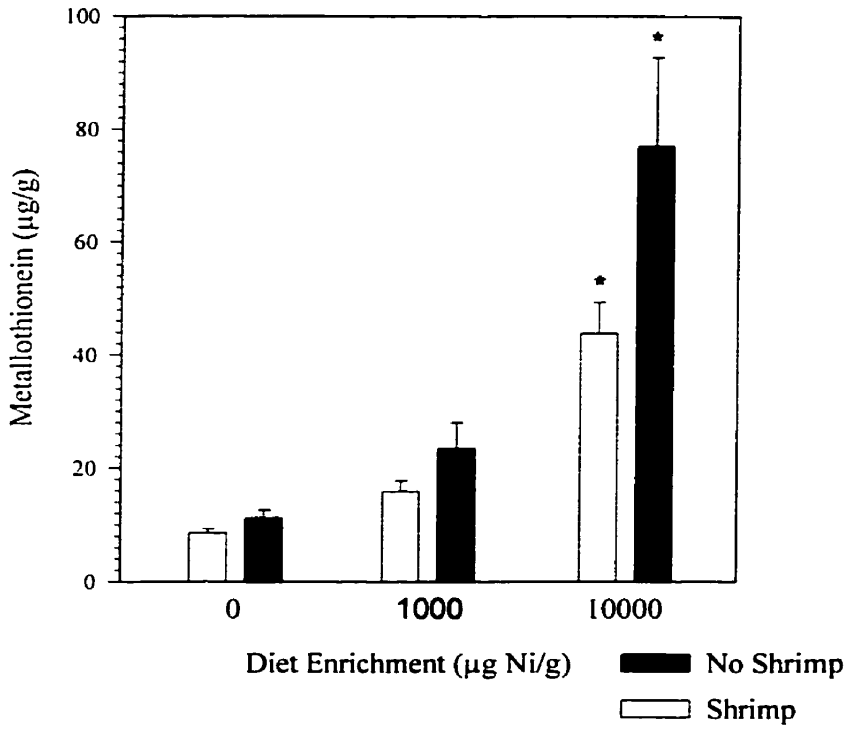

c) Gill
Lake trout
Lake whitefish
d) Muscle
Lake trout
Lake whitefish
Ni Concentration in Tissue (µg Ni/g ww)
2.0
1.5
1.0
0.5
0.0
0.4
0.3
0.2
0.1
0
1000
10000
Diet Enrichment (µg Ni/g)
Shrimp
No Shrimp

Figure 2. Metallothionein concentrations in the kidney of lake whitefish and lake trout fed diets containing 0, 1000, and 10000 μg Ni/g processed with and without brine shrimp. Data are expressed as the mean (± SE). Asterisks represent significant differences ($p<0.05$) observed between control and treatment groups for each diet type.

Figure 2

a) Lake whitefish



b) Lake trout

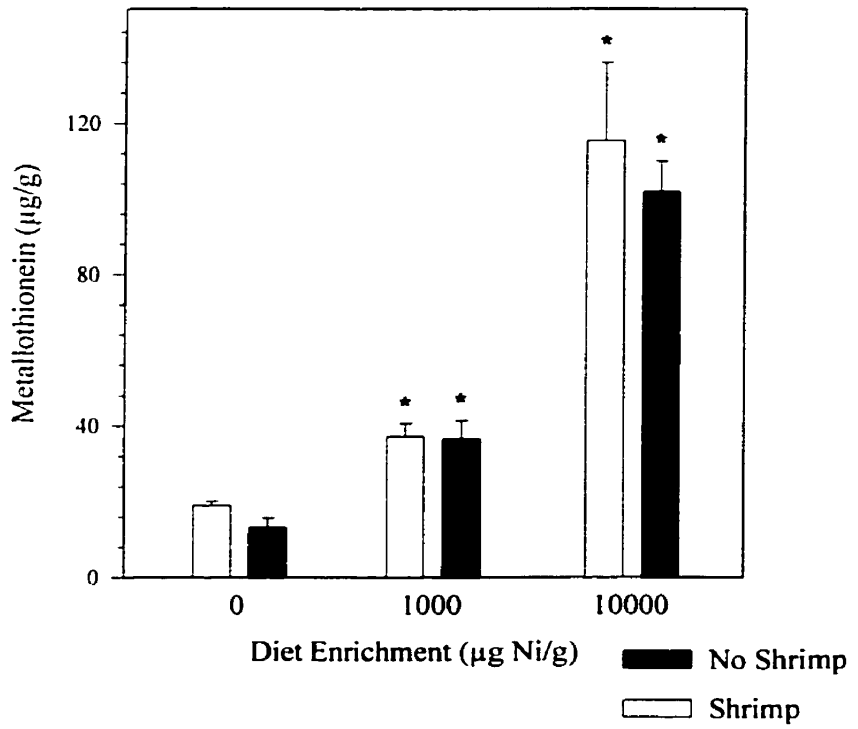
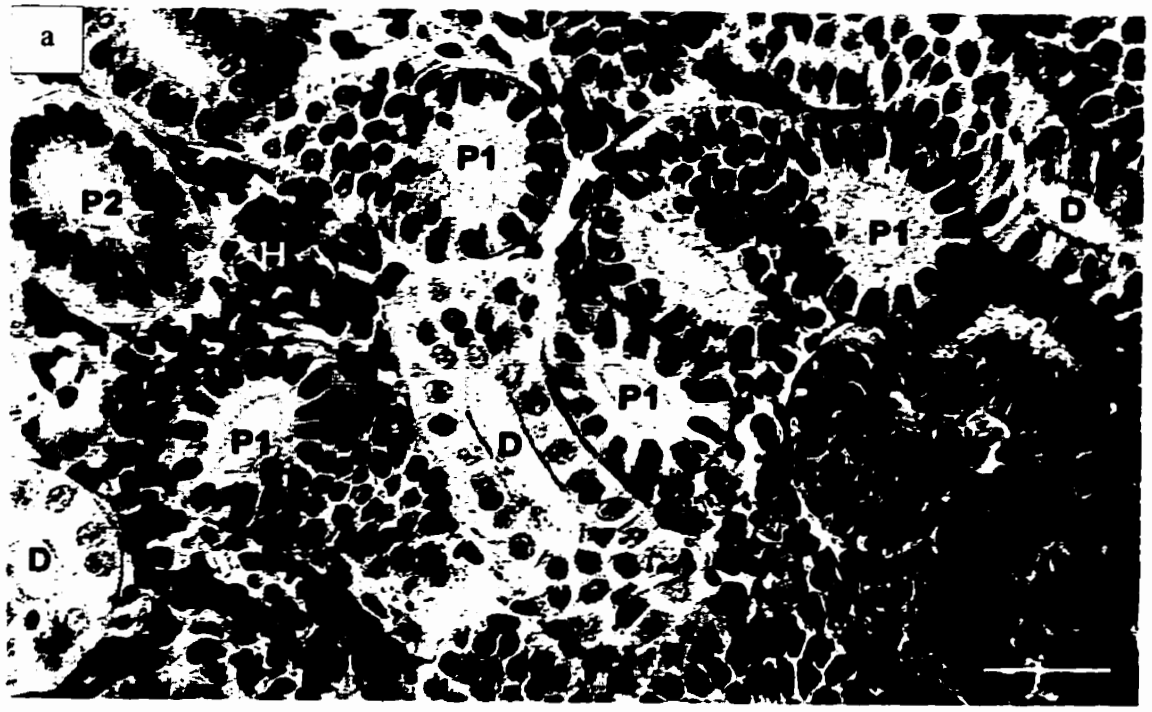


Figure 3. Posterior kidney photomicrographs. **a)** Posterior kidney of a lake whitefish fed a control diet. Bar = 30 μm. H&E stain. D, Distal tubule; P1, 1st segment of the proximal tubule; P2, 2nd segment of the proximal tubule; H, hematopoietic tissue. **b)** Posterior kidney of a lake whitefish fed 10000 μg Ni/g (NS). Alterations, including necrotic tubular epithelial cells (NC), accumulation of cellular debris in lumen of tubules (DE), dilated and degenerating tubules (segment unidentifiable) (U), and depletion of hematopoietic tissue (DH), are observed. Bar = 30 μm. H&E stain. D, Distal tubule; P2, 2nd segment of the proximal tubule; H, hematopoietic tissue.

Figure 3



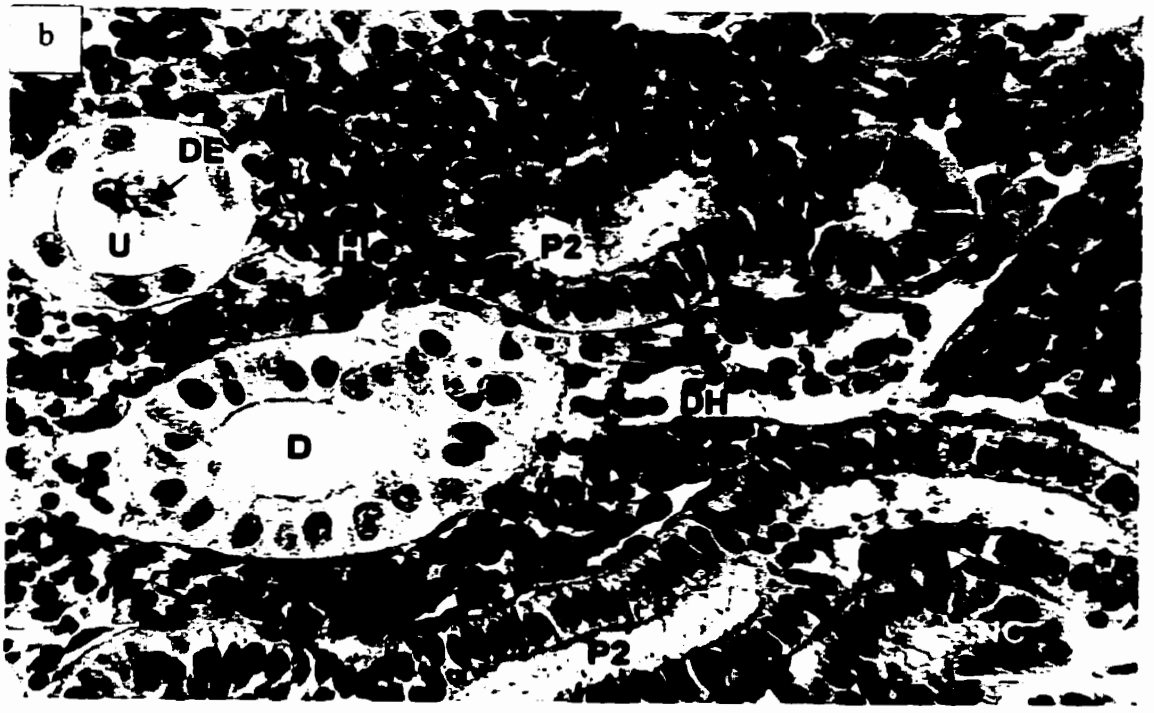


Figure 4. Liver photomicrographs. **a)** Low magnification photomicrograph of the liver of a lake whitefish fed a control diet. Bar = 80 μm. H&E stain. **b)** Low magnification photomicrograph of a lesion in the liver of a lake whitefish fed 10000 μg Ni/g (NS) (between arrows). Bar = 80 μm. H&E stain. **c)** High magnification photomicrograph of a liver of a lake whitefish fed a control diet. Bar = 30 μm. H&E stain. Normal cellular architecture, with 2-cell thick cords (between arrows) of polygonal hepatocytes (H) separated by sinusoids, is observed. **d)** High magnification photomicrograph of a lesion in the liver of a lake whitefish fed 10000 μg Ni/g (NS). Lesion is characterized by the presence of ruptured hepatocytes (RH), hepatocytes with darkened nuclei and eosinophilic cytoplasm (H), the accumulation of cellular debris (DE), cellular dissolution, and altered cord structure. Bar = 30 μm. H&E stain.

Figure 4

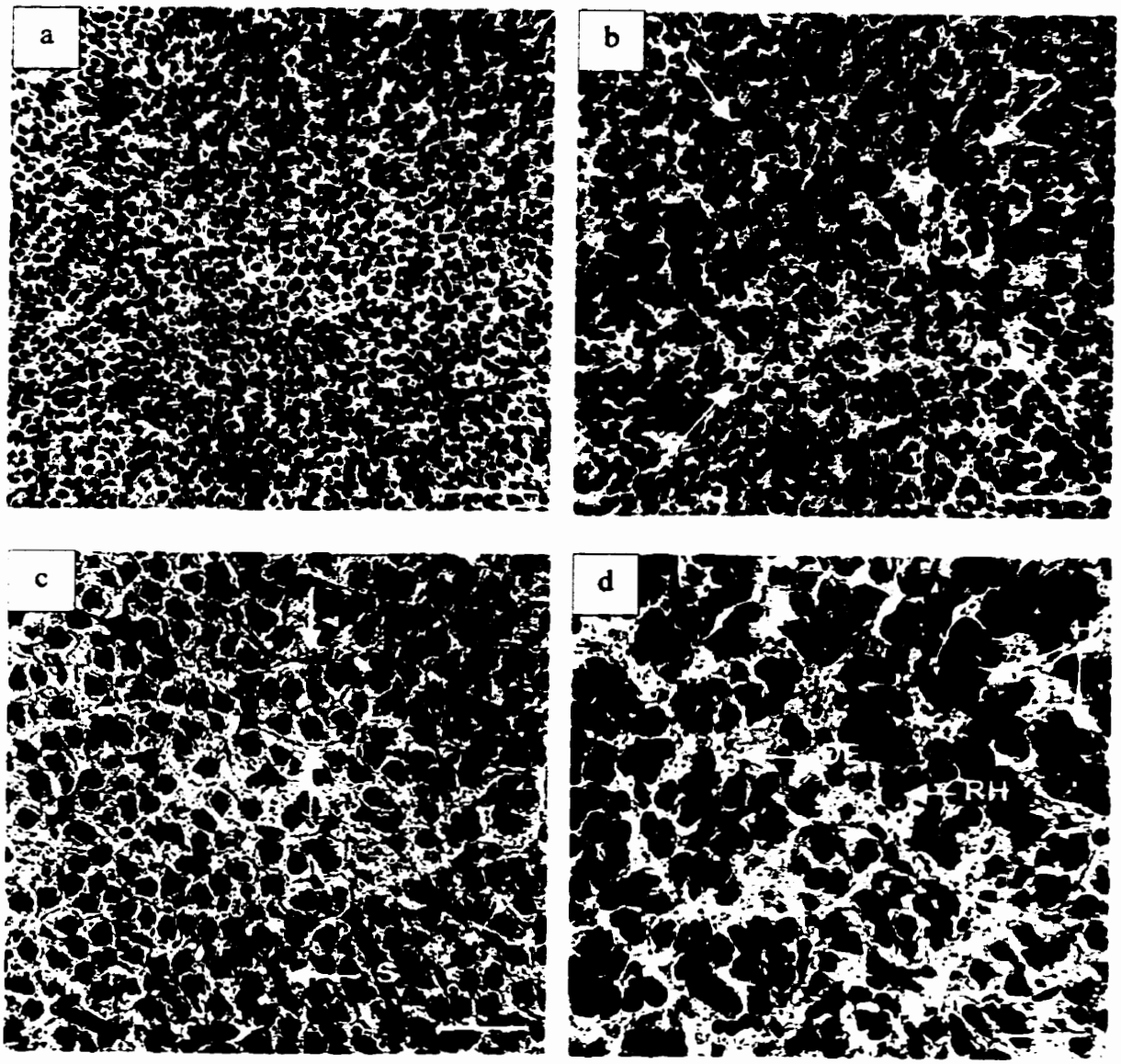


Figure 5. Intestine photomicrographs. **a)** Low magnification photomicrograph of a cross-section of intestine of a lake whitefish fed a control diet. Bar = 500 μm. H&E stain. ML, muscularis longnitudinalis; MC, muscularis circularis; S, stratum granulosum and compactum; LP, lamina propria ; LE, lamina epithlialis; L, lumen.
b) Low magnification photomicrograph of a cross-section of intestine of a lake whitefish fed 10000 μg Ni/g (WS). Bar = 500 μm. H&E stain. Note accumulation of debris in lumen (between block arrows) and expansion of lamina propria (LP). **c)** High magnification photomicrograph of the debris in the intestinal lumen of a lake whitefish fed 10000 μg Ni/g (WS). Bar = 30 μm. H&E stain. G, goblet cell; LE, lamina epithelialis. **d)** Photomicrograph of a cross-section of intestine of a lake whitefish fed a control diet. Bar = 80 μm. H&E stain. LP, lamina propria; LE, lamina epithlialis; L, lumen, G, goblet cell. **e)** Photomicrograph of a cross-section of intestine of a lake whitefish fed 10000 μg Ni/g (WS). Degenerative changes, including the desquamation of the lamina epithelialis (mucosal epithelium), presence of necrotic cells at the tips of the intestinal folds (NC), and accumulation of debris in lumen (DE), are apparent. Bar = 80 μm. H&E stain.

Figure 5

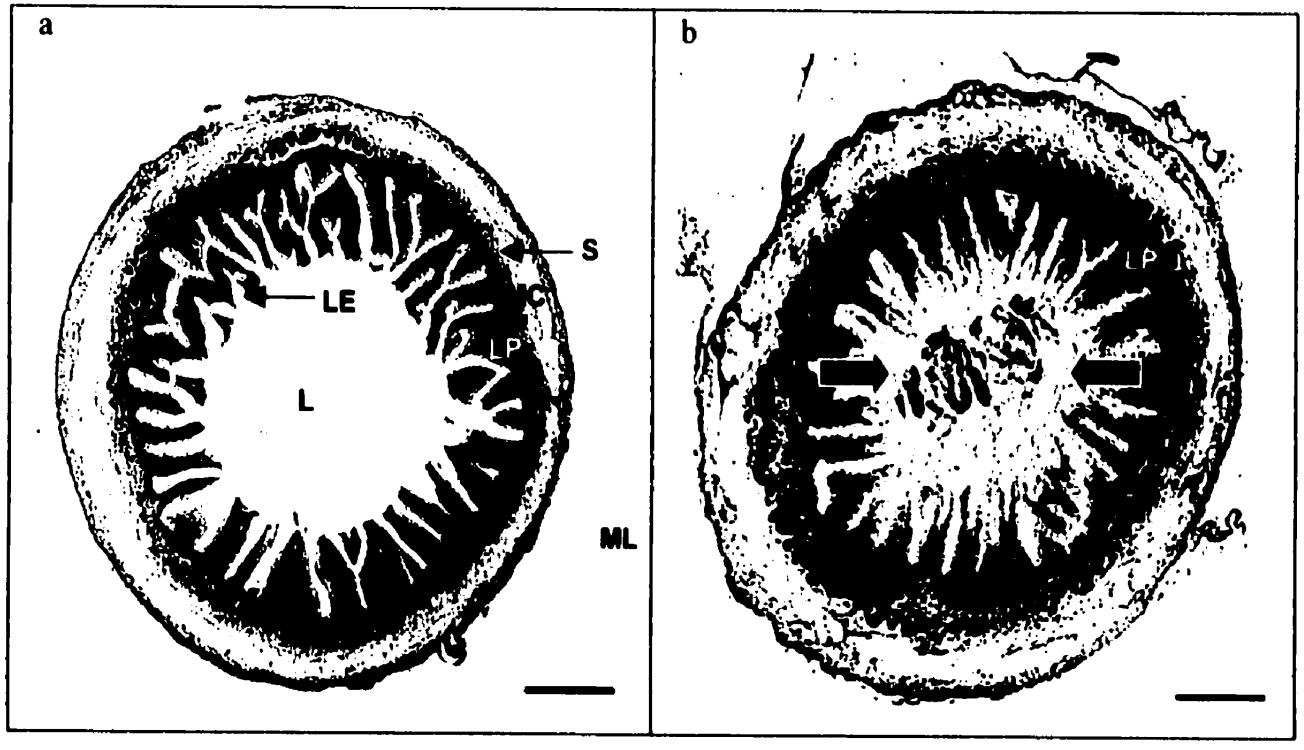


Figure 5

Chapter Two

Accumulation and distribution of dietary nickel in lake whitefish (*Coregonus clupeaformis*).

1) ABSTRACT

Benthic-feeding fish residing in Ni-contaminated systems are exposed to Ni through ingestion of contaminated food items and sediments. Laboratory based research is needed to provide insight into the potential impacts of the chronic exposure of freshwater fish to dietary Ni. Lake whitefish (*Coregonus clupeaformis*) were fed diets containing 0, 10, 100, and, 1000 μg Ni/g for 10, 31, and 104 days. Stomach, pyloric caeca, intestine, kidney, liver, gall bladder, gonad, gill, bone, muscle, skin, and scales were analyzed to evaluate the accumulation and distribution of Ni. Fish fed the medium and high dose diets accumulated significant amounts of Ni in a majority of the tissues sampled, even after only 10 d of exposure. Ni concentrations were highest in intestine and pyloric caeca of whitefish fed 1000 μg Ni/g on day 10, but decreased on subsequent sampling days, possibly due to protective mechanisms. Ni accumulation in stomach, kidney, liver, gill, skin, and scales was dose and duration-dependent. Ni concentrations measured in bone, gall bladder, gonad, and muscle of fish fed the control diet for 10 days and fish fed the high dose diet for all durations appeared to increase in a duration dependent manner. Exposure to Ni was also observed to alter the concentrations of Cu and Zn in tissues of lake whitefish. However, Cu and Zn concentrations in the tissues analyzed were variable and did not follow a common pattern or trend. The tissues that best assess dietary Ni bioavailability are kidney and scales.

2) INTRODUCTION

Mining, smelting, refining, metal processing, fuel combustion, and waste incineration activities release significant amounts of nickel (Ni) into the freshwater environment, through atmospheric deposition and in liquid effluents and leachates (Chau and Kulikovsky-Cordeiro, 1995). Elevated concentrations of Ni have been observed in surficial sediments near Canadian base-metal, gold, and uranium mining operations (Environment Canada, 1994). For example, surficial sediments collected downstream from gold mining operations located near Red Lake, Ontario contained Ni concentrations as high as 1100 μg Ni/g (dry weight, dw) (J.F. Klaverkamp, unpublished data). In another field study, Bradley and Morris (1986) collected sediment samples from lakes located near base-metal mining smelters in Sudbury, Ontario, and discovered that the greatest Ni concentrations, up to 4490 μg Ni/g (d.w), were observed in surficial sediments of lakes located closest to smelters.

Numerous field studies have also reported elevated concentrations of Ni in invertebrates and fish collected from Ni-contaminated areas (Hutchinson et al., 1976; Ney and Hassel, 1983; Dallinger and Kautzky, 1985; Bradley and Morris, 1986; Mastala *et al.*, 1992; Sharif *et al.*, 1993; Tariq *et al.*, 1993; Kashulin and Reshetnikov, 1995; Moiseenko *et al.*, 1995; Maletin *et al.*, 1996; Allen-Gil *et al.*, 1997; Brotheridge *et al.*, 1998; Klavins *et al.*, 1998a; Klavins *et al.*, 1998b; Klaverkamp *et al.*, 2000a). For example, zooplankton collected from the Wanapitei River, located near base-metal mining operations in Sudbury, Ontario, exhibited whole body Ni concentrations as high as 27 μg Ni/g (wet weight, ww) (Hutchinson *et al.*, 1976). Ni concentrations in the surficial sediments of the Wanapitei River were 224 μg Ni/g (d.w.). Hutchinson *et al.*

(1976) also reported Ni concentrations as high as 51.6 μg Ni/g (ww) in kidneys of yellow pickerel.

Fish residing in polluted freshwater systems are exposed to Ni, primarily, through the ingestion of contaminated food and sediments (Dallinger and Kautzky, 1985). A limited number of studies have investigated Ni uptake in fish through laboratory-based aqueous (Tjalve *et al.*, 1988; Ray *et al.*, 1990; Sreedevi *et al.*, 1992) and dietary (Chapter 1) exposures. Further research investigating the exposure of fish to dietary Ni is needed to elucidate the potential impacts of chronic dietary Ni exposure on natural populations of freshwater fish.

To address these research needs, a laboratory-based study was conducted to assess the accumulation and distribution of Ni in a freshwater fish species exposed to Ni via the diet. Lake whitefish (*Coregonus clupeaformis*) were fed control and Ni-contaminated diets for 3 durations. The concentrations of Ni selected for the diets were environmentally relevant, as they were based on the concentrations of Ni reported to occur in oxic sediments near Canadian base-metal, gold, and uranium mining and milling operations. Lake whitefish were selected for use in the experiments based on their sensitivity to Ni exposure (Chapter 1), economic importance, distribution, and because they feed on benthic organisms (Bodaly, 1986; DFO, 1997; Cooley and Klaverkamp, 2000; Pedlar and Klaverkamp, 2000). The results of this study will be used to evaluate which tissues in fish best assess the biological availability of Ni.

3) MATERIALS AND METHODS

3.1) Fish

Adult lake whitefish were obtained from stocks at the Freshwater Institute, Winnipeg, Manitoba. Seventy-two lake whitefish, 4 years in age with weights and fork lengths (mean ± SE) of 272 ± 5.86 g and 27.5 ± 0.206 cm, respectively, were randomly distributed into 12 tanks. The fish were acclimated for two weeks. During the acclimation period fish were fed a ration of a commercially prepared control diet equal to 0.5% of the total body weight per tank every Monday, Wednesday and Friday.

3.2) Tanks

Twelve 200 L fiberglass tanks, with transparent, plexi-glass lids were used. Aerated, de-chlorinated, treated municipal water was supplied to the tanks (Wagemann *et al.*, 1987). Photoperiod was controlled by a timed artificial lighting system; with 11.5 hours of light and 11.5 hours of darkness, separated by two 30-minute periods of intermediate light.

3.3) Diets

Diets were prepared at the Freshwater Institute, Winnipeg, Manitoba. Four diets containing nominal concentrations of 0, 10, 100, and 1000 μg Ni/g were prepared. The form of Ni used in the diets was nickel sulphate hexahydrate, $NiSO_4 \cdot 6H_2O$ (Fisher Scientific, Fairlawn, NJ). Commercial sinking pellets (Martin Feed Mills, Elmira, ON), containing 42% crude protein, 16% crude fat, 2% crude fiber, 5% ash, and 0.9% calcium,

were ground in a feed mill to produce flour. The control diet mixture was prepared by homogenizing a mixture of flour and deionized, distilled water in a large stainless steel food mixer. The contaminated diet mixtures were prepared in the food mixer by combining an appropriate quantity of $NiSO_4 \cdot 6H_2O$ with flour to produce nominal concentrations of 10, 100, and 1000 μg Ni/g. De-ionized, distilled water was added to the contaminated diet mixtures and homogenized using the mixer. Mixtures were further processed using an extruder to produce noodles. The noodles were dried to remove water, broken, sorted into No.3 size pellets using a particle sorter, and stored at -20° C. To prevent contamination between diet mixtures, diets were produced in ascending order of Ni concentrations and all equipment used to prepare diets was thoroughly cleaned.

Diets were analyzed in triplicate to verify Ni concentrations, with a detection limit of 0.05 μg Ni/g (ww). Diets, with nominal concentrations of 0, 10, 100, and 1000 μg Ni/g contained 1.1 ± 0.02, 12 ± 0.12, 110 ± 0.68 μg Ni/g, and 1100 ± 3.0 μg Ni/g (w.w), respectively, expressed as mean ± SE.

3.4) Water Quality

The water quality of each tank was monitored every Monday, Wednesday, and Friday for the duration of the experiment. Dissolved oxygen (92 ± 0.67 % saturation), pH (7.7 ± 0.03), and temperature (10.7 ± 0.103 ° C) were measured, expressed as mean ± SE for all tanks. Concentrations of major ions, total anion and cations, organic acids, total dissolved inorganic and organic carbon, total suspended solids, conductivities, and

alkalinities of water supplied to the tanks are presented in Cooley and Klaverkamp (2000).

To minimize the exposure of fish to Ni-contaminated waste products, solid waste was siphoned from tanks daily, tanks were flushed weekly, and tanks received water at a flow rate of 1 L every 54 seconds. This flow rate resulted in 90 % replacement of tank water in 7 hours and 99% replacement of tank water in 15 hours (Sprague, 1973). Additionally, tanks were brushed frequently to deter algal growth.

To confirm that fish were not being exposed to high concentrations of Ni through the aqueous route, water samples were collected from each tank and analyzed. One day prior to each sampling day, water samples were collected in an acid-washed container, acidified (0.5% nitric acid) and stored in the refrigerator until analyzed.

3.5) Experimental Design and Procedures:

Each of the 12 tanks were exposed to a combination of two treatments, with n=6 fish/tank. Treatment 1 is the concentration of Ni in the diet, at 0, 10, 100, and 1000 μg Ni/g and treatment 2 is the duration of exposure, at 10, 31, and 104 days. Days 10, 31, and 104 occurred on April 3, April 24, and July 6 of 1998.

Each tank was presented with a food ration equal to 0.5% total body weight per tank every Monday, Wednesday and Friday. Feeding activity was monitored visually throughout the experiment.

One individual from the treatment group fed 100 μg Ni/g for 104 days was sampled prior to day 104, due to feed refusal, and was excluded from all data sets.

3.6) Sampling:

On days 10, 31, and 104, fish from one tank representing each dose group were sampled. Fish were anesthetized with a pH-neutralized solution of tricaine methane sulfate (MS-222) (Sigma Chemical Co., St. Louis, MO) at a concentration of 500 mg MS-222/L. Fish were removed from the anesthetic after 1-2 minutes, when there was no response to a tail pinch. Fork lengths and weights were measured. Blood was removed from the caudal artery and vein, using a 20-gauge needle and 3cc syringe pre-treated with ammonium heparin (Sigma Chemical Co., St. Louis, MO).

Tissues were dissected using methods described in Cooley and Klaverkamp (2000) and were prepared for metals analyses after each sampling day, as described below. Plasma, gill, intestine, kidney, liver, stomach, and pyloric caeca were stored at – 90 °C for metals and biochemical analyses. Bile, bone, gall bladder, muscle, scales, and skin were stored - 40 °C for metals analyses.

Tissues were prepared for metals analyses as follows. Gill filaments were removed from the gill arch. Scales, with attached epidermal layer, and muscle were separated from the skin. Opercular bones were scraped to remove overlying tissue. Gall bladders were cut longitudinally. Scales, skin, opecular bones, and gall bladders were rinsed with deionized, distilled water and blotted. Stomach and intestine (small and large pooled) samples were separated, cut longitudinally, lightly scraped using a spatula, rinsed with saline solution, and blotted dry.

3.7) Metals Analyses

All analytical work was conducted in the Environmental Chemistry Lab at the Freshwater Institute, Winnipeg, MB.

Ni concentrations were measured in the feeds and gill, intestine, kidney, liver, pyloric caeca, scales, skin, and stomach of lake whitefish. Samples were digested in 4 ml of trace metal, analytical- grade nitric acid and heated at 135 ° C until dried. The digestion process was repeated twice, using 2.5 ml and 1.0 ml of nitric acid, respectively. Charred samples were treated with 2 drops of 30% hydrogen peroxide, following the addition of nitric acid. Tubes were cooled, 150 µl of nitric acid was added, and volumes were diluted to 12.5 ml with deionized, distilled water, and heated for 1 hour at 80 ° C. After cooling, volumes were diluted to 25 ml with deionized, distilled water and mixed with a vortex.

Samples were analyzed using flame atomic absorption spectroscopy (Varian SpectrAA-20 Atomic Absorption Spectrometer) to detect the higher range of Ni concentrations (25 - 2000 µg/L). Lower concentrations of Ni (1-50 µg/L) were measured using graphite furnace atomic absorption spectroscopy (Hitachi Polarized Zeeman Atomic Absorption Spectrometer Model 8200). Samples were diluted if necessary. Detection limits, based on tissue weights, are listed in Table 1.

Analyses of bone (operculum), gall bladder, and muscle samples were also conducted, using the methods described above, in fish fed the control diet for 10 days and fish fed the high dose diet for all durations. Statistical analyses were not conducted on these partial data sets.

Zinc, copper, and cadmium concentrations were measured in gill, intestine, kidney, liver, and pyloric caeca tissues using flame atomic absorption spectroscopy (Varian SpectrAA-20 Atomic Absorption Spectrometer). Detection limits for the other metals for each tissue analyzed are given in Table 1. All Cd concentrations were below the detection limits of the instrument.

Analytical accuracy was verified using certified biological reference materials (National Research Council Canada) and reagent blanks. Reagent blanks, dogfish muscle (dorm-2), dogfish liver (dolt-2), and lobster hepatopancreas (tort-2) were included in each set of samples. All reference materials measured were within 95% confidence limits.

In the discussion section, where results regarding Ni accumulation in tissues from this study were compared to results from other studies, it was often necessary to convert concentrations given as dry weights to wet weights, to correct for the percentage moisture present in tissue samples. A calculated wet weight concentration, denoted as "μg Ni/g (ww calc)", was determined by multiplying the documented dry weight concentration by 0.2 (Jarvinen and Ankley, 1999). All other data for metal concentrations in tissues are expressed as μg Ni/g wet weight (ww).

3.8) Statistics

All statistical analyses described below were performed using SPSS v. 9.0 software and were based on methods described in Neter *et al.* (1990), SPSS (1999), and Stevens (1992).

3.81 Ni, Cu, and Zn Accumulation

One-way ANOVAs and appropriate multiple comparison techniques were used to test for differences between fish fed control and Ni-contaminated diets. First, a two-way ANOVA was conducted to test for dose and duration effects and interaction. Because significant dose effects and/or interactions were observed, separate one-way ANOVAs were conducted for each duration (10, 31, or 104 days). First, the assumptions of the one-way ANOVA were tested and transformations were applied as necessary. When assumptions of normality were met data were analyzed using a one-way ANOVA, with $p< 0.05$. If the results from the one-way ANOVA were significant, significant differences ($p<0.05$) between control and treated groups were identified using an appropriate multiple comparison technique: a) if assumptions of equal variance were met Dunnett's test was used, and b) if assumptions of equal variance were not met Dunnett's T3 test was used. When the assumptions of normality were not met, a non-parametric Kruskal-Wallis test was used to test for differences between control and treated groups. If results from that test were significant, data were ranked, analyzed using a one-way ANOVA and tested using Dunnett's test.

3.82 Dose and Duration Dependency

Linear regression was used to assess how well Ni concentrations in the tissues were explained by the dose and duration of exposure. The significance of a linear relationship between the dependent (Ni accumulation in tissue) and the independent variables (dose and duration) was tested using $p<0.05$. Prior to conducting the regression the assumptions of the model were checked and transformations were applied as

necessary. The actual concentrations of Ni in the diet were substituted for the nominal concentrations of Ni in the diets, so if necessary, transformations could be applied. The combined R^2 values represent the ability of the independent variables (dose and/or duration) to predict the dependent variable (Ni concentrations in tissues). The partial R^2 values represent the predictive power of an independent variable, when the linear effect of the other independent variable in the model is removed. In some cases the independent variable, duration, was not found to have a significant linear relationship with the dependent variable and was excluded from the model.

3.83 Outliers

A kidney sample from one fish fed the control diet for 104 days was not included in the data analyses because sample contamination was suspected.

4) RESULTS

4.1) Water Quality

On days 9, 30, and 101, Ni concentrations in the water of tanks holding fish fed 0, 10, and 100 μg Ni/g were < 2.0 μg Ni/L. Water collected from tanks holding fish fed 1000 μg Ni/g on days 9 and 30, and 101 contained 2.9 ± 0.44 μg, 4.8 ± 0.82, and <2.0 μg Ni/L, respectively. These results confirm that Ni concentrations in the water were not considerably elevated.

4.2) Feeding and Growth

Feeding behavior and growth were similar for fish in all treatment groups, with the exception of one individual. One female, weighing 165 g, was sacrificed on day 56, because it refused to eat the 100 μg Ni/g diet. Analyses were not conducted on this fish. The following observations provide evidence that all other fish continued to eat the control and contaminated diets and to grow: 1) fish were observed eating the food, 2) there was evidence of food in the intestinal tract of fish when they were sampled, and 3) similar weight gains were observed in all treatment groups (Figure 1). Dietary rations were not adjusted during the experiment to compensate for fish growth. As a result, dose rates to fish decreased as the experiment continued because of fish growth (Table 2).

4.3) Nickel Accumulation in Lake Whitefish Tissues

Concentrations of Ni measured in stomach, pyloric caeca, intestine, kidney, liver, gill, skin, and scales of lake whitefish fed diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days are presented in Figure 2. Fish fed low dose diets only exhibited elevated concentrations of Ni in pyloric caeca on day 31, and in the intestine and kidney on day 104. Fish fed medium dose diets, however, accumulated higher concentrations of Ni in a majority of tissues sampled, with the exceptions of gill, liver, scales, and skin on day 10, and skin on day 31. Fish fed the high dose diets exhibited increased Ni concentrations in all 8 tissues on all sampling days.

The highest mean Ni concentrations were observed in intestine (70 μg Ni/g (ww)) and pyloric caeca (8.2 μg Ni/g (ww)) of fish fed the high dose diet for 10 days (Figures

2b and c). After day 10, Ni concentrations in the pyloric caeca and intestine decreased considerably, but continued to accumulate in a dose-dependent manner (Table 3).

In gills, kidney, liver, scales, skin, and stomach, the greatest Ni concentrations were observed in lake whitefish fed the high dose diet for 104 days. Ni accumulation in these tissues on day 104 was as follows: kidney (5.9 μg Ni/g (ww)) > scales (4.3 μg Ni/g (ww)) > skin (2.7 μg Ni/g (ww)) > gill (1.6 μg Ni/g (ww)) > stomach (1.4 μg Ni/g (ww)) > liver (0.51μg Ni/g (ww). Regression models indicated that Ni concentrations in gill (R^2 = 0.85), kidney (R^2 = 0.76), liver (R^2 = 0.66), scales (R^2 = 0.81), skin (R^2 = 0.86), and stomach (R^2 = 0.87) increased with the dose and duration of exposure (Table 3). Dose was a much strong predictor of Ni concentrations in these tissues compared to duration, as evidenced by the following partial R^2 values observed in the regression models: gill (R^2 = 0.83), kidney (R^2 = 0.73), liver (R^2 = 0.66), scales (R^2 = 0.80), skin (R^2 = 0.86), and stomach (R^2 = 0.87). Although duration was incorporated into the regression models, it can only be considered a weak predictor of Ni concentrations in these tissues.

Ni concentrations in bone, gall bladder, gonad, and muscle of fish fed high dose diets for 10, 31, and 104 days are presented in Table 4. Although these results could not be analyzed statistically, Ni concentrations appeared to increase in all tissues of whitefish fed the high dose diets, except gonads, in a duration dependent manner.

Similar patterns of Ni accumulation and distribution were observed in treatment groups fed similar cumulative doses of Ni. For example, fish that were fed 1000 μg Ni/g for 10 days and 100 μg Ni/g for 104 days consumed 22 and 24 μg Ni/g (ww), respectively (Table 2). These fish exhibited similar Ni concentrations in the gills, liver, scales, and skin (Figure 3). Significantly higher concentrations of Ni, however, were

observed in the kidneys and lower concentrations were observed in stomachs of fish fed 100 μg Ni/g for 104 days, compared to fish fed 1000 μg Ni/g for 10 days. In this comparison, only tissues that accumulated Ni in a dose and duration dependent manner were evaluated.

4.4) Copper and Zinc Accumulation in Lake Whitefish Tissues

Concentrations of Cu and Zn were measured in kidney, liver, gill, intestine, and pyloric caeca tissues of lake whitefish fed diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days. On day, 104, significantly decreased Zn concentrations were observed in pyloric caeca of fish fed 1000 μg Ni/g, compared to control fish (Figure 4). The results for Zn accumulation in gill were highly variable, with mean concentrations fluctuating from 31 -140 μg Zn/g (ww), without any consistent patterns (data not shown). Zn concentrations in intestine, kidney, and liver were similar between the treatment groups fed control and contaminated diets, with concentrations ranging from 160-240, 23-33, 25-31 μg Zn/g (ww), respectively (data not shown). Cu concentrations in these tissues were variable and did not follow a common pattern or trend (Figure 5). Decreases in Cu concentrations, however, were observed in several tissues of fish fed Ni-contaminated diets.

5) DISCUSSION

Lake whitefish consumed the Ni-contaminated diets and exhibited similar feeding behavior to fish fed control diets. Consumption of the diets did not appear to be affected by addition of nickel sulfate. However, in a related study, when 10000 μg Ni/g was

administered to lake trout (*Salvelinus namaycush)* and lake whitefish feed refusal was observed (as described in results section of Chapter 1).

Fish growth was unaffected by exposure to dietary Ni. These results contrast with those documented in a number of mammalian studies, which report decreased growth in rats fed concentrations of Ni ranging from 500 – 2500 μg Ni/g (Eisler, 1998).

A small number of field studies have reported tissue distributions of Ni in natural populations of whitefish that were consistent with those observed in the current study, indicating that whitefish exposed to natural diet items and prepared Ni-contaminated diets take up Ni in a similar manner (Kashulin and Reshetnikov, 1995; Moiseenko *et al.*, 1995; Klaverkamp *et al.*, 2000a). For example, lake whitefish (*C. clupeaformis*) collected from Little Macdonald Lake, which received treated mine water from a uranium mine in Key Lake, Saskatchewan, had elevated concentrations of Ni in kidney and liver, at mean concentrations of 4.7 μg Ni/g and 0.57 μg Ni/g, respectively (Klaverkamp *et al.*, 2000a). These concentrations were 7x and 9x higher, respectively, than concentrations found in kidney and liver of lake whitefish from a reference lake. Concentrations of Ni, reaching 690 μg Ni/g (dw), were also observed in the surficial sediments of Little Macdonald Lake. In the current study, similar Ni concentrations were observed in lake whitefish fed 1000 μg Ni/g for 31 days and 104 days, with mean concentrations of 4.9 and 5.9 Ni/g in kidney and 0.44 and 0.51 μg Ni/g in liver, respectively. In another example, whitefish (*Coregonus lavaretus*) were collected from a lake, with concentrations of 2227 μg Ni/g (d.w.) in the surficial sediments, located near Russian base-metal mining smelters. These whitefish accumulated 28 μg Ni/g (d.w) in the kidney, 9.6 μg Ni/g (d.w.) in the gill, and 7.5, 2.7, and 1.3 μg Ni/g (d.w.) in the skeleton,

liver, and muscle, respectively (Kashulin and Reshetnikov, 1995). Ni concentrations observed in this study, in kidney, gill, skeleton, liver, and muscle of lake whitefish fed 1000 µg Ni/g for 104 days, at mean concentrations of 5.9, 1.6, 2.4, 0.51, and 0.20 µg Ni/g, respectively.

Patterns of Ni accumulation and distribution observed in this study were also similar to those observed in other species of freshwater fish collected from Ni-contaminated areas (Hutchinson et al., 1976; Ney and Hassel, 1983; Dallinger and Kautzky, 1985; Bradley and Morris, 1986; Mastala *et al.*, 1992; Sharif *et al.*, 1993; Tariq *et al.*, 1993; Maletin *et al.*, 1996; Allen-Gil *et al.*, 1997; Brotheridge *et al.*, 1998; Klavins *et al.*, 1998a; Klavins *et al.*, 1998b). For example, Maletin *et al.* (1996) observed elevated concentrations of Ni in benthic-feeding Prussian carp (*Carassius auratus*) sampled from the Danube River in Yugoslavia, with concentrations of 1.55, 0.80, 0.71, and 0.48 µg Ni/g observed in gills, gonads, muscle, and liver, respectively. In the current study, lake whitefish fed 1000 µg Ni/g for 104 days accumulated similar concentrations of Ni in gills, liver, and gonads, however, much lower concentrations were observed in muscle. In another study, Bradley and Morris (1986) collected lake trout (*Salvelinus namaycush*) from Lake Nelson, near mining operations in Sudbury, Ontario, and observed a mean concentration of 1.0 µg Ni/g (ww calc.) in kidney, and concentrations less than 0.4 µg Ni/g (ww calc.) in liver and muscle. Surficial sediments of Lake Nelson contained 444 µg Ni/g (dw). The concentrations of Ni observed in trout kidney were similar to those observed in kidney (1.6 µg Ni/g) of lake whitefish fed 1000 µg Ni/g for 10 days in this study. The concentrations of Ni in liver and muscle of lake trout cannot

be compared with the current study because Bradley and Morris (1986) documented higher detection limits.

The field studies described above demonstrate that freshwater fish residing in polluted areas accumulate the highest concentrations of Ni in kidney, with gills, bone, and liver serving as other important sites of Ni accumulation. Typically, field studies do not evaluate Ni accumulation in the stomach, pyloric caeca, and/or intestine of fish residing in impacted systems, even though these fish are exposed to Ni predominantly through the diet. Dallinger and Kautzky (1985), however, investigated the importance of contaminated food in the uptake of heavy metals in natural populations of rainbow trout (*Salmo gairdneri*). Rainbow trout collected from the Augraben River in Italy accumulated the greatest amounts of Ni in the gastrointestinal tract contents, followed by gills, gastrointestinal tract tissues, kidney, muscle, liver, and gonads, at concentrations of 3.6, 2.9, 1.6, 1.6, 1.2, 1.2, and 0.7 μg Ni/g (ww calc.), respectively. The high Ni concentrations observed in the contents of the gastrointestinal tract, confirm that natural populations of fish, residing in Ni-contaminated environments, are exposed to Ni through the ingestion of contaminated diet items. The Ni concentrations observed in kidney and gonads of these rainbow trout were similar to those observed in the current study in lake whitefish fed 1000 μg Ni/g for 10 d, at concentrations of 1.6 and 0.59 μg Ni/g, respectively. Concentrations of Ni observed in gills, muscle, and liver of rainbow trout exceeded the highest concentrations observed in these tissues in this study. Tissues (stomach, pyloric caeca, and intestine) of the gastrointestinal tract of rainbow trout were analyzed as a combined sample, so individual tissue comparisons cannot be made with this study.

Other field studies report patterns of Ni accumulation and distribution in fish that were different from those observed in this study. In a number of studies, concentrations of Ni observed in fish tissues were higher than those observed in the dietary exposure, particularly in muscle, gill, and liver (Hutchinson *et al.*, 1976; Sharif *et al.*, 1993; Tariq *et al.*, 1993; Klavins *et al.*, 1998a; Klavins *et al.*, 1998b). For example, northern pike, yellow pickerel, northern rock bass, brown bull-head, redhorse sucker, and white sucker collected from the Wanapitei River, located near Sudbury, Ontario, had Ni concentrations ranging from 11.8-51.6, 11.1-31.7, 10.7-17.0, and 9.5-13.8 μg Ni/g in kidney, gill, liver, and muscle, respectively (Hutchinson *et al.*, 1976). In studies by Sharif *et al.* (1993), Tariq *et al.* (1993), and Klavins *et al.* (1998b), elevated concentrations of Ni as high as 1.22 (ww calc.), 2.73, and 0.89 μg Ni/g, respectively, were observed in muscle of fish collected from polluted waters. In some field studies, comparisons could not be made with the current study because Ni concentrations were reported for the whole body rather than for individual tissues (Johnson, 1987; Winger *et al.*, 1990; Hatcher *et al.*, 1992).

Comparisons made between this study and a preliminary, laboratory based study (Chapter 1), that investigated dietary Ni uptake in lake trout and lake whitefish, reveal similar patterns of Ni accumulation. In the preliminary experiment, lake whitefish and lake trout were fed diets containing 0, 1000, and 10000 μg Ni/g, prepared with and without brine shrimp, over a period of 18 days. Lake whitefish fed 1000 μg Ni/g (diet without shrimp) accumulated similar Ni concentrations (expressed as μg Ni/g) in kidney (4.0), gill (0.64), gall bladder (1.2), stomach (1.2), and muscle (0.11) to those observed in this study, in lake whitefish fed 1000 μg Ni for 10 and 31 days. Lake trout fed 1000 μg Ni/g for 18 days accumulated higher concentrations of Ni (expressed as μg Ni/g) in

stomach (10), liver (1.1), gall bladder (3.1), gonads (1.6), and muscle (0.29) and similar concentrations of Ni in gill (1.5), bone (0.77), and liver (1.1) to those observed in this study in lake whitefish fed 1000 μg Ni/g for 104 days. Both lake whitefish and lake trout fed 1000 μg Ni/g accumulated lower concentrations of Ni in bone and higher concentrations of Ni in gonads than those observed in this study.

Similar patterns of Ni accumulation and distribution were also observed in lake whitefish exposed to dietary Ni and in freshwater fish exposed to waterborne Ni (Tjalve *et al.*, 1988; Ray *et al.*, 1990). In aqueous exposures, Ni tends to accumulate primarily in kidney, with gills, liver, and intestine serving as other important sites of Ni uptake. For example, Tjalve *et al.* (1988) exposed brown trout (*Salmo trutta*) to water containing 0.0001 and 0.01 mg/L of $^{63}Ni^{2+}$ for 1 and 3 weeks. Trout exposed to 0.01 mg $^{63}Ni^{2+}$/L for 3 weeks accumulated the highest concentrations of Ni in kidneys, followed by gills, and liver, at concentrations of 0.233, 0.177, and 0.160 μg $^{63}Ni^{2+}$/g, respectively. Similar concentrations of Ni were observed in this study, in kidney and gills of lake whitefish fed 100 μg Ni/g for 10 d. Mean concentrations of 0.42 and 0.14 μg Ni/g were observed in kidney and gills, respectively, while a lower mean concentration of 0.05 μg Ni/g was observed in liver. The accumulation of Ni in liver, kidney, gills, and intestine of catfish (*Clarias batrachus*) exposed to water containing 5, 10, 15, 20, and 30 mg/L of nickel sulfate for 4 and 30 days was investigated by Ray *et al.* (1990). Concentrations of Ni observed in intestine (8.3 μg Ni/g) of catfish exposed to 30 mg/L for 30 days were similar to those observed, in this study, in whitefish fed 100 μg Ni/g for 30 days (9.7 μg Ni/g). Concentrations of Ni observed in kidney of whitefish fed 1000 μg Ni/g for 104 d (5.9 μg Ni/g) were similar to those observed in catfish exposed to 10 mg/L for 4 days and 5 mg/L

for 30 days, at concentrations of 6.2 μg Ni/g and 6.9 μg Ni/g, respectively. Baseline concentrations of Ni in liver and gill of control catfish were greater than the concentrations of Ni observed in whitefish fed 1000 μg Ni/g for 104 days.

The greatest Ni concentrations were observed in intestine and pyloric caeca of lake whitefish fed the high dose diet on day 10, with concentrations in intestine as high as 70 μg Ni/g. After day 10, nickel concentrations in intestine and pyloric caeca decreased substantially, but continued to demonstrate dose-dependency. These decreased Ni concentrations could not be attributed to feed refusal because whitefish in all treatment groups continued to feed on the experimental diets and grow. The decreased Ni concentrations in these tissues were likely attributed to protective mechanisms that were controlling Ni uptake.

The ability of aquatic and terrestrial animals to regulate Ni uptake has been widely reported (WHO, 1991; Environment Canada, 1994). In mammals, a majority of ingested Ni is eliminated in feces, with only 1-10% of Ni being absorbed through the intestine and subsequently eliminated in urine (WHO, 1991). In the current study, the strong dose dependent pattern of Ni accumulation observed in kidney, liver, stomach, gills, skin, and scales and the substantial decreases in Ni concentrations observed in the intestine and pyloric caeca on days 31 and 104 provide evidence that a protective mechanism is controlling Ni uptake.

The regulatory mechanisms that control Ni absorption in the intestine are not well understood. In intestines of mammals, Ni is absorbed and regulated by the absorptive mechanism for Fe (Forth and Rummel, 1971; Luckey and Vengopal, 1977; Tallkvist and Tjalve, 1997). Ni is chemically similar to Fe and competes for the same carrier proteins

in the intestinal mucosa (Luckey and Vengopal, 1977; Tallkvist and Tjalve, 1997). When these carrier proteins become saturated, Ni uptake is limited. Accordingly, Ni bound to these carrier proteins remains in the mucosal cells, which are desquamated and excreted after 2-3 days (Luckey and Vengopal, 1977). Intestinal absorption of Ni is decreased through this protective mechanism, resulting in reduced Ni accumulation and toxicity.

The kidney was a principal site of Ni accumulation in lake whitefish, with scales, skin, bone, gills and stomach serving as other important sites of accumulation. Mechanisms for Ni uptake in kidney are poorly understood in fish, but have been investigated in mammals. Mammalian researchers suggest that Ni likely enters the kidney as a low molecular weight Ni-histidine complex through the glomerular capillaries, and following filtration Ni binds to a Ni-sequestering renal glycoprotein (Abdulwajid and Sarkar, 1983). This non-inducible glycoprotein is thought to be a fragment of the renal basement membrane (Abdulwajid and Sarkar, 1983). Ni bound to this glycoprotein may accumulate or may be subsequently excreted in the urine (Abdulwajid and Sarkar, 1983).

The current study, as well as a depuration study (Tjalve *et al.*, 1988), provides evidence that the kidney is an important pathway for Ni excretion in fish. Tjalve *et al.* (1988) exposed brown trout (*Salmo trutta*) to water containing 0.0001 and 0.01 mg/L of $^{63}Ni^{2+}$ for 1 and 3 weeks, followed by a 1 and 3 week depuration period. After the 3-week depuration period the kidneys retained proportionally more $^{63}Ni^{2+}$, compared to other tissues. The authors suggested that the retention of Ni in the kidney reflects excretion of the metal via this pathway. In the current study, lake whitefish fed 100 μg Ni/g for 104 days and 1000 μg Ni/g for 10 days accumulated 3.8 and 1.6 μg Ni/g,

respectively, in the kidney. The higher concentrations of Ni present in the kidneys of fish fed the medium dose diet for 104 days, may be due the movement of Ni from other tissues to the kidney for excretion. Additionally, the increased Ni concentrations and histological alterations (Chapter 3) observed in kidneys of lake whitefish exposed to Ni-contaminated diets provides further evidence that renal excretion is an important route of Ni excretion in lake whitefish.

Similarly, the accumulation of elevated concentrations of Ni in the liver and gall bladder and the presence of hepatic lesions (Chapter 3) in lake whitefish fed Ni-contaminated diets provides evidence that biliary excretion may be an important route of Ni excretion in freshwater fish. Elevated Ni concentrations were observed in gall bladder and liver, reaching concentrations up to 2.3 μg and 0.51 μg Ni/g, respectively, in lake whitefish fed 1000 μg Ni/g for 104 days. Field studies also document elevated concentrations of Ni, as high as 17 μg Ni/g, in livers of fish collected from polluted areas (Hutchinson *et al.*, 1976). The concentrations of Ni in the bile were not measured in lake whitefish in this study. However, in a preliminary experiment (Chapter 1), whitefish administered 10000 μg Ni/g for 18 days had average Ni concentrations in the bile as high as 14 μg Ni/g (Chapter 1). Hepatic lesions were also observed in lake whitefish fed Ni-contaminated diets (Chapter 3). The role of liver in the detoxification and excretion of Ni in fish needs to be investigated further.

The skeleton and scales of fish appear to serve as important storage sites for Ni. Results from this study indicate that lake whitefish exposed to dietary Ni accumulate significant amounts of Ni in bone and scales. Lake whitefish fed 1000 μg Ni/g for 10, 31, and 104 days accumulated Ni concentrations, ranging from 1.9-4.3 μg Ni/g, in scales,

and slightly lower concentrations in bone, ranging from 1.3-2.4 μg Ni/g. Field studies have also reported elevated concentrations of Ni in skeletons of fish collected from polluted areas (Kashulin and Reshetnikov, 1995; Moiseenko *et al.*, 1995).

Bone and scales may accumulate Ni by a related mechanism due to their similarity in composition. The mechanisms involved in Ni uptake in mineralized tissues of fish, however, are not well understood. In fish, scales have also been observed to incorporate other heavy metals, like Zn, Pb, and U into the mineralized matrix (Sauer and Watabe, 1988; Yoshitomi *et al.*, 1998; Sauer and Watabe, 1989a; Sauer and Watabe, 1989b; Cooley and Klaverkamp, 2000). Ni may accumulate in fish scales, in a similar manner, by binding to Ca receptors on osteoblast cells or may accumulate through lysosomal degradation of metal-binding protein in the osteoblast cells. Additionally, lysosomal degradation of metal-binding proteins, like albumin, in osteoblasts represents a possible mechanism for storing and/or detoxifying excess metal ions in fish (Sauer and Watabe, 1989b). Because lake whitefish were exposed to Ni through the diet, accumulation observed in the scales is likely attributed to deposition within the calcified scale matrix, rather than absorption to the epidermal surface (Sauer and Watebe, 1989a; Yoshitomi *et al.*, 1998). However, this cannot be established as the molecular sites of Ni binding were not examined in this study.

The ability of fish scales to accumulate heavy metals, including Ni, has the potential for application to environmental monitoring programs (Sauer and Watebe, 1989a; Cooley and Klaverkamp, 2000;). A number of factors make the use of scales advantageous for use in bio-monitoring programs. First, scales can be removed from fish in field collections without sacrificing individuals from the population. Second, new

scales regenerate rapidly to replace those removed (Yoshitomi *et al.*, 1998). Third, scales can also provide information regarding the age and population of the fish (Sauer and Watabe, 1989a). Furthermore, the chemical composition of scales is stable over long periods of time (Yoshitomi *et al.*, 1998).

Exposure to Ni was also observed to alter the concentrations of Cu and Zn in tissues of lake whitefish. However, Cu and Zn concentrations in the tissues analyzed were variable and did not follow a common pattern or trend. In other studies, Ni has been observed to alter the endogenous concentrations of Cu and Zn in fish (Ghazaly, 1992) and mammals (Schroeder et al., 1974; Chmielnicka *et al.*, 1982; Rosenberg and Kappas, 1989). For example, increased Zn concentrations were observed in liver of lake whitefish fed 10000 μg Ni/g in the preliminary experiment (Chapter 1). In another study, Ghazaly (1992) observed increases in Cu and Zn in *Tilapia nilotica* exposed to waterborne Ni. Increased endogenous concentrations of Cu and Zn have been shown to result in increased production of metallothionein in aquatic organisms (Roesijadi, 1992). No relationship between metallothionein production and Cu and Zn concentrations was observed in this study, as discussed in the following chapter.

6) CONCLUSIONS AND RECOMMENDATIONS

The results obtained from this research can be used to guide field bio-monitoring efforts to assess Ni bioavailability in fish. In particular, the use of kidney and scales is strongly recommended. Additionally, the analyses of the tissues and contents of the gastrointestinal tract are recommended to elucidate the route of exposure to Ni.

As this is the first study to investigate Ni accumulation, distribution, and toxicity associated with exposure of fish to dietary Ni, a number of fundamental questions need to be examined in future research efforts. First, research is needed to elucidate the mechanisms involved in the absorption and cellular uptake, transport, metabolism, and detoxification of Ni in fish. Second, to further understand the significance of this laboratory exposure to natural populations of fish, research should be conducted comparing the bioavailability of Ni in prepared contaminated diets to natural diet items. Third, additional laboratory studies are recommended to examine the uptake and depuration of dietary Ni in fish, specifically for longer periods of duration. Fourth, the relationship between Fe and Ni in fish and the underlying mechanisms involved in the regulation of Ni in intestine should be investigated. Finally, the relationships between Ni accumulation in tissues and the toxicity observed at the molecular and cellular levels need to be investigated.

Table 1. Calculated detection limits (μg/g) for Ni, Cu, and Zn in 8 tissues of lake whitefish.

Tissue	Calculated detection limits[a] (μg/g)					
	Ni†		Cu††		Zn†	
	Mean	SE	Mean	SE	Mean	SE
Gill	0.06	0.00	0.71	0.02	0.71	0.02
Intestine	0.29	0.01	3.6	0.17	3.6	0.17
Kidney	0.22	0.00	2.7	0.03	2.7	0.03
Liver	0.18	0.00	2.2	0.02	2.2	0.02
Pyloric Caeca	0.07	0.00	0.87	0.02	0.87	0.02
Scales	0.10	0.00	-	-	-	-
Skin	0.09	0.00	-	-	-	-
Stomach	0.08	0.00	-	-	-	-

† = analyzed using graphite furnace atomic absorption spectroscopy
†† = analyzed using flame atomic absorption spectroscopy
"-" indicates that metal concentration was not measured

a. Calculated mean detection limit:

$$\frac{\text{detection limit for instrument (μg/L)} * \text{volume of sample (L)}}{\text{tissue weight (g)}}$$

Table 2: Exposure rates and cumulative total of ingested Ni in lake whitefish fed 0, 10, 100, and 1000 µg Ni/g for 10, 31 and 104 days. The decrease in exposure due to growth is expressed as a percentage decrease from the initial exposure rate.

Duration (days)	Dose	Exposure Rate (µg Ni/g body weight /day)[1]		Decrease in Exposure Due to Growth[3] (% decrease from initial exposure)	Calculated Cumulative Total of Ingested Ni [2] (µg Ni/g body weight)	
		Using initial BW	Using final BW		Using initial BW	Using final BW
10	Control	0.00	0.00	0.00	0.02	0.02
	Low	0.02	0.02	0.00	0.24	0.24
	Medium	0.22	0.21	4.5	2.2	2.1
	High	2.2	2.1	4.5	22	21
31	Control	0.00	0.00	0.00	0.07	0.07
	Low	0.03	0.02	33	0.78	0.69
	Medium	0.23	0.21	8.7	7.2	6.6
	High	2.3	2.1	8.7	72	66
104	Control	0.00	0.00	0.00	0.24	0.20
	Low	0.02	0.02	0.00	2.6	2.1
	Medium	0.23	0.17	26	24	18
	High	2.3	1.8	22	240	190

Where:
initial BW = wet body weight (g) start of experiment
final BW = wet body weight (g) on sampling day

Calculations:

1. Exposure Rate = (feed consumption per tank of fish per exposure period (g)/initial BW or final BW (g)) * 1/exposure period (days) * measured dietary Ni concentration (µg Ni/g)
2. Calculated Cumulative total of ingested Ni (µg Ni/g body weight) = (feed consumption per tank of fish per exposure period (g)/initial BW or final BW) * measured dietary Ni concentration (µg Ni/g)
3. % decrease in exposure = (exposure rate (based on initial BW) – exposure rate (based on final BW)) /exposure rate (based on initial BW) * 100

Table 3. Estimated Regression Models with combined R^2 values for the model and partial R^2 values for each independent variable included in the model.

Tissue	Estimated Regression Model	Combined R^2	Partial R^2	
			Dose	Duration
Gill	Square root [Ni] in the gill = -7.1 E-02 + 2.7 E-02(square root [Ni] in diet) + 3.9 E-02(square root duration)	0.85	0.83	0.36
Intestine*	Log [Ni] in the intestine = -0.48 + 0.61(log [Ni] in diet)	0.71	-	-
Kidney	Square root [Ni] in the kidney = -0.20 + 5.1 E-02(square root [Ni] in diet) + 8.8 E-02(square root duration)	0.76	0.73	0.29
Liver	Square root [Ni] in the liver = 9.5 E-02 + 1.4 E-02(square root [Ni] in diet) + 1.1 E-02(square root duration)	0.66	0.66	0.06
Pyloric Caeca*	Log [Ni] in the pyloric caeca = -1.6 + 0.63(log [Ni] in diet)	0.74	-	-
Scales	[Ni] in the scales = 0.20 + 2.4 E-03([Ni] in diet) + 8.0 E-03(duration)	0.81	0.80	0.25
Stomach	Square root [Ni] in the stomach = 0.14 + 2.8 E-02(square root [Ni] in diet) + 1.4 E-02(square root duration)	0.87	0.87	0.08
Skin	Square root [Ni] in the skin = -6.0 E-02 + 3.7 E-02(square root [Ni] in diet) + 3.8 E-02(square root duration)	0.86	0.86	0.25

* In some cases the independent variable, duration, was not found to have a significant linear relationship with the dependent variable and was excluded from the model.

The combined R^2 values represent the ability of the independent variables (dose and/or duration) to predict the dependent variable (Ni concentrations in tissues). The partial R^2 values represent the predictive power of an independent variable, when the linear effect of the other independent variable in the model is removed.

Table 4. Ni accumulation in bone (operculum), gall bladder, gonads, and muscle samples (μg Ni/g ww). Ni concentrations were measured in fish fed the control diet for 10 days and fish fed the high dose diet for all durations.

	Mean (± SE) Ni Concentration (μg Ni/g ww)			
	Treatment Group			
	0 μg Ni/g	1000 μg Ni/g		
Tissue	10 d	10 d	31 d	104 d
bone[a]	1.0 (0.16)	1.3 (0.07)	2.1 (0.05)	2.4 (0.06)
gall bladder[b]	0.03 (0.00)	0.69 (0.11)	1.7 (0.20)	2.3 (0.10)
gonads[a]	0.03 (0.00)	0.59 (0.08)	0.68 (0.01)	0.58 (0.01)
muscle[a]	0.04 (0.00)	0.08 (0.00)	0.14 (0.01)	0.20 (0.01)

a: n=6

b: n=4

Figure 1. Weight Gain in lake whitefish fed diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days. The mean weight gain observed for each treatment group is plotted.

Figure 1.

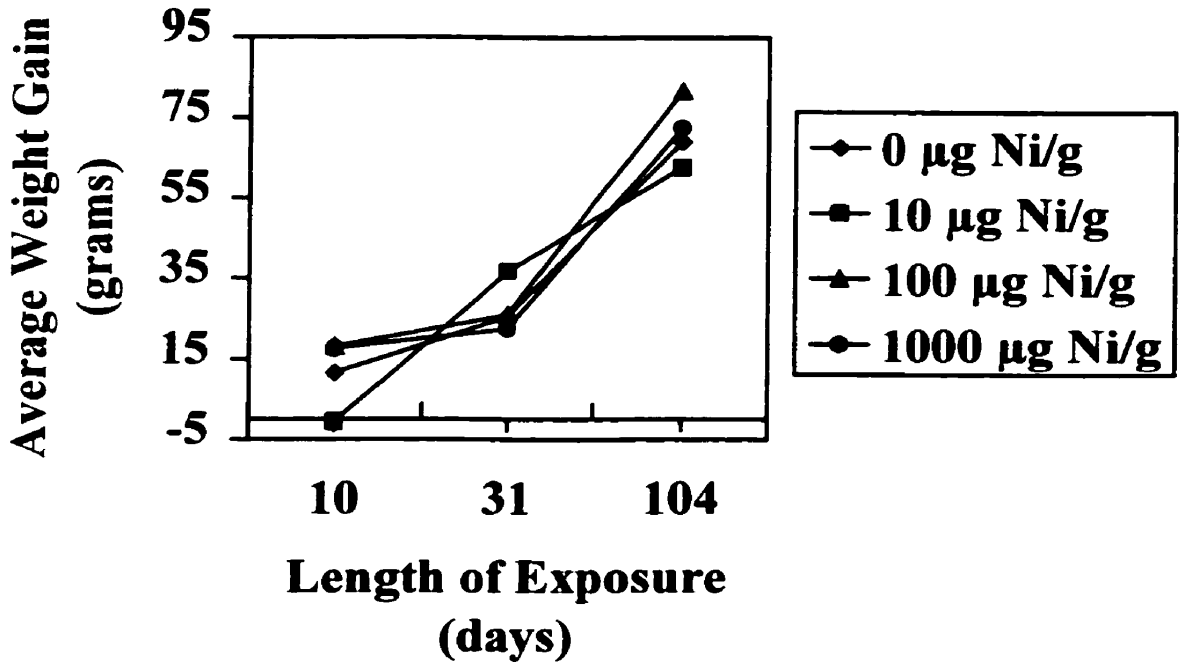

Average Weight Gain (grams)
95
75
55
35
15
-5
10
31
104
Length of Exposure (days)
0 µg Ni/g
10 µg Ni/g
100 µg Ni/g
1000 µg Ni/g

Figure 2. Ni accumulation in eight tissues of lake whitefish fed 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days: a) stomach, b) pyloric caeca, c) intestine, d) kidney, e) liver, f) gill, g) skin, and h) scales. Data expressed as mean (± SE). Asterisks represent significant differences observed between control and treatment groups for each duration ($p<0.05$).

Figure 2

a) Stomach

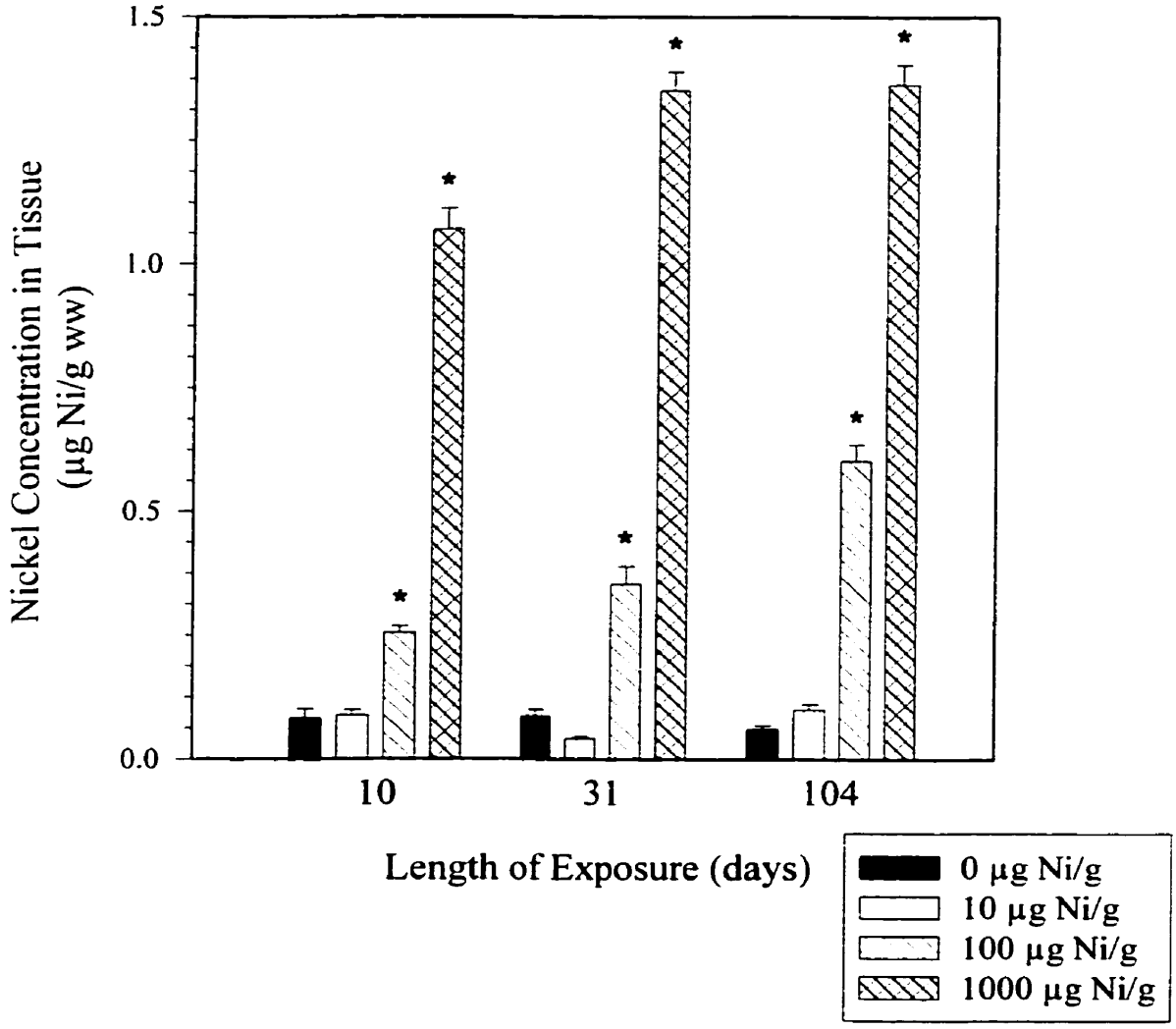


Figure 2
b) Pyloric Caeca

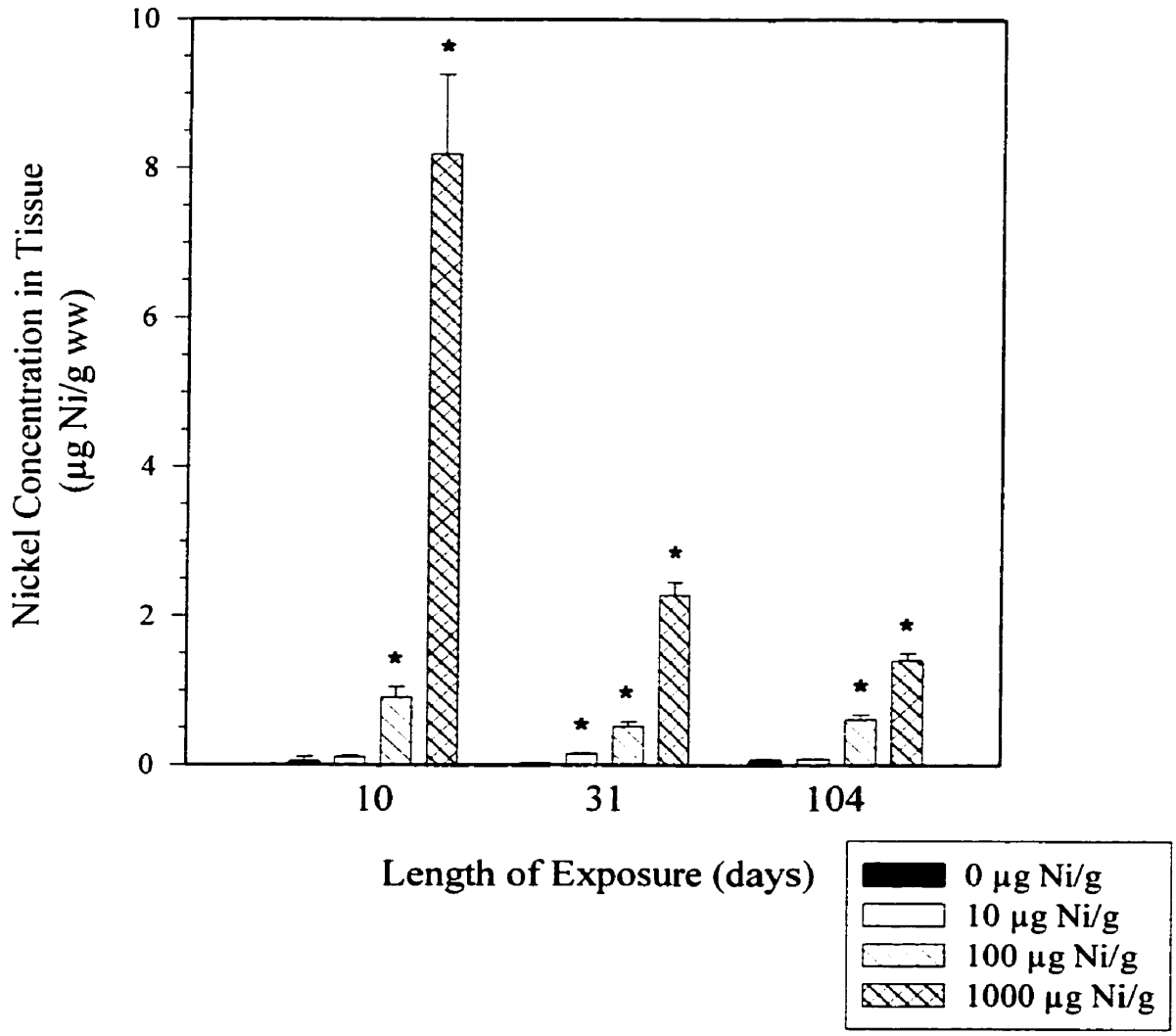


Figure 2
c) Intestine

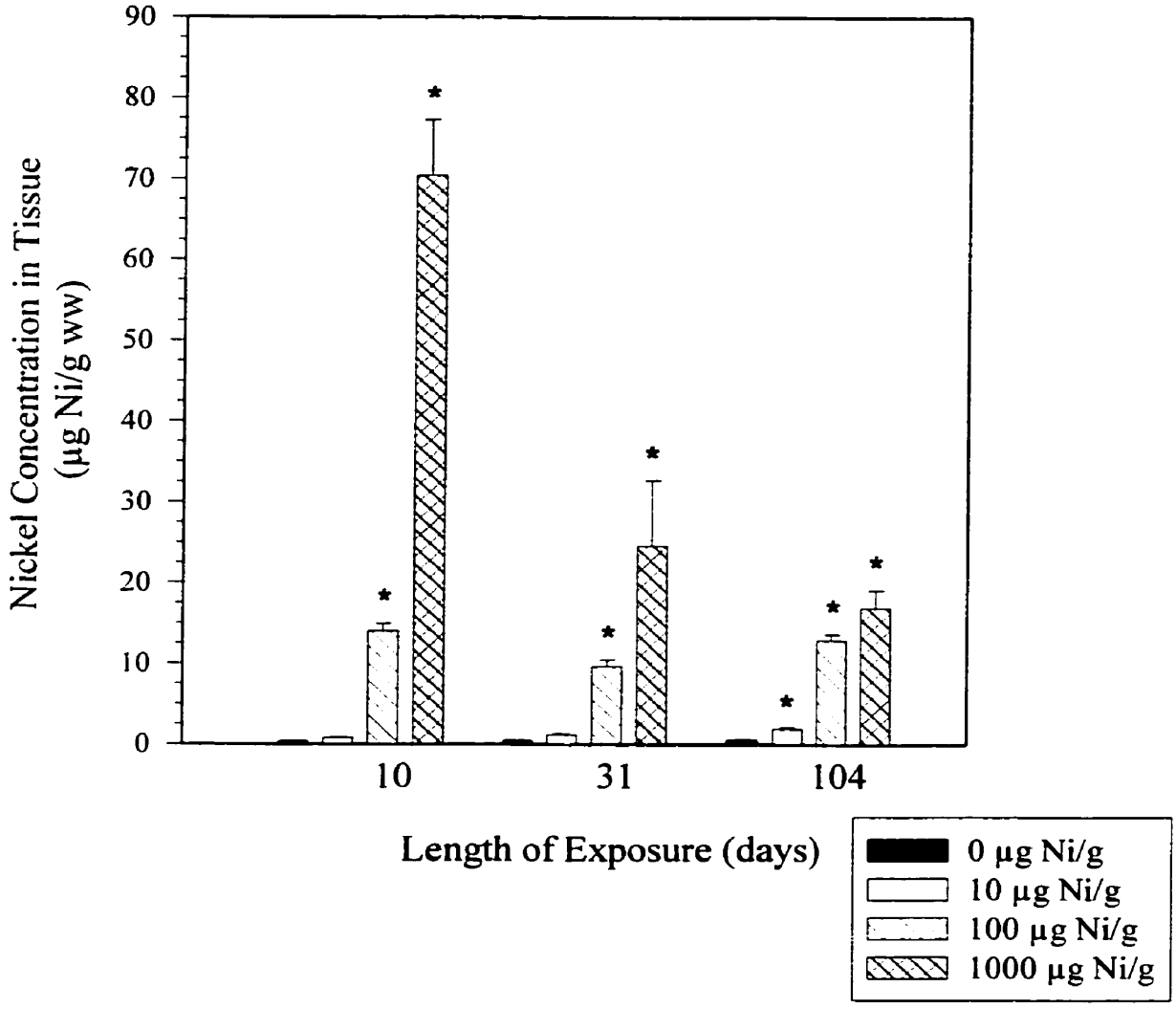


Figure 2
d) Kidney

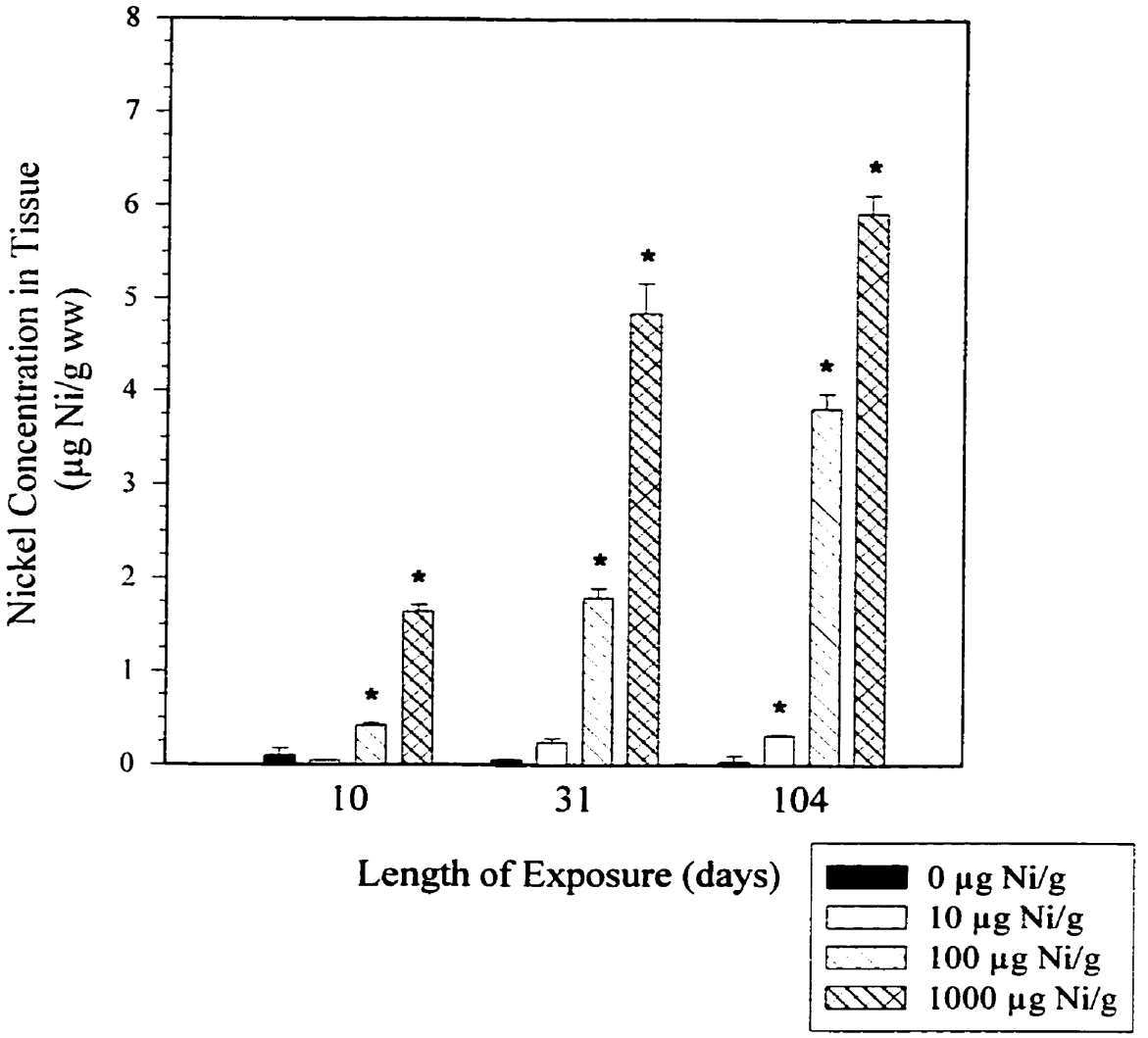


Figure 2
e) Liver

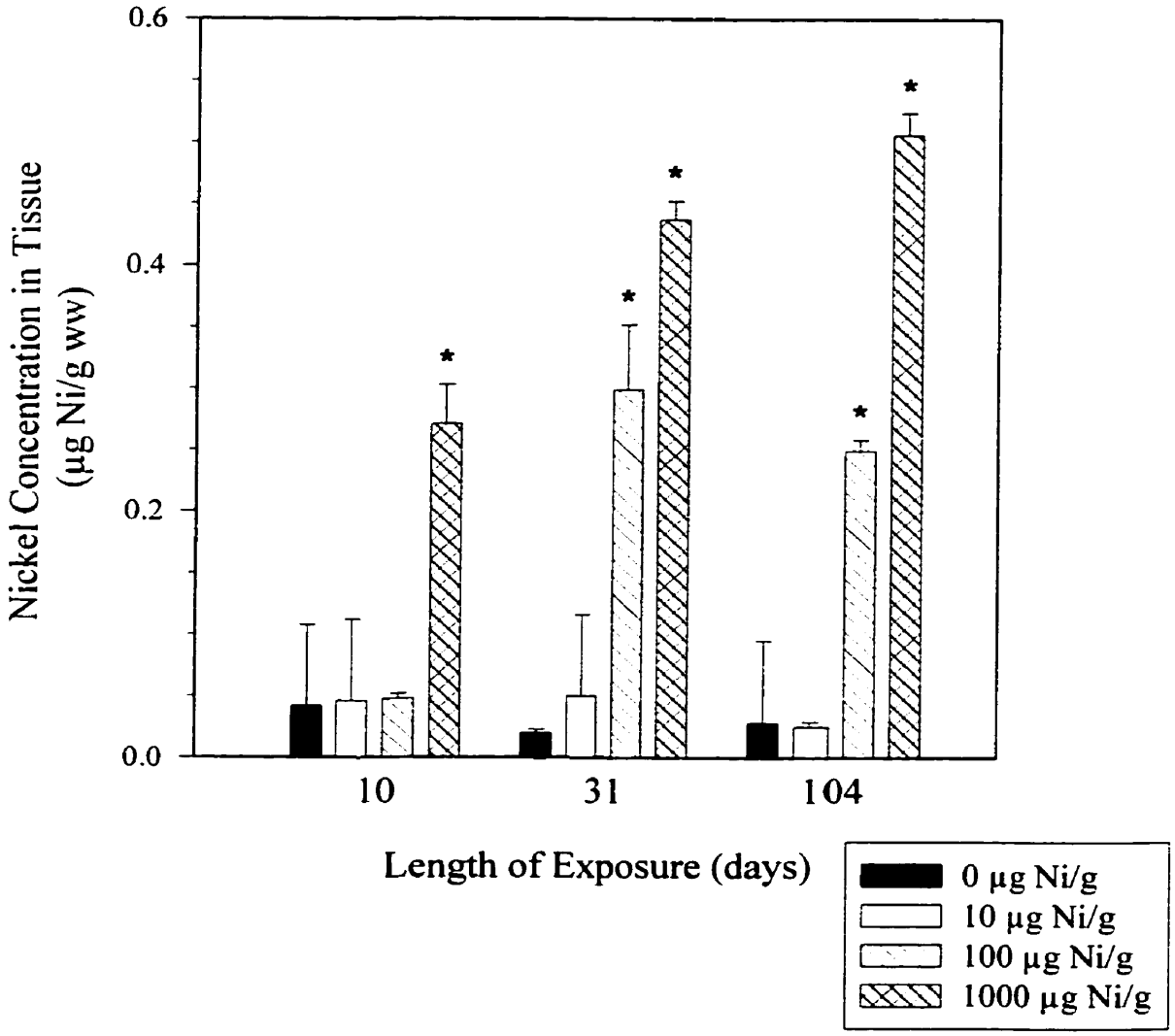


Figure 2
f) Gill

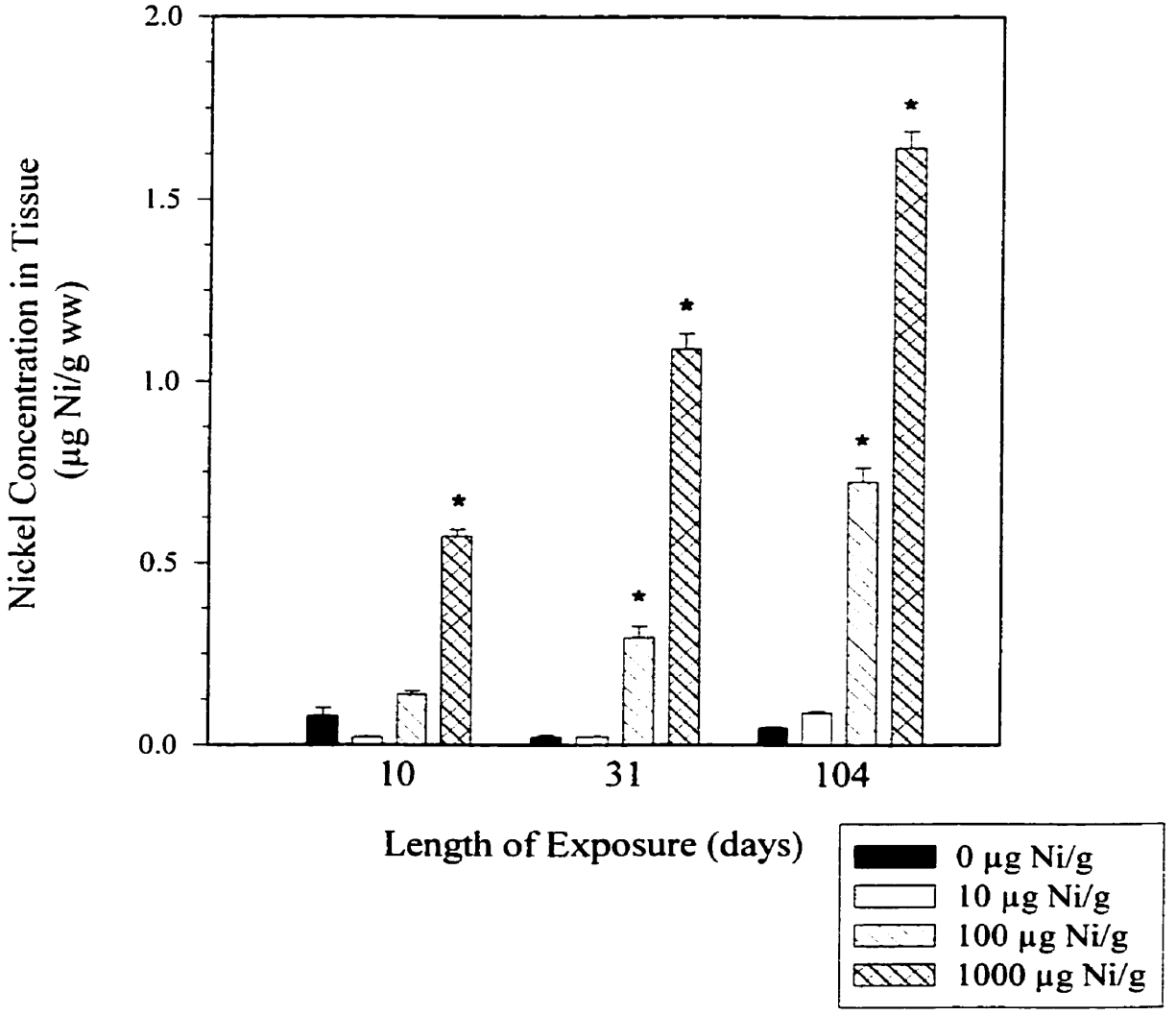


Figure 2
g) Skin

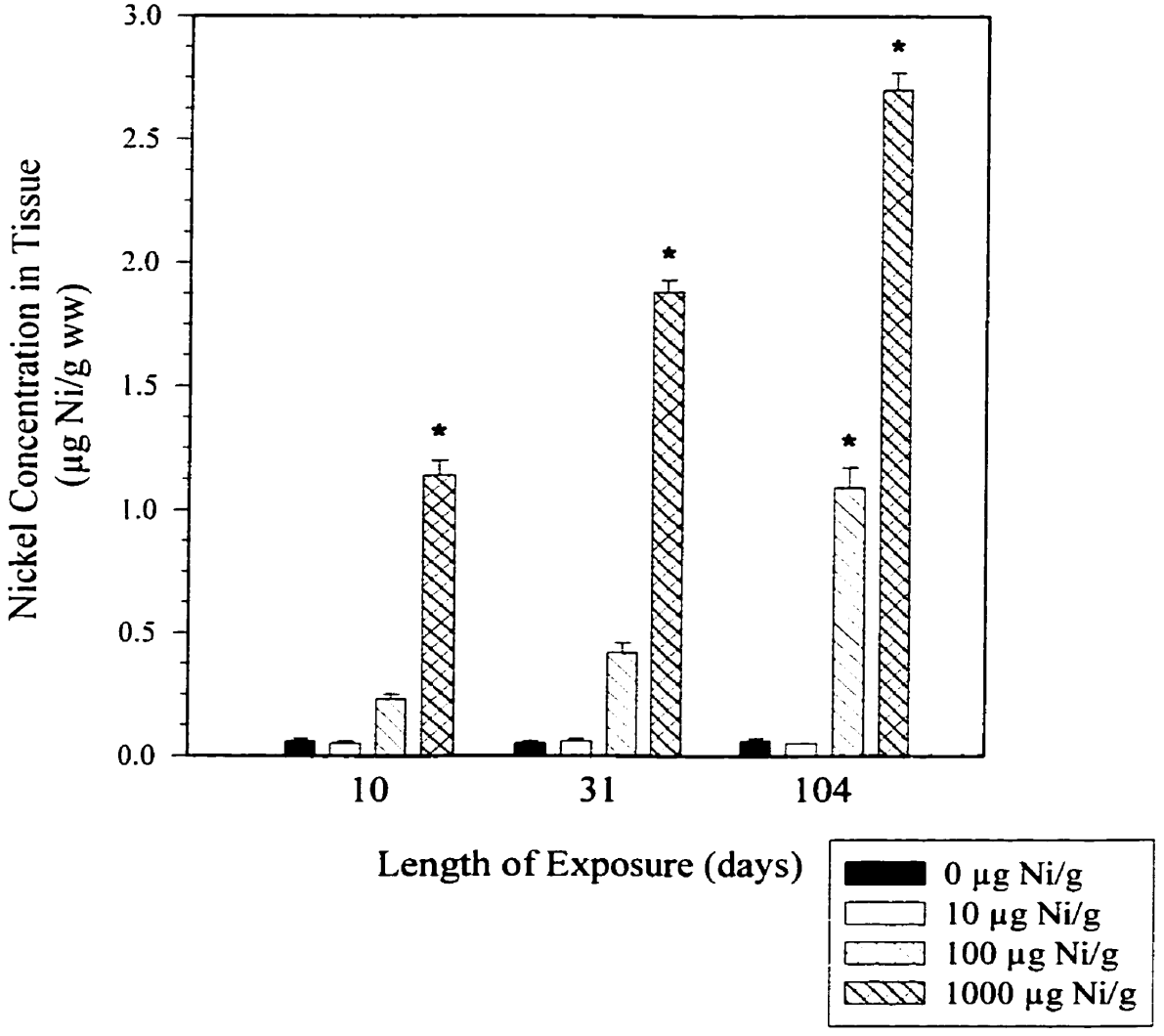


Figure 2
h) Scales

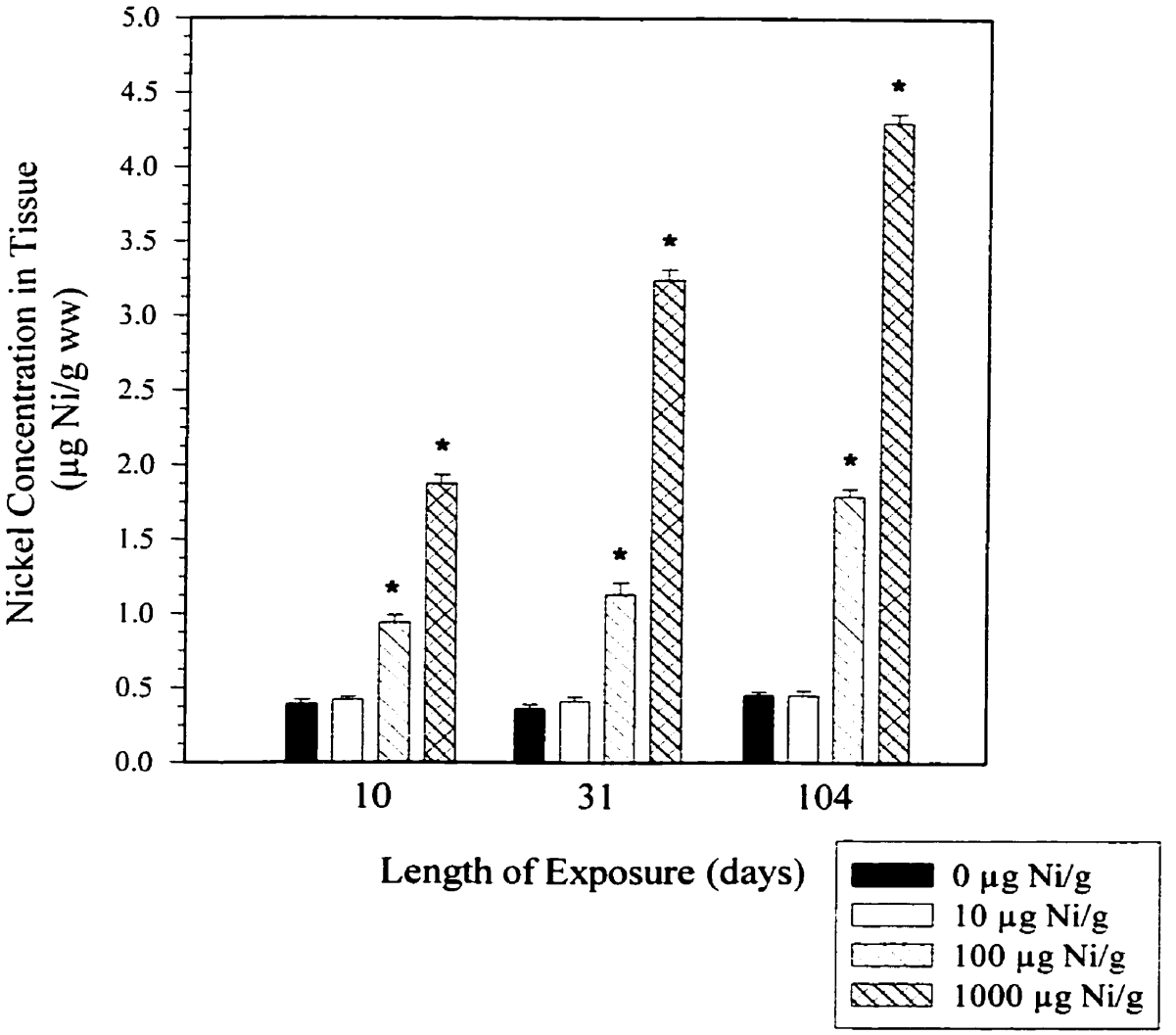


Figure 3: Ni accumulation in 6 tissues of control lake whitefish and treated lake whitefish fed similar cumulative concentrations of Ni. Data are expressed as mean ± SE.

Figure 3

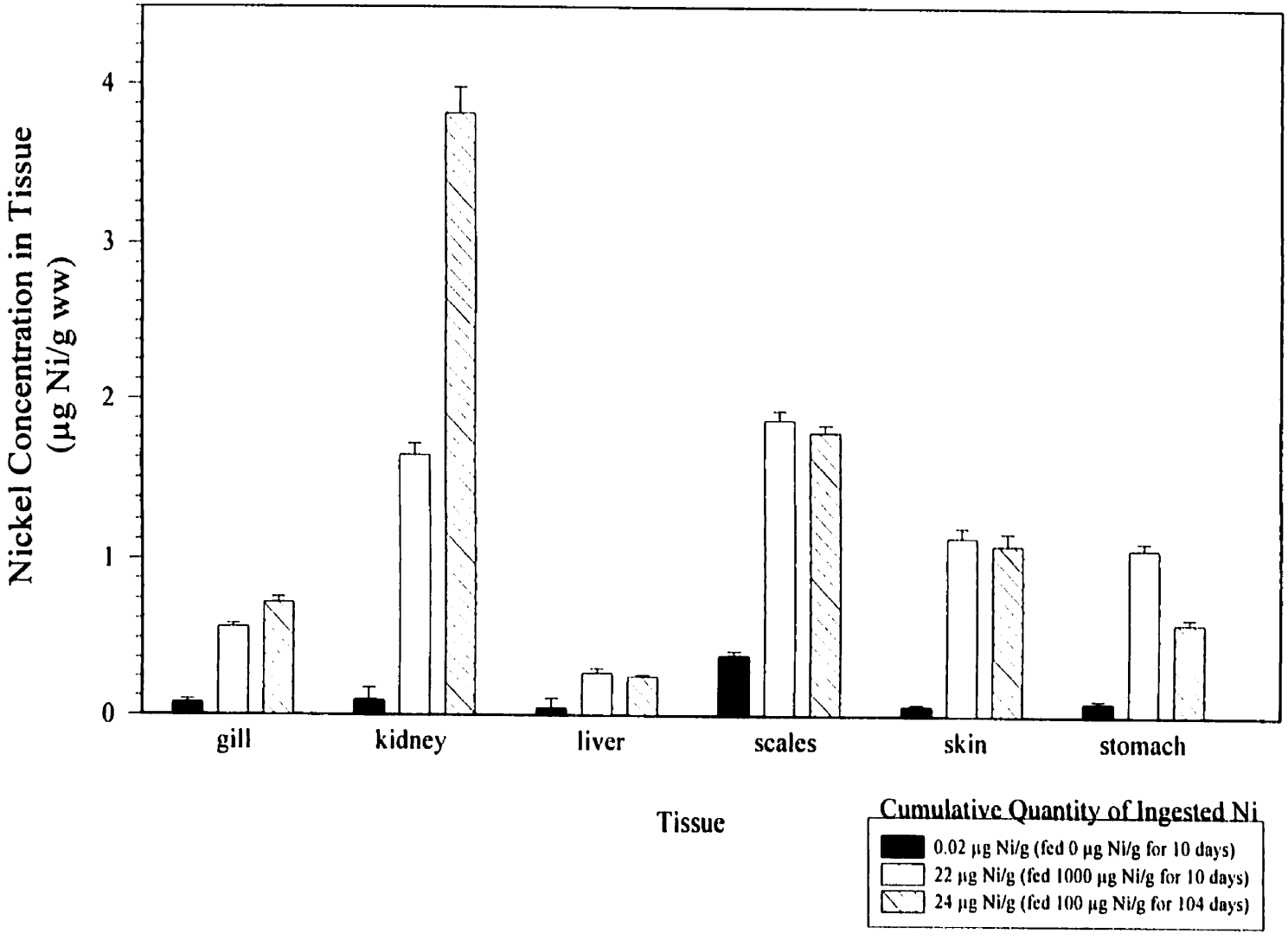

Nickel Concentration in Tissue
(µg Ni/g ww)
0
1
2
3
4
gill
kidney
liver
scales
skin
stomach
Tissue
Cumulative Quantity of Ingested Ni
0.02 µg Ni/g (fed 0 µg Ni/g for 10 days)
22 µg Ni/g (fed 1000 µg Ni/g for 10 days)
24 µg Ni/g (fed 100 µg Ni/g for 104 days)

Figure 4. Zn accumulation in pyloric caeca of lake whitefish fed diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days. Data are expressed as the mean (± SE). Asterisks represent significant differences ($p<0.05$) observed between control and treatment groups for each diet type.

Figure 4

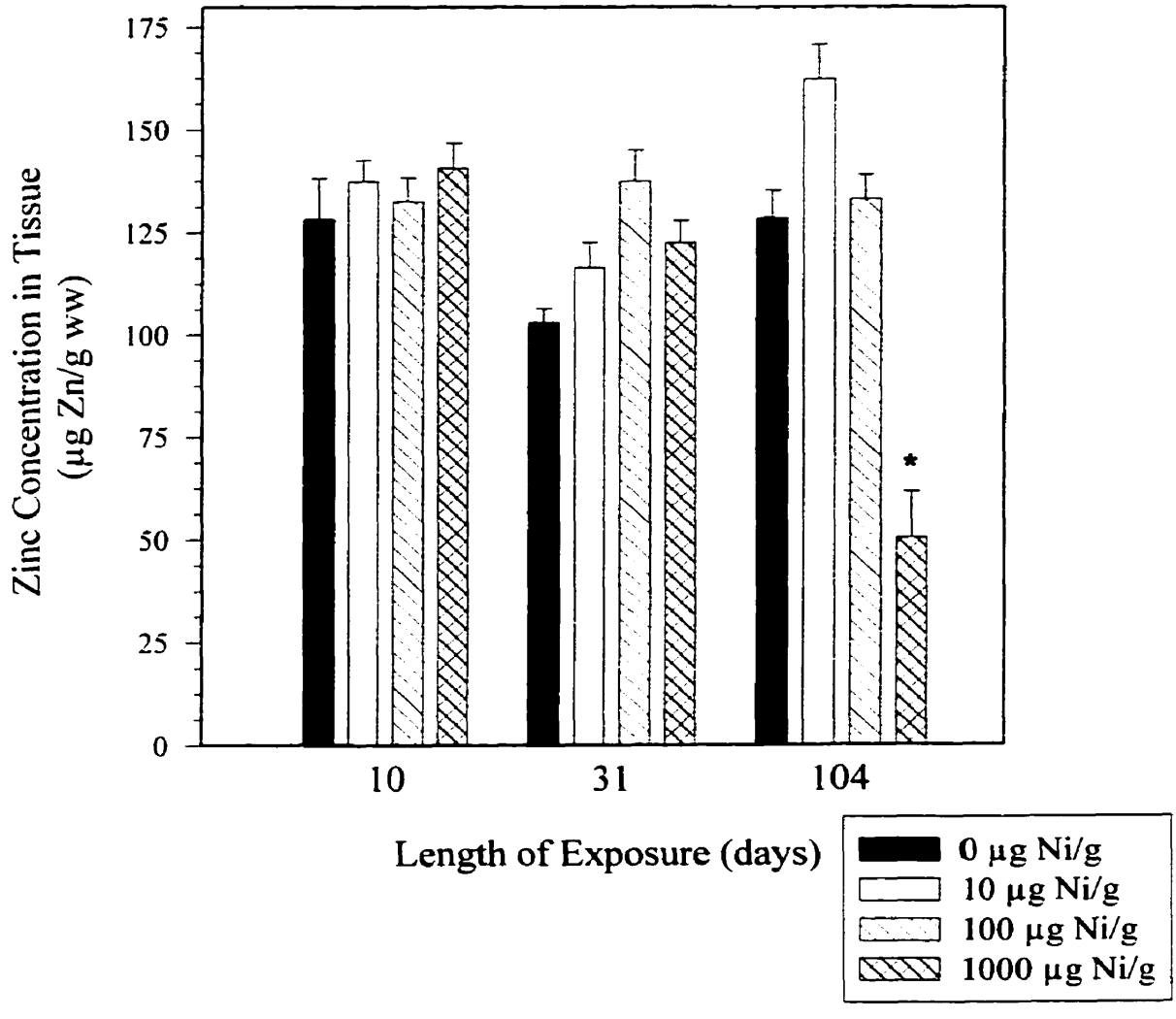

Zinc Concentration in Tissue
(µg Zn/g ww)
175
150
125
100
75
50
25
0
10
31
104
Length of Exposure (days)
*
0 µg Ni/g
10 µg Ni/g
100 µg Ni/g
1000 µg Ni/g

Figure 5. Cu accumulation in five tissues of lake whitefish fed diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days: a) gill, b) intestine, c) kidney, d) liver, and e) pyloric caeca. Data are expressed as the mean (± SE). Asterisks represent significant differences ($p<0.05$) observed between control and treatment groups for each diet type.

Figure 5

a) Gill

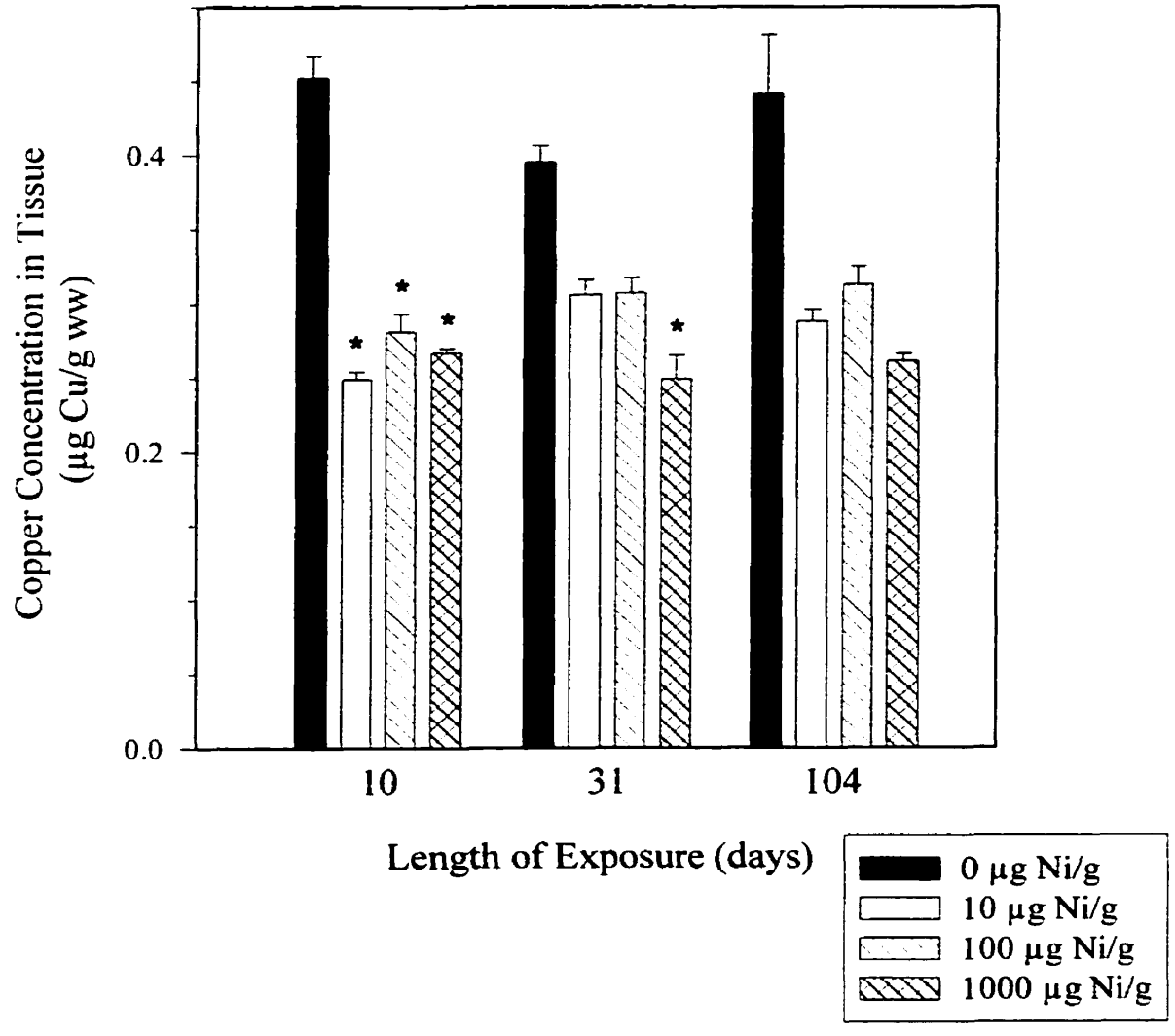


Figure 5
b) Intestine

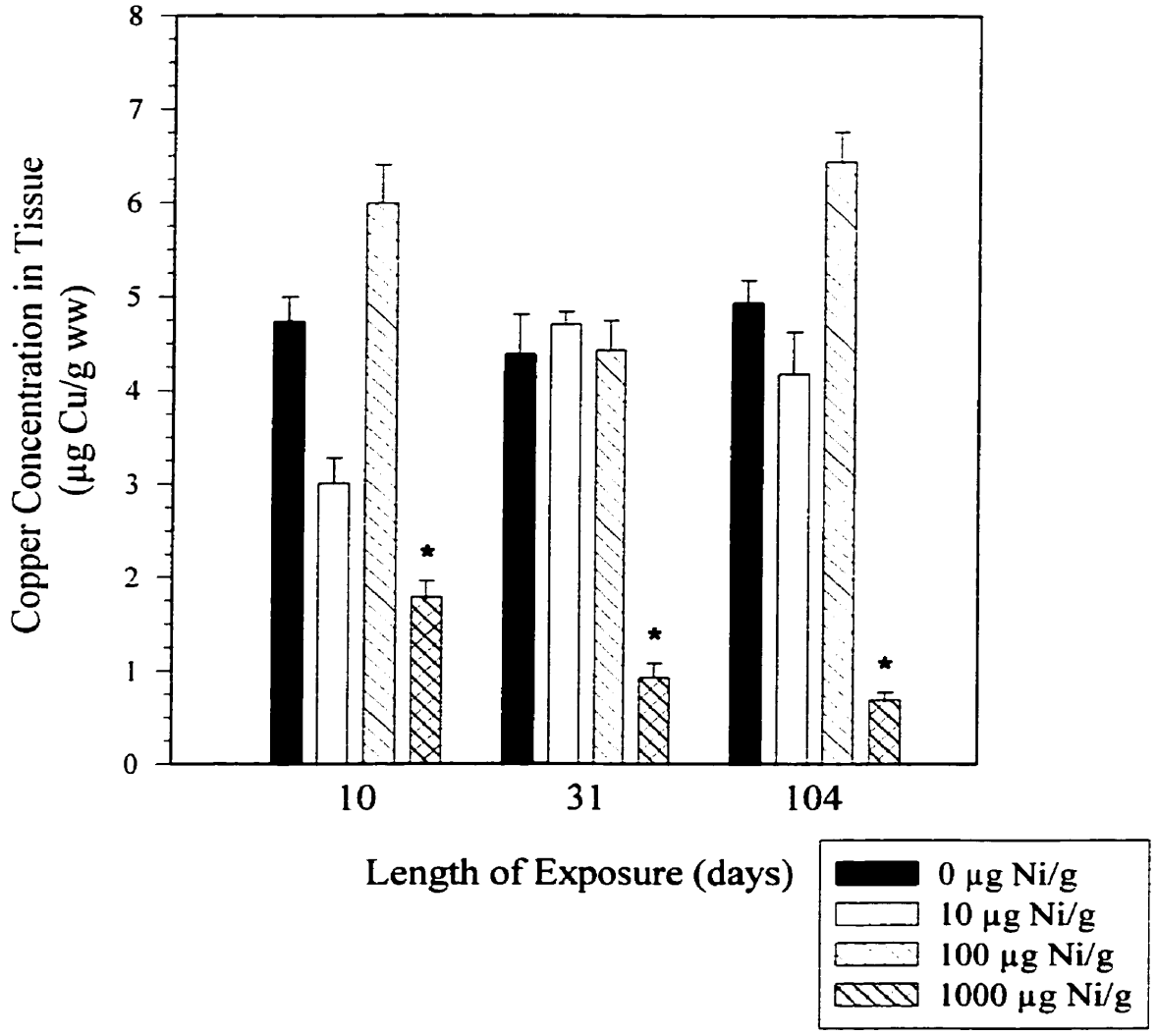


Figure 5
c) Kidney

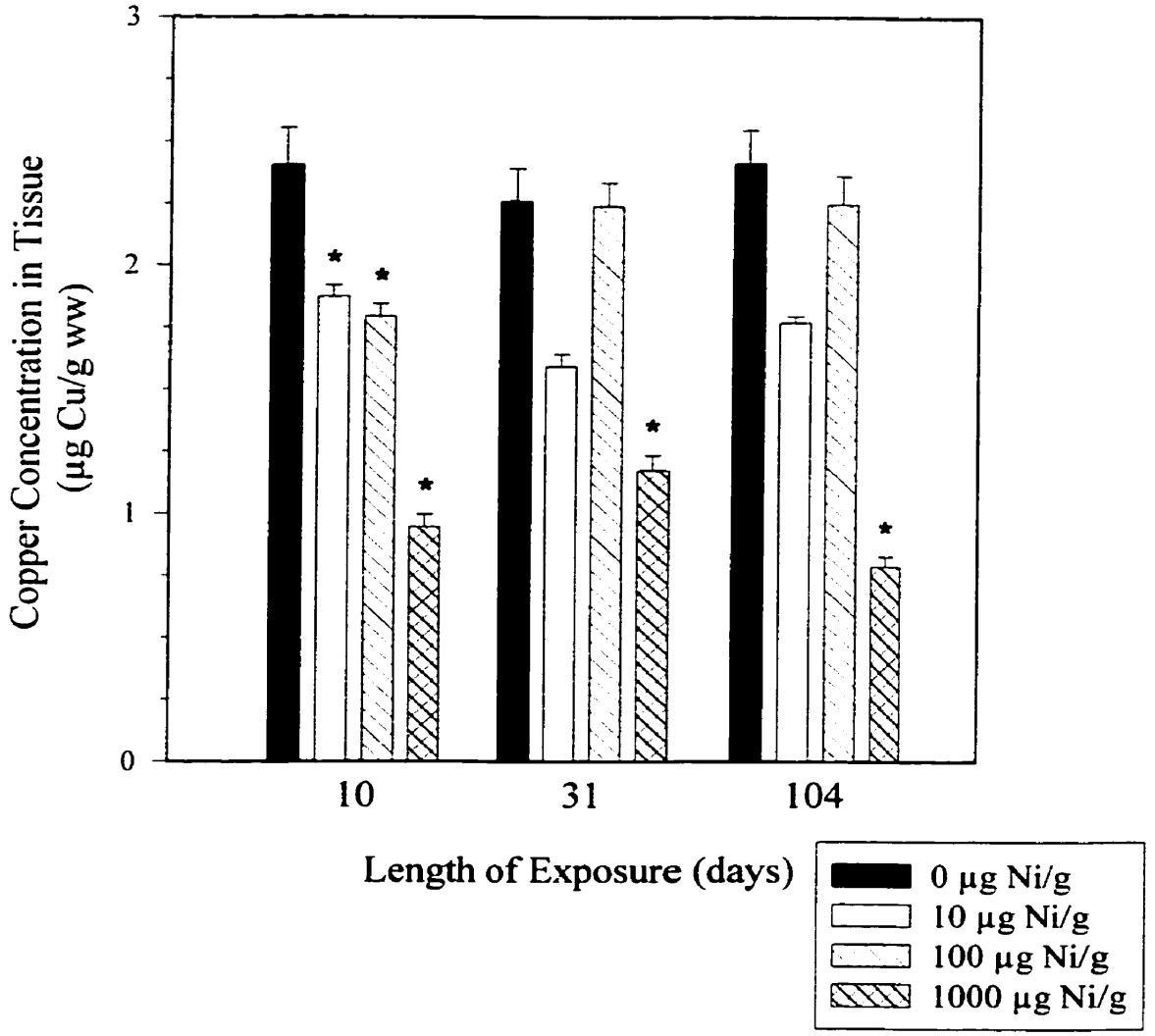


Figure 5
d) Liver

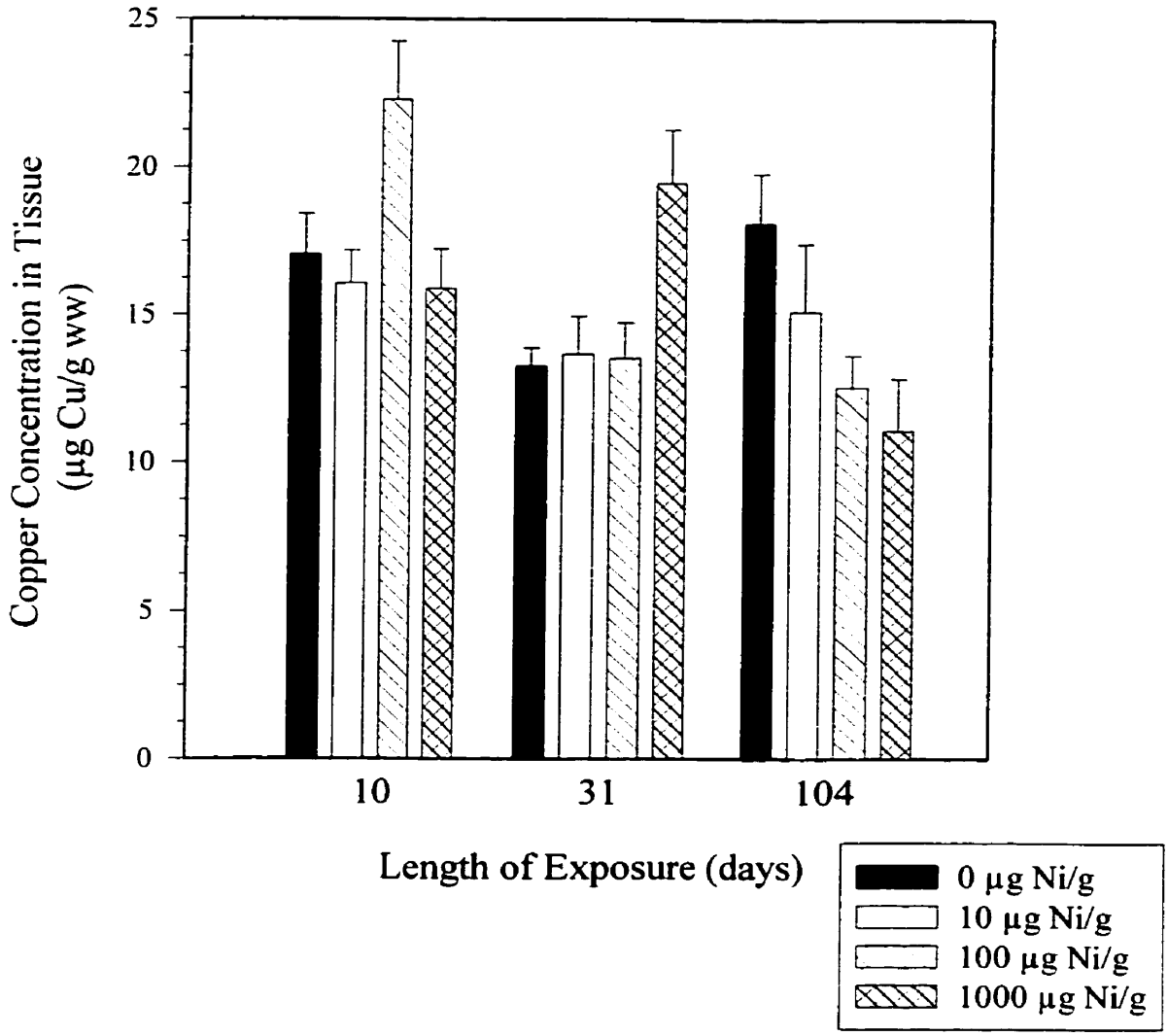
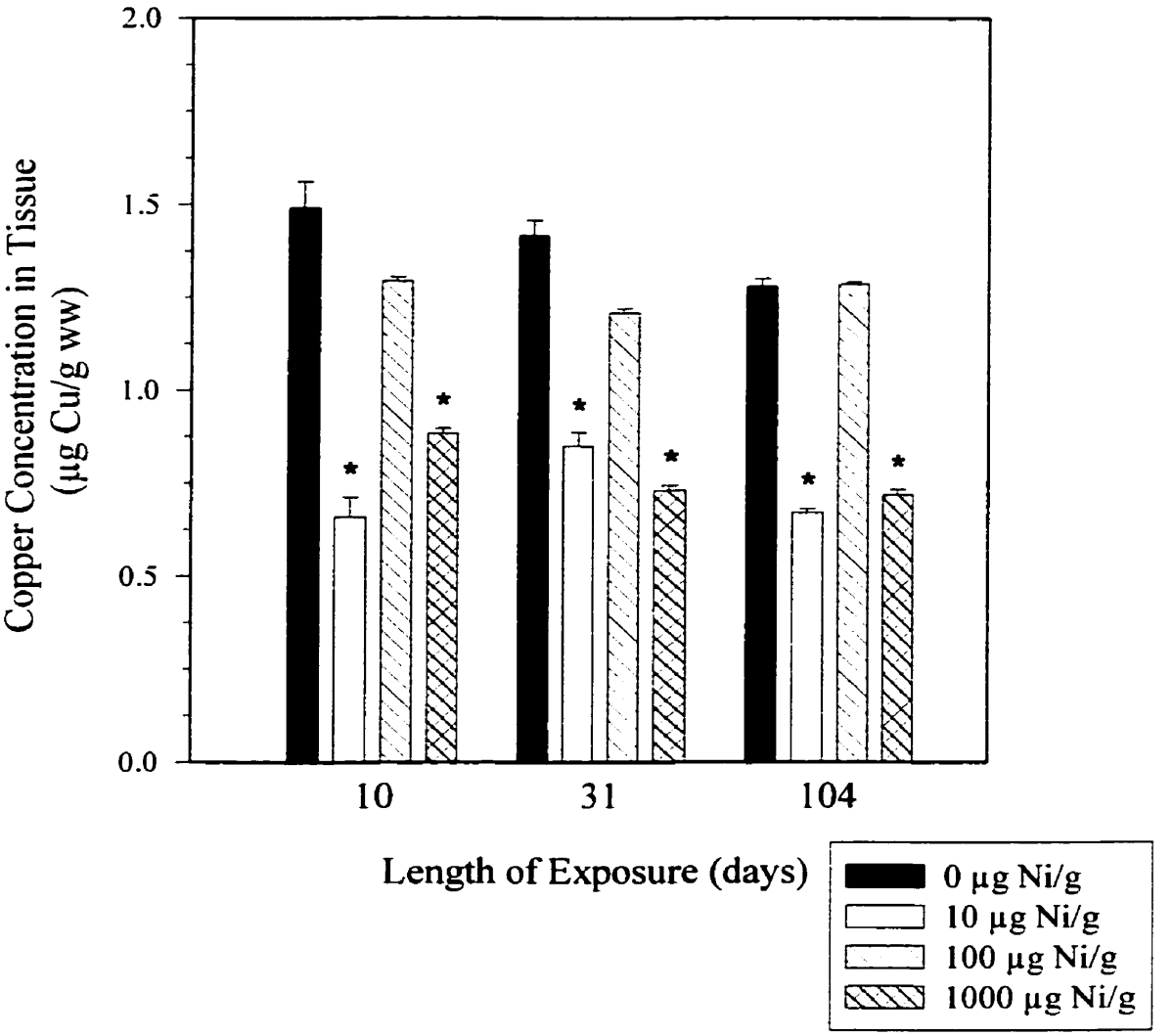


Figure 5
e) Pyloric Caeca



Chapter Three

Toxicology of dietary nickel

in lake whitefish (*Coregonus clupeaformis*).

1) ABSTRACT

The sublethal toxicity associated with exposure of adult lake whitefish (*Coregonus clupeaformis*) to diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days was assessed through the measurement of responses, through a range of levels of biological organization. Molecular and tissue level hematological parameters, including concentrations of glucose and hemoglobin and hematocrit, were not different in control and treated fish. Organ and whole organism parameters, including LSI, growth, and condition factor, were also unaffected. Histopathological lesions in kidney and liver proved to be the most sensitive and reliable indicators of Ni exposure. In liver of treated fish, areas of focal necrosis and altered bile ducts were observed. Histological alterations were observed throughout the posterior kidneys, in glomeruli, tubules, collecting ducts, and hematopoietic tissue, of fish fed medium and high dose diets. Renal alterations observed in fish fed medium and high dose diets included necrotic epithelial cells in tubules and collecting ducts, disintegrating tubules, glomerular alterations, debris in lumen of tubules, collecting ducts, and hematopoietic tissue, depletion of hematopoietic tissue, increased presence of pigmented macrophages and altered staining. Of the 1st and 2nd segments of the posterior and distal tubule cross-sections evaluated, the most frequent alterations were observed in distal tubules. In whitefish kidneys, the frequency (%) of altered distal tubules and fields of views with alterations increased with the dose and duration of exposure. At the molecular level, significant increases in MT concentrations were observed in intestine of whitefish fed the high dose diet on day 10, but these increases were not sustained. Significant increases in lipid peroxide concentrations were also observed in plasma of whitefish fed the high dose diet on day 31, but were not

observed on day 104. These molecular level responses may be important indicators of Ni exposure in fish, but require further evaluation. The assessment of renal and hepatic histopathology and tissue residues (discussed in Chapter 2) is recommended for use in field bio-monitoring programs, to evaluate exposure of natural populations of fish to Ni.

2) INTRODUCTION

The sublethal toxicity of Ni to freshwater fish has been investigated in a number of laboratory-based aqueous exposures (Department of Environment, 1971; Agrawal *et al.*, 1979; Hughes *et al.*, 1979; Gill and Pant, 1981; Chaudhry and Nath, 1984; Nath and Kumar, 1989; Nath and Kumar 1990; Ghazaly, 1992; Alkahem, 1994; Jha *et al.*, 1994; Jha and Jha, 1994; Pyle, 1999). Waterborne exposures are conducted to provide data relevant to natural populations of freshwater fish residing in systems where Ni concentrations are elevated in the water. However, in most Ni-contaminated systems, concentrations of Ni are highest in sediments (Hutchinson *et al.*, 1976; Dallinger and Kautzky, 1985; Mastala *et al.*, 1992; Tariq *et al.*, 1993; Moiseenko *et al.*, 1995; Klavins *et al.*, 1998b; Nriagru *et al.*, 1998). As a result, benthic-feeding fish are exposed to Ni, predominantly, through the ingestion of contaminated food items and sediments (Dallinger and Kautzky, 1985; Dallinger *et al.*, 1987; Handy, 1996). Research investigating the exposure of fish to dietary Ni is needed to provide data most relevant to chronic exposures that are occurring in the environment.

To address these research needs, first, a preliminary experiment was conducted investigating the sublethal toxicity and uptake of Ni in adult lake whitefish and lake trout (*Salvelinus namaycush*) fed diets containing 0, 1000, and 10000 μg Ni/g for a short

duration (Chapter 1). The results of this study demonstrated that both species of fish could readily absorb and accumulate Ni and that lake whitefish were more susceptible to Ni-induced cellular and subcellular injuries.

To better understand the accumulation and distribution of Ni in lake whitefish exposed to dietary Ni a longer-term experiment was conducted. Lake whitefish were fed 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days. The sublethal toxicity associated with these exposures was assessed through the measurement of responses at various levels of biological organization and is presented in this chapter. Responses were evaluated at the molecular (metallothionein induction and lipid peroxide production, hematological parameters (glucose and hemoglobin), cellular and tissue (histopathological and hematological parameters (hematocrit)), and organ and whole organism (LSI, condition factor, and growth) levels. The results from this research can be used to identify toxic effects of dietary Ni in lake whitefish and to determine the most sensitive and reliable indicators of Ni exposure.

3) MATERIALS AND METHODS

Information on fish, tanks, water quality, photoperiod, and diets is provided in Chapter 2.

3.1) Experimental Design and Procedures:

Lake whitefish were exposed to 0, 10, 100, and 1000 μg/g of dietary Ni for 10, 31, and 104 days. Lake whitefish were presented with a food ration equal to 0.5% total body weight per tank every Monday, Wednesday and Friday. Each treatment group,

representing one of twelve combinations of dose and duration of exposure, had a sample size of 6.

One individual from the treatment group fed 100 μg Ni/g for 104 days was sampled prior to day 104, due to feed refusal. Analyses were not conducted on this fish.

3.2) Sampling:

On days 10, 31, and 104, fish from one tank representing each dose group were sampled. After fish were anesthetized (as described in materials and methods section in Chapter 2), blood was removed from the caudal artery and vein, using a 20-gauge needle and 3 cc syringe pre-treated with ammonium heparin. A drop of whole blood was transferred to a blood glucose electrode and glucose concentrations were measured using a blood glucose sensor (Medisense, Inc., UK). Hematocrit content in the whole blood was determined by transferring a small amount of whole blood to a capillary tube, sealing the tube, centrifuging for 5 min at 8000 rpm using a Damon/IEC Division Clinical Centrifuge (Needham, MA), and measuring hematocrit using a Damon/IEC Division microcapillary hematocrit reader (Needham, MA). A 20 μl sample of blood was transferred to a tube containing Drabkin's solution for hemoglobin measurements. The remaining quantity of the whole blood sample was transferred into a vacutainer, pre-treated with EDTA, and centrifuged for 3 minutes at 10000 rpm. The resultant plasma was pipetted into labeled microcentrifuge tubes and frozen at –90 °C for lipid peroxide analyses.

Tissues were dissected and prepared for analyses using the methods described in Cooley and Klaverkamp (2000). Gill, intestine, kidney, liver, stomach, and pyloric caeca were stored at –90 °C for biochemical analyses.

3.3) Toxicological Analyses

3.31 Molecular Responses

i) Metallothionein Analysis

MT concentrations were measured in kidney, liver, intestine, pyloric caeca, and gill tissue, using the mercury displacement assay described in Klaverkamp *et al.* (2000b). A subset of stomach samples was analyzed to determine MT concentrations. However, MT concentrations in stomach were less than analytical detection limits.

ii) Lipid Peroxide Analysis

Lipid peroxides in plasma were analyzed using the K-Assay LPO-CC kit (Kamiya Biomedical Company, Seattle, WA).

iii) Hematology

Hemoglobin concentrations were measured using the Sigma Diagnostics Total Hemoglobin Procedure No. 525 (St. Louis, MO). Methods for determining concentrations of glucose and hematocrit are described above. Hematocrit is a tissue

level parameter; however, in subsequent sections it will be included with molecular level hematological parameters.

3.32 Cellular Responses

Histopathology

After 48 hours, posterior kidney and liver samples immersed in Bouin's fixative were washed in 3 changes of 70% ethanol over 3 days and stored in 70% ethanol. Tissues were processed using an ethanol/butanol series in an IL MVP automated tissue processor. Tissues were embedded in paraffin (Tissue Prep II). Paraffin blocks of liver and posterior kidney were sectioned into 7 μm thick sections. Several tissue sections were mounted on glass slides and stained using Harris' hematoxylin and eosin series (Edwards, 1967). All chemicals used to fix, process, and stain tissues were obtained from Fisher Scientific (Fair Lawn, NJ). Qualitative and semi-quantitative assessments of histological alterations were conducted using a Zeiss Photomicroscope III light microscope. To ensure consistency, qualitative and semi-quantitative assessments of each kidney were conducted on the same tissue section.

Histological alterations in kidney of whitefish were assessed qualitatively. The centermost tissue section on a slide was scanned in its entirety, to assess histological alterations. If folding or excessive tearing was evident in the centermost section an alternate section was used. Sections were examined for alterations in tubules, collecting ducts, blood vessels, hematopoietic tissue, and glomeruli. A description of the alterations characteristic of each dose group is provided in the results section.

Two semi-quantitative approaches were used to evaluate histological alterations in posterior kidneys. The first approach assessed alterations in cross sections of the 1st and 2nd segments of proximal tubules (P1 and P2, respectively), distal tubules (D), and glomeruli. First, the centermost coordinates of each tissue section were determined using a vernier scale and the section was divided into four quadrants. Next, each of the four quadrants was scanned, starting at the center of the section. The first 3 P1, P2, D tubules, and glomeruli cross-sections encountered in each quadrant were examined for histological alterations. Tubules were examined for the following alterations: epithelial cell alterations, including pyknotic nuclei, presence of vacuoles and deposits in cytoplasm, ruptured cells, brush border damage, and the presence of cellular debris in lumina. The degrees of alteration observed in tubule cross-sections were categorized as slightly altered (if 1 epithelial cell was altered), moderately altered (if >1 epithelial cell – 50 % of all epithelial cells in the tubule were altered), and severely altered (if > 50 % of epithelial cells in the tubule were altered). Severely altered tubule cross-sections that could not be identified were excluded. The average % of altered tubules (for each degree of alteration), % of altered P1, P2, and D tubules (total % and % for each degree of alteration), and % of altered glomeruli were determined for each treatment group.

To provide an additional assessment of the extent of renal alteration, a second approach was used to assess how frequently alterations in tubules and collecting ducts were observed. A rectangular field of view, measuring 355 x 230 μm, was used to scan the entire tissue section. The percentage of fields of view with 1 or more altered tubule(s) and/or collecting duct(s) was determined for each fish. The number of fields of view evaluated in each tissue section ranged from 87-330. Fields of view lacking tubules

or collecting ducts were not assessed. The average % of altered fields of views was determined for each treatment group.

Histological alterations in liver of whitefish were assessed qualitatively, using methods described above. Sections were examined for alterations in hepatocyte appearance, bile ducts, staining, and cord structure.

Photomicrographs representing typical control and treated kidneys and livers were taken using a Zeiss Photomicroscope III and a Kodak DC-120 digital camera, using the Kodak Microscopy Documentation System (MDS120), edited using Adobe Photodeluxe 2.0, and printed.

3.33 Organ and Whole Organism Measurements

Measurements of liver somatic indices (LSI), growth, % change in length, and condition factor were made using conventional approaches. LSI were calculated using the formula: (liver weight x 100)/(final body weight-liver weight). Growth (percent change in weight) was calculated using the formula: ((final body weight/initial body weight)-1) x 100. Percent change in length was calculated using a similar calculation. Condition factors were calculated using the formula: (final body weight/final fork length^3) x 100.

3.4) Statistics

All statistical analyses described below were performed using SPSS v. 9.0 software, using methods described in Neter *et al.* (1990), SPSS (1999), and Stevens (1992).

3.41 Toxicological Parameters

One-way ANOVAs and appropriate multiple comparison techniques were used to test for differences between fish fed control and Ni-contaminated diets. First, a two-way ANOVA was conducted to test for dose and duration effects and interaction. Because significant dose effects and/or interactions were observed, separate one-way ANOVAs were conducted for each duration (10, 31, or 104 days). First, the assumptions of the one-way ANOVA were tested and transformations were applied as necessary. When assumptions of normality were met data were analyzed using a one-way ANOVA, with $p< 0.05$. If the results from the one-way ANOVA were significant, significant differences ($p<0.05$) between control and treated groups were identified using an appropriate multiple comparison technique: a) if assumptions of equal variance were met Dunnett's test was used, and b) if assumptions of equal variance were not met Dunnett's T3 test was used. When the assumptions of normality were not met, a non-parametric Kruskal-Wallis test was used to test for differences between control and treated groups. If results from that test were significant, data were ranked, analyzed using a one-way ANOVA and tested using Dunnett's test.

3.42 Dose and Duration Dependency

Linear regression was used to assess whether concentrations of MT and lipid peroxides, and frequency (%) of histological alterations were explained by the dose and/or duration of exposure. The significance of a linear relationship between the dependent variable (toxicological parameter) and the independent variables (dose and/or duration) was tested with $p<0.05$. Prior to conducting the regression, the assumptions of

the model were checked and transformations were applied as necessary. The actual concentrations of Ni in the diet were substituted for the nominal concentrations of Ni in the diets, so if necessary, transformations could be applied. The correlations between MT concentrations and dose or Ni concentrations in intestine and the correlations between lipid peroxide concentrations and dose were tested separately for each sampling day, as these parameters were significantly different from controls only on 1 of the 3 sampling days. Histological parameters were tested for dose and duration dependency. The combined R^2 values obtained from the regression model represent the ability of the independent variables (dose and duration) to predict the dependent variable (histological parameter); whereas, the partial R^2 values represent the predictive power of the independent variable, when the linear effect of the other independent variable in the model is removed.

4) RESULTS

4.1) Molecular Responses

i) Metallothionein

Concentrations of metallothionein (MT) in kidney, gill, intestine, liver and pyloric caeca of lake whitefish are presented in Table 1. On day 10, significantly higher concentrations of MT were observed in intestines of lake whitefish fed the high dose diet for 10 days. These increases were correlated with dose ($R^2 = 0.57$) and the concentrations of Ni observed in intestine ($R^2 = 0.43$) on this sampling day (Table 2). MT concentrations in intestines of treated fish were not elevated, relative to control fish, on days 31 and 104; although, on day 31 a weak, but significant, correlation was

observed between MT concentrations and concentrations of Ni in intestine ($R^2 = 0.25$) (Table 2). In pyloric caeca concentrations of MT were variable. On day 31, significantly higher MT concentrations were observed in pyloric caeca of whitefish fed the low dose diet. Conversely, significantly lower MT concentrations were observed in pyloric caeca of whitefish fed the high dose diet for 104 days. Concentrations of MT in kidney, gill, and liver of treated fish were not significantly different from concentrations observed in control fish.

Cu and Zn concentrations were also measured in kidney, gill, liver, pyloric caeca, and intestine of lake whitefish to determine if any alterations in Cu and Zn concentrations were associated with altered MT concentrations (data presented in Chapter 2). Exposure to Ni was also observed to alter the concentrations of Cu and Zn in tissues of lake whitefish. However, Cu and Zn concentrations in the tissues analyzed were variable and did not follow a common pattern or trend.

ii) Lipid Peroxides

Concentrations of lipid peroxides in plasma of lake whitefish are presented in Figure 1. On day 31, significant increases in lipid peroxide concentrations were observed in whitefish fed the high dose diet. These increases were weakly correlated with dose ($R^2 = 0.29$) (Table 2). Concentrations of lipid peroxides did not differ between control and treated fish on days 10 and 104.

iii) Hematological Parameters

Concentrations of hemoglobin and glucose and hematocrit were unaffected by exposure to Ni (Table 3).

4.2) Cellular Responses

Histopathology

No gross renal or hepatic pathologies were observed in control and treated lake whitefish.

Alterations were infrequent and minor in the tubules, hematopoietic tissue, and glomeruli of kidneys of control lake whitefish (Figures 2a, c, and e). Slight alterations observed were characterized by the presence of necrotic epithelial cells in P1, P2, and/or D tubules, usually with only 1 necrotic cell observed per tubule cross-section, and accumulation of debris in the lumen of P1, P2, D, and collecting tubules.

Slight alterations were also observed in kidneys of whitefish fed the low dose diet. These alterations were similar to those described for control fish, including the appearance of necrotic epithelial cells and accumulation of debris in lumen of collecting ducts.

Increased alterations were observed in kidneys of whitefish fed the medium dose diet. These alterations were generally characterized by the presence of swollen, ruptured, and necrotic epithelial cells in P1, P2, D tubules, with 1 or > 1 necrotic cell observed per tubule cross-section, necrotic epithelial cells in collecting ducts and tubules, desquamated epithelial cells, debris in Bowman's space of glomeruli, debris in the lumen of P1, P2, D, and collecting tubules, collecting ducts, and hematopoietic tissue, and altered staining.

Kidneys of whitefish fed the high dose diet exhibited the most noticeable and frequent alterations (Figure 2b). These alterations were generally characterized by increases in the presence of swollen, ruptured, and necrotic epithelial cells in P1, P2, D

tubules, and collecting tubules and ducts, with 1 to greater than half of the epithelial cells in a tubule exhibiting alterations, disintegrating tubules, appearance of debris in Bowman's space of glomeruli, debris in the lumen of P1, P2, D, and collecting tubules, collecting ducts, and hematopoietic tissue, depletion of hematopoietic tissue, increased presence of pigmented macrophages and altered staining.

Whitefish fed the high dose diet exhibited the highest mean percentages of slightly and moderately altered tubule cross-sections on days 31 and 104 (Table 4). Whitefish fed the medium dose diet exhibited increased, albeit lower, frequencies (%) of slight and moderate alterations on days 31 and 104, and days 10 and 31, respectively. Tubules of whitefish fed low dose and control diets were not significantly altered.

Significant increases in the number of glomeruli with debris-filled Bowman's space were observed in kidneys of whitefish fed the high dose diet for 104 days, with an average of 51% of the glomeruli being affected (Figure 2d, Figure 3a). Higher percentages of glomerular alterations were observed in lake whitefish fed high dose diets on days 10 and 31 but were not statistically significant, due to the high degree of variability between fish.

Of the P1, P2, and D tubule cross-sections evaluated, the most frequent alterations were observed in D tubules (Figures 3b-d). The most commonly observed alterations in D tubules were epithelial cell alterations, including the presence of pyknotic and shrunken nuclei and vacuoles in cytoplasm, swollen and ruptured epithelial cells, desquamated epithelial cells, altered staining, and the presence of cellular debris in lumen (Figures 2f and g). The frequency of altered D tubules was correlated with the dose and duration of exposure ($R^2 = 0.48$) (Table 2). Although, it is apparent, from the partial R^2

values, that dose (R^2=0.48) is a much stronger predictor of the percentage of altered D tubule cross sections than duration (R^2=3.0E-03). Increased mean percentages, ranging from 33%-71%, of altered D tubules were observed in whitefish fed medium and high dose diets, (Figure 4b). P2 tubules exhibited a lower frequency of alteration than D tubules. Higher mean percentages of altered P2 tubules, ranging from 43-47%, however, were observed in whitefish fed the medium and high dose diets for 31 and 104 days (Figure 3c). P1 tubules were the least affected by Ni exposure. Increased alterations in P1 tubules were observed only in whitefish fed the high dose diet for 104 days (Figure 3d).

Fish fed Ni-contaminated diets exhibited a higher frequency of slight and moderate alterations in D, P1, and P2 tubules. More frequent slight alterations were observed in D tubules of fish fed the diets containing 100 μg Ni/g for 10 and 104 days and 1000 μg Ni/g for all durations and P1 tubules of fish fed the diets containing 1000 μg Ni/g for 104 days (Table 5). More frequent moderate alterations were observed in distal tubules of fish fed medium and high dose diets for all durations and P2 tubules of fish fed the medium dose diet for 31 days and the high dose diet for 10 days. The number of severe alterations observed in P1, P2, and D tubules was not significantly elevated in treated fish (Table 5). However, because many of the severely altered tubule segments encountered could not be identified (Figure 2h), the numbers of severely altered tubules were underestimated.

Significant increases in the percentage of fields of view with alterations were observed in kidneys of whitefish fed medium and high dose diets for all durations (Figure 4). The percentages of altered fields of view were strongly correlated with the dose and

duration of exposure ($R^2 = 0.75$) (Table 2). Although both variables were incorporated into the model ($p<0.05$), it is evident from the partial R^2 values that dose ($R^2=0.75$) is a much stronger predictor of percentage of altered fields of view than duration ($R^2=4.0E-03$).

A qualitative assessment of liver pathology demonstrated that whitefish fed Ni-contaminated diets exhibited a greater degree of cellular alteration. The livers of control lake whitefish typically consist of cords of polygonal hepatocytes, which are separated by sinusoids (Figures 5a and c). Alterations in livers of control fish were infrequent and minor. In some control fish small areas of focal necrosis (described below) located adjacent to bile ducts and veins were observed, which were attributed to normal cell turnover. In lake whitefish fed low and medium dose diets, areas of normal parenchyma were interspersed with more frequent and/or larger areas of necrotic tissue. These areas of focal necrosis were most commonly characterized by swollen and ruptured hepatocytes, altered positioning of nuclei in hepatocytes, hepatocytes with pyknotic nuclei and granular cytoplasm, the presence of debris in sinusoids, altered cellular architecture, cellular dissolution, inflammation characterized by lymphocytic infiltration, and altered staining. These necrotic areas were located throughout the parenchyma, including but not limited to, areas bordering bile ducts and veins. The livers of lake whitefish fed the high dose diet were most noticeably altered. Areas of focal necrosis were observed throughout the tissue section, surrounded by areas of normal parenchyma (Figures 5b and d). Fatty degeneration was suspected in some individuals because of the presence of round, sharp-edged vacuoles; however, this could not be confirmed by alternate staining methods, as lipids were removed during tissue processing. The

alterations observed in livers of fish fed Ni-contaminated diets appeared to increase in severity and frequency with both dose and duration. Bile ducts were slightly altered in fish fed medium and high dose diets. Alterations observed include necrotic epithelial cells and lumenal debris.

4.3) Organ and Whole Organism Parameters

Liver and gonad somatic indices, growth (% change in weight), and % change in fork length, and condition factors were unaffected by exposure to Ni (Table 6). Sex ratios are also provided in Table 6. Growth results are also presented and discussed in Chapter 2.

5) DISCUSSION

Molecular responses of aquatic organisms to contaminants usually represent the

valuable monitoring tool for the assessment of metal exposure in natural populations of fish (Petering *et al.*, 1990; Roesijadi, 1992; Klaverkamp *et al.*, 1997; Klaverkamp *et al.*, 2000a).

Nearly all that is known about the ability of Ni to induce MT production has been discovered through mammalian studies. However, because of contradictory results reported in these studies, the status of Ni as an MT inducer is open to question. For example, in several studies it has been reported that Ni exposure has resulted in increased concentrations of hepatic and renal MT in laboratory animals (Sunderman and Fraser, 1983;Khandelwal *et al.*, 1989; Fleet *et al.*, 1990; Arizono *et al.*, 1991), whereas, in studies by Nation *et al.* (1985) and Srivastava *et al.* (1995) Ni did not alter renal and hepatic MT concentrations. The contrasting results observed in mammalian studies may be linked to differences in experimental protocol, including route of exposure, form of Ni used, analytical method used, differences between experimental animals used (age, sex, size, species, and nutritional status), and effects of other environmental and physiological factors which can induce MT production, including reproductive status, stress, and inflammation (Sunderman and Fraser, 1983; Robertson *et al.*, 1988; Fleet *et al.*, 1990; Hildalgo *et al.*, 1990; Petering *et al.*, 1990; Hylland *et al.*, 1994; McNamara and Buckley, 1994; Srivastava *et al.*, 1995).

MT concentrations in intestines of whitefish fed the high dose diet were 56% higher than concentrations observed in intestines of control fish on day 10. The increases that occurred in intestinal MT, on day 10, were correlated with both the doses of Ni administered in the diet and the concentrations of Ni observed in intestine. It was on this sampling day that the highest mean concentration of Ni (70 μg Ni/g) was observed in the

intestine of whitefish fed the high dose diet (as described in results section of Chapter 2). On days 31 and 104, MT concentrations in intestine were not different between control and treated fish and lower mean concentrations of Ni, of 25 and 17 μg Ni/g, respectively, were observed in intestines of whitefish fed the high dose diet. On day 31, a correlation between concentrations of MT and concentrations of Ni in intestine was observed. These decreases in Ni concentrations observed in intestine, after day 10, were likely due to the regulation of Ni, through increased excretion and controlled uptake. Consequently, after day 10, due to regulatory processes the concentrations of Ni in the intestine may have not been high enough to sustain MT production. Increased MT production observed in intestine on day 10 was not related to changes in intestinal Cu or Zn concentrations.

The only studies that evaluate the relationship between Ni exposure and MT induction in fish include this study, a preliminary study (Chapter 1), and a waterborne exposure conducted by Pyle (1999). The findings of this study and the preliminary study are similar. In the preliminary experiment, no significant differences in renal and hepatic MT were observed between control and treated lake whitefish exposed to 1000 μg Ni/g for 18 days (Chapter 1). Increases in renal MT were observed, however, in lake trout fed 1000 μg Ni/g, indicating that MT induction differs between fish species. Significant increases were observed in renal and hepatic MT in whitefish and trout fed 10000 μg Ni/g for 19 days; however, increases observed in lake trout may have been attributed to starvation. Comparisons cannot be made regarding MT concentrations in intestine, because intestinal MT concentrations were not measured in the preliminary study.

Results from this dietary exposure and a waterborne exposure conducted by Pyle (1999) provide evidence that Ni-induced MT synthesis in fish is related to the route of

exposure. When fish are exposed to metals via the diet, the intestine is the primary site of metal uptake (Luckey and Vengopal, 1977). Consequently, if fish exposed to dietary Ni can bind metals in the intestine, they may be afforded greater protection against metal toxicity. When fish are exposed to metals in water, the gills are the primary site of metal uptake. In the waterborne exposure conducted by Pyle (1999), juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to 6.6 mg Ni/L for 7 days showed elevated concentrations of MT in gills relative to control fish. The results of these two studies indicate that MT is binding Ni at the initial sites of uptake, as a protective mechanism, and that intestine and gill should be analyzed in field and laboratory studies assessing Ni-induced MT production in fish.

A second molecular response examined was lipid peroxide production. Lipid peroxidation is recognized as an important molecular mechanism for cellular injury (Muriel, 1997). Lipid peroxides are formed from a process, in which free radicals react with polyunsaturated fatty acids associated with lipids of cell membranes, endoplasmic reticulum, and mitochondria. The end result of this self-propagating process is the disruption of essential cellular components including cell membranes (Muriel, 1997).

Numerous mammalian studies have shown that lipid peroxidation can be induced by Ni (Sunderman *et al.*, 1989; Knight and Voorhees, 1990; Misra *et al.*, 1990; Sole *et al.*, 1990; Srivastava *et al.*, 1990; Misra *et al.*, 1991; Rodriguez *et al.*, 1991; Iscan *et al.*, 1992; Stinson *et al.*, 1992; Novelli *et al.*, 1995; Srivastava *et al.*, 1995; Chen *et al.*, 1998; Chakrabarti and Bai, 1999). Ni has also been observed to lower concentrations of antioxidant enzymes, like glutathione, in mammals, resulting in a diminished cellular defense system and increased cellular damage (Misra *et al.*, 1990; Srivastava *et al.*, 1990;

Misra *et al.*, 1991;Rodriguez *et al.*, 1991; Iscan *et al.*, 1992). The exact mechanisms involved in the Ni-induced generation of lipid peroxides and the resultant cellular damage have not been identified and have been subject to much speculation. Mammalian studies also indicate that lipid peroxides may induce and be scavenged by MT (Hildalgo *et al.*, 1988; Fleet *et al.*, 1990; Srivastava *et al.*, 1995) however, no correlation between these two responses was observed in this study.

The cellular injuries observed in kidneys and livers of lake whitefish fed the high dose diet were accompanied by significant increases in lipid peroxide concentrations on day 31. The concentrations of plasma lipid peroxides in lake whitefish fed the high dose diet were 57% higher than concentrations observed in control fish on day 31. These results are similar to the findings of Cooley *et al.* (2000), who observed an association between lipid peroxide production and histological alterations observed in the kidney and liver of lake whitefish exposed to dietary U. However, in this experiment, on day 104, despite the continued prevalence of histological lesions, lipid peroxide concentrations did not differ between control and treated fish. Perhaps, a stronger association might have been seen between lipid peroxide production and cellular injury in lake whitefish, if lipid peroxides were measured directly in kidney and liver homogenate instead of in plasma. However, it is also possible that these two responses are not directly related to each other, as some researchers report that Ni-induced damage to DNA and other cellular components occurs prior to and independently of lipid peroxidation (Stinson *et al.*, 1992; Chakrabarti and Bai, 1999). More research is needed to understand the relationship between cellular and sub-cellular injuries and lipid peroxidation in fish.

The only studies that evaluated the relationships between Ni exposure and lipid peroxidation in fish include this study and the preliminary study (Chapter 1). When comparing results on short-term exposures, the findings of both these studies were similar. In this study, lipid peroxide concentrations were not elevated in plasma of whitefish fed 1000 μg 4 times, during a 10-day period. Similarly, in the preliminary experiment, lipid peroxides concentrations in plasma did not differ between control and treated lake whitefish fed 1000 μg Ni/g 7 times, during a 18-day period. Increases in lipid peroxides were observed in lake trout fed 10000 μg Ni/g, but may have been due to starvation.

Other molecular level responses investigated were the concentrations of glucose and hemoglobin in the blood. Hematocrit, a tissue level response, was also measured. All of these hematological parameters were unaffected by Ni exposure. Generally, the use of hematological responses as indicators of exposure of fish to contaminants is not recommended, as transient alterations of these parameters can occur due to stress related to capture, anesthesia, and bleeding, and other environmental and physiological events (Heath, 1995). A small number of studies have been conducted investigating hematological alterations in fish exposed to waterborne (Agrawal *et al.*, 1979; Gill and Pant, 1981; Chaudhry and Nath, 1984; Ghazaly, 1992; Alkahem, 1994; Jha and Jha, 1994) and dietary Ni (Chapter 1) in the laboratory. Because many of the results reported in these studies are contradictory, no conclusions can be made regarding the effects on Ni on these hematological parameters.

Histopathological responses can provide valuable information regarding the sublethal and chronic effects associated with contaminant exposure in fish (Hinton *et al.*,

1992; Meyers and Hendricks, 1995; Schwaiger *et al.*, 1997; Bernet *et al.*, 1999). A sparse amount of research has been conducted investigating the histopathology associated with waterborne (Department of Environment, 1971; Hughes *et al.*, 1979; Nath and Kumar, 1989; Nath and Kumar 1990; Jha *et al.*, 1994) and dietary exposures (Chapter 1) of fish to Ni.

The kidney plays a principal role in the accumulation, detoxification, and excretion of Ni and is considered to be a target organ for Ni toxicity (Sunderman, 1977; WHO, 1991; Eisler, 1998). Histological alterations observed in kidneys of whitefish fed Ni-contaminated diets are similar to those reported in the preliminary study (Chapter 1) and in mammalian studies (Gitlitz *et al.*, 1975; Pereira *et al*, 1997; USPHS, 1997). In the preliminary study, similar renal alterations, including the presence of necrotic epithelial cells in proximal tubules and accumulation of debris in the lumen of tubules, were observed in the kidneys of whitefish fed 1000 µg Ni/g and 10000 µg Ni/g for 18 days, but not in lake trout. In mammalian studies, rats and mice exposed to Ni via the diet and through injection exhibited renal alterations, characterized by tubules with degenerating epithelial cells, tubular necrosis, and glomerular abnormalities (Gitlitz *et al.*, 1975; Pereira *et al*, 1997; USPHS, 1997).

Toxic materials that are transported via the blood, like Ni, are common causes of nephrosis, particularly in the renal epithelium (Meyers and Hendricks, 1985). In this exposure, histological alterations were observed throughout the kidneys, in glomerulus, tubules, and collecting ducts, of fish fed medium and high dose diets. Alterations were most prominent in the posterior end of the nephron, in distal tubules and collecting ducts. Because excretory processes occur in the posterior segments of the nephron (Takashima

and Hibiya, 1995), the increased alterations observed are likely attributed to the routing of Ni to these segments for excretion. In glomeruli of treated fish, acellular debris was observed in Bowman's space of glomeruli. This debris may have been due to overproduction of acellular mesangial material, which is produced as a non-specific toxicological response (Burkitt *et al.*, 1996). Histopathological parameters measured in kidney represent the only parameters evaluated in this study that exhibited dose and duration dependent responses. The frequency (%) of fields of views with alterations was strongly correlated (R^2=0.75) with dose and duration. This strong linear relationship suggests that the overall extent of alteration observed in kidney can be reliably predicted by the dose and duration of Ni administered.

The presence of altered bile ducts and hepatic lesions in whitefish fed medium and high dose diets suggest that bile excretion may be an important route for Ni excretion in fish. The presence of similar hepatic lesions in fish exposed to waterborne (Department of Environment, 1971; Jha *et al.*, 1994) and dietary Ni (Chapter 1) supports this hypothesis. For example, lake whitefish fed 10000 μg Ni/g for 18 days exhibited minor areas of focal necrosis, similar to those described in this experiment (Chapter 1). In another study, perch exposed to waterborne Ni also exhibited areas of focal necrosis, located adjacent to central veins, hepatocytes with altered nuclear positioning and pyknotic nuclei, ruptured hepatocytes, and loss of cord structure (Jha *et al.*, 1994). Liver degeneration was observed in rainbow trout exposed to waterborne Ni (Department of Environment, 1971). Additional research is needed to assess the importance of liver in Ni detoxification in fish.

Organ and whole organism level responses including liver somatic indices, growth (% change in weight), and fork length, and condition factor were unaffected by Ni exposure. These parameters were not altered in lake whitefish in the preliminary experiment (as described in the results section of Chapter 1). Waterborne exposures of fish to Ni are conducted over a short period of time, usually ranging from a few hours up to a month in duration; consequently, alterations to liver somatic indices, % change in weight and fork length, and condition factor are not reported in these studies. Because of relationships between renal histopathology and the dose and duration of exposure to Ni, exposures longer than 104 days are required to evaluate whether organ and whole-organ responses are affected by renal alterations.

6) CONCLUSIONS AND RECOMMENDATIONS

The results obtained from this research can be used to guide field bio-monitoring efforts to assess Ni toxicity in natural populations of fish. The use of qualitative and semi-quantitative methods to assess histological alterations in kidney and liver of fish is strongly recommended, as these parameters represent the most sensitive and reliable indicators of Ni exposure. Molecular responses, including MT induction in intestine and production of lipid peroxides in plasma, may prove to be important responses to Ni exposure, but require further evaluation.

As this is the first study to investigate the toxicity associated with exposure of fish to dietary Ni, a number of fundamental questions need to be examined in future research efforts. First, research is needed to determine if sublethal toxic effects (i.e.

histopathologies) associated with dietary Ni exposure would be sustained and result in effects at the organ and whole organism levels in fish exposed for a longer duration. Second, the relationships between MT induction, lipid peroxidation, and dietary Ni exposure in fish need to be investigated further. Third, the underlying mechanisms involved in the generation of lipid peroxides and histopathological alterations resulting from Ni exposure should be investigated to determine if these two responses are causally related. Finally, research assessing the mutagenic and carcinogenic effects of dietary Ni exposure in fish is also recommended, as Ni has been shown to cause cancer and mutations in mammals (Eisler, 1998).

Table 1. Metallothionein concentrations in kidney, liver, gill, intestine, and pyloric caeca in lake whitefish fed diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days. Asterisks represent significant differences observed between control and treatment groups for each duration ($p<0.05$).

Duration	Dose	Mean Metallothionein Concentrations (± SE) (μg/g)				
		Kidney	Liver	Gill	Intestine	Pyloric Caeca
10	0 μg Ni/g	15.5 (2.02)	355 (48.0)	12.5 (1.89)	53.8 (6.75)	77.3 (10.5)
	10 μg Ni/g	17.9 (3.59)	339 (77.5)	11.9 (0.513)	34.5 (8.85)	92.6 (16.5)
	100 μg Ni/g	18.3 (2.31)	423 (98.3)	14.5 (1.87)	43.6 (5.05)	71.2 (12.3)
	1000 μg Ni/g	22.1 (2.02)	292 (46.4)	15.0 (1.62)	95.7 (11.5)*	106 (14.7)
31	0 μg Ni/g	21.2 (3.21)	263 (30.8)	15.9 (0.992)	48.0 (5.90)	82.7 (8.83)
	10 μg Ni/g	24.8 (1.75)	265 (40.2)	13.7 (1.59)	42.2 (3.17)	127 (12.7)*
	100 μg Ni/g	19.8 (2.07)	349 (77.1)	13.9 (1.49)	60.7 (14.1)	58.4 (10.1)
	1000 μg Ni/g	28.7 (4.46)	319 (54.6)	19.0 (2.83)	49.6 (10.7)	60.9 (5.73)
104	0 μg Ni/g	20.7 (1.43)	402 (56.0)	20.2 (3.97)	48.1 (9.11)	88.5 (7.53)
	10 μg Ni/g	22.0 (1.87)	263 (83.1)	15.8 (2.59)	49.2 (4.93)	86.7 (11.2)
	100 μg Ni/g	21.9 (3.79)	265 (37.4)	20.2 (4.17)	58.5 (7.09)	55.8 (10.1)
	1000 μg Ni/g	26.6 (4.90)	237 (73.4)	24.4 (6.63)	58.8 (18.4)	53.5 (9.76)*

Table 2. Estimated Regression Models with combined R^2 values for the model and partial R^2 values for each independent variable included in the model.

Toxicological Parameter	Estimated Regression Model	Combined R^2	Partial R^2	
			Dose	Duration
[Metallothionein] (intestine) day 10[a]	[metallothionein] in intestine on day 10 = 43 + 4.8 E-02([Ni] in diet)	0.57	-	-
[Metallothionein] (intestine) day 10[a]	[metallothionein] in intestine on day 10 = 46 + 0.55([Ni] in intestine on day 10)	0.43	-	-
[Metallothionein] (intestine) day 31[a]	[metallothionein] in intestine on day 31 = 46 + 0.41([Ni] in intestine on day 31)	0.25	-	-
[lipid peroxides] day 31[a]	Square root [lipid peroxides] on day 31 = 1.2 + 1.7(square root [Ni] in diet)	0.29	-	-
% altered distal tubules[b]	% of altered distal tubule cross sections = 24 + 4.0E-02([Ni] in diet) + 2.4E-02 (duration)	0.48	0.48	3.0E-03
% altered fields of view[b]	Square root (% of altered fields of view) = 3.6 + 0.11(square root [Ni] in diet) + 1.7E-02(square root duration)	0.75	0.75	4.0E-03

a: tested for dose or accumulation (Ni) dependency for a specified duration

b: tested for dose and duration dependency

The combined R^2 values represent the ability of the independent variables (dose and/or duration) to predict the dependent variable (Ni concentrations in tissues).

The partial R^2 values represent the predictive power of an independent variable, when the linear effect of the other independent variable in the model is removed.

Table 3. Hematological parameters in lake whitefish fed diets containing 0, 10, 100, and 1000 µg Ni/g for 10, 31, and 104 days. No significant differences were observed between control and treatment groups for each duration ($p<0.05$).

Duration	Dose	Mean Hemoglobin Concentration (± SE) (g/dl)	Mean Glucose Concentration (± SE) (mmol/L)	Mean Percent Hematocrit (± SE)
10	0 µg Ni/g	11 (0.60)	4.5 (0.17)	38.5 (2.30)
	10 µg Ni/g	10 (0.32)	4.3 (0.24)	36.3 (1.15)
	100 µg Ni/g	11 (0.63)	4.8 (0.40)	40.0 (1.64)
	1000 µg Ni/g	10 (0.63)	4.2 (0.14)	35.2 (2.14)
31	0 µg Ni/g	9.7 (0.23)	4.0 (0.22)	40.1 (1.48)
	10 µg Ni/g	10 (0.69)	3.9 (0.18)	37.7 (2.58)
	100 µg Ni/g	10 (0.55)	4.0 (0.22)	38.3 (2.49)
	1000 µg Ni/g	9.8 (0.48)	3.9 (0.14)	38.1 (2.42)
104	0 µg Ni/g	9.1 (0.94)	4.1 (0.15)	38.4 (2.97)
	10 µg Ni/g	9.5 (0.65)	4.2 (0.09)	40.8 (1.22)
	100 µg Ni/g	8.5 (0.55)	4.9 (0.35)	36.7 (1.63)
	1000 µg Ni/g	8.9 (0.53)	4.1 (0.07)	34.8 (1.30)

Table 4. Frequency of slightly, moderately, and severely altered tubules (P1, P2, and D) in kidneys of lake whitefish fed diets containing 0, 10, 100, and 1000 µg Ni/g for 10, 31, and 104 days. Slight, moderate, and severe alterations are defined in the Materials and Methods section. Asterisks represent significant differences observed between control and treatment groups for each duration ($p<0.05$).

Duration	Dose	Mean % of altered tubules (± SE)		
		Slightly altered	Moderately altered	Severely altered
10	0 µg Ni/g	7.4 (2.8)	0 (0)	0 (0)
	10 µg Ni/g	21 (7.8)	0 (0)	0 (0)
	100 µg Ni/g	19 (2.3)	5.1 (1.3)*	0.46 (0.46)
	1000 µg Ni/g	25 (1.5)	13 (4.2)	0.46 (0.46)
31	0 µg Ni/g	7.9 (1.7)	0 (0)	0 (0)
	10 µg Ni/g	12 (2.8)	1.9 (1.4)	0 (0)
	100 µg Ni/g	27 (3.8)*	12 (3.2)*	0.93 (0.59)
	1000 µg Ni/g	29 (2.7)*	12 (2.3)*	2.3 (0.85)
104	0 µg Ni/g	4.6 (1.3)	0 (0)	0 (0)
	10 µg Ni/g	12 (2.0)	0.46 (0.46)	0 (0)
	100 µg Ni/g	26 (1.9)*	9.4 (2.4)	0 (0)
	1000 µg Ni/g	31 (3.0)*	16 (2.9)*	1.4 (0.95)

Table 5. Frequency (%) of slight, moderate, and severe alterations in first (P1) and second (P2) segments of the proximal, and distal tubule cross-sections in kidneys of lake whitefish fed diets containing 0, 10, 100, and 1000 µg Ni/g for 10, 31, and 104 days. Slight, moderate, and severe alterations are defined in the Materials and Methods section. Asterisks represent significant differences observed between control and treatment groups for each duration ($p<0.05$).

		Mean % of altered tubules (± SE)								
		P1 Tubules			P2 Tubules			D Tubules		
Duration	Dose	Slightly altered	Moderately altered	Severely altered	Slightly altered	Moderately altered	Severely altered	Slightly altered	Moderately altered	Severely altered
10	0 µg Ni/g	4.2 (2.9)	0 (0)	0 (0)	6.9 (6.9)	0 (0)	0 (0)	11 (2.8)	0 (0)	0 (0)
	10 µg Ni/g	10 (4.0)	0 (0)	0 (0)	25 (16)	0 (0)	0 (0)	29 (10)	0 (0)	0 (0)
	100 µg Ni/g	10 (2.6)	0 (0)	0 (0)	22 (5.6)	6.9 (2.6)	1.4 (1.4)	25 (2.2)*	8.3 (2.2)*	0 (0)
	1000 µg Ni/g	8.3 (3.0)	1.4 (1.4)	0 (0)	26 (2.6)	18 (6.6)*	0 (0)	39 (1.8)*	21 (7.1)*	1.4 (1.4)
31	0 µg Ni/g	5.6 (2.8)	0 (0)	0 (0)	1.4 (1.4)	0 (0)	0 (0)	17 (5.3)	0 (0)	0 (0)
	10 µg Ni/g	10 (1.4)	0 (0)	0 (0)	10 (6.9)	2.8 (0)	0 (0)	17 (5.0)	3 (0)	0 (0)
	100 µg Ni/g	21 (7.7)	2.8 (1.8)	0 (0)	24 (6.9)	14 (3.5)*	1.4 (1.4)	36 (6.3)	18 (5.9)*	1.4 (1.4)
	1000 µg Ni/g	10 (4.0)	2.8 (1.8)	0 (0)	29 (8.8)	13 (3.5)	5.6 (2.8)	49 (6.9)*	19 (5.6)*	1.4 (1.4)
104	0 µg Ni/g	1.4 (1.4)	0 (0)	0 (0)	2.8 (1.8)	0 (0)	0 (0)	10 (3.3)	0 (0)	0 (0)
	10 µg Ni/g	8.3 (3.0)	0 (0)	0 (0)	8.3 (3.7)	0 (0)	0 (0)	19 (4.7)	1.4 (1.4)	0 (0)
	100 µg Ni/g	6.9 (2.6)	1.4 (1.4)	0 (0)	24 (7.3)	5.6 (2.8)	0 (0)	33 (8.1)*	17 (6.1)*	0 (0)
	1000 µg Ni/g	22 (4.1)*	8.3 (0.00)	0 (0)	33 (8.9)	5.6 (3.5)	4.2 (2.9)	36 (5.6)*	35 (9.2)*	0 (0)

Table 6. Liver somatic indices, % change in weight, % change in length, condition factors, and sex ratios of lake whitefish fed diets containing 0, 10, 100, and 1000 µg Ni/g for 10, 31, and 104 days.

Duration	Dose	Liver Somatic Index[a]	% Change in Weight[a]	% Change in Length[a]	Condition Factor[a]	Sex Ratio (females:males)
10	0 µg Ni/g	0.73 (0.02)	4.22 (0.765)	2.01 (0.449)	1.34 (0.023)	4:2
	10 µg Ni/g	0.72 (0.02)	-0.221 (0.687)	-0.079 (0.576)	1.26 (0.016)	4:2
	100 µg Ni/g	0.70 (0.02)	6.54 (0.595)	1.16 (0.517)	1.29 (0.009)	4:2
	1000 µg Ni/g	0.67 (0.02)	5.75 (0.472)	1.40 (0.622)	1.31 (0.850)	3:3
31	0 µg Ni/g	0.73 (0.01)	10.7 (0.862)	11.5 (2.41)	1.15 (0.043)	4:2
	10 µg Ni/g	0.63 (0.02)	13.2 (1.02)	4.67 (0.660)	1.30 (0.014)	4:2
	100 µg Ni/g	0.61 (0.02)	9.23 (1.55)	2.85 (0.736)	1.30 (0.010)	3:3
	1000 µg Ni/g	0.60 (0.01)	8.87 (0.442)	3.58 (0.495)	1.25 (0.016)	0:6
104	0 µg Ni/g	0.71 (0.02)	23.3 (0.749)	7.55 (0.348)	1.31 (0.010)	3:3
	10 µg Ni/g	0.63 (0.01)	26.1 (0.796)	6.09 (0.369)	1.27 (0.007)	2:4
	100 µg Ni/g	0.70 (0.03)	35.1 (2.68)	9.49 (1.20)	1.25 (0.019)	3:2[b]
	1000 µg Ni/g	0.57 (0.01)	27.0 (0.640)	9.60 (0.847)	1.36 (0.024)	1:5

[a] Data are expressed as mean (± SE). No significant differences were observed between control and treatment groups for each duration ($p<0.05$).

[b] One individual from this treatment group was sampled prior to day 104, due to feed refusal

Figure 1. Concentrations of lipid peroxides in plasma of lake whitefish fed diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days. Data are expressed as mean (± SE). An asterisk indicates that significant differences were observed between control and treatment groups for each duration ($p<0.05$). "DL" indicates that lipid peroxide concentrations were below the analytical detection limits and a value of 1.0 nmol/ml, equal to one half of the detection limit, was assigned.

Figure 1

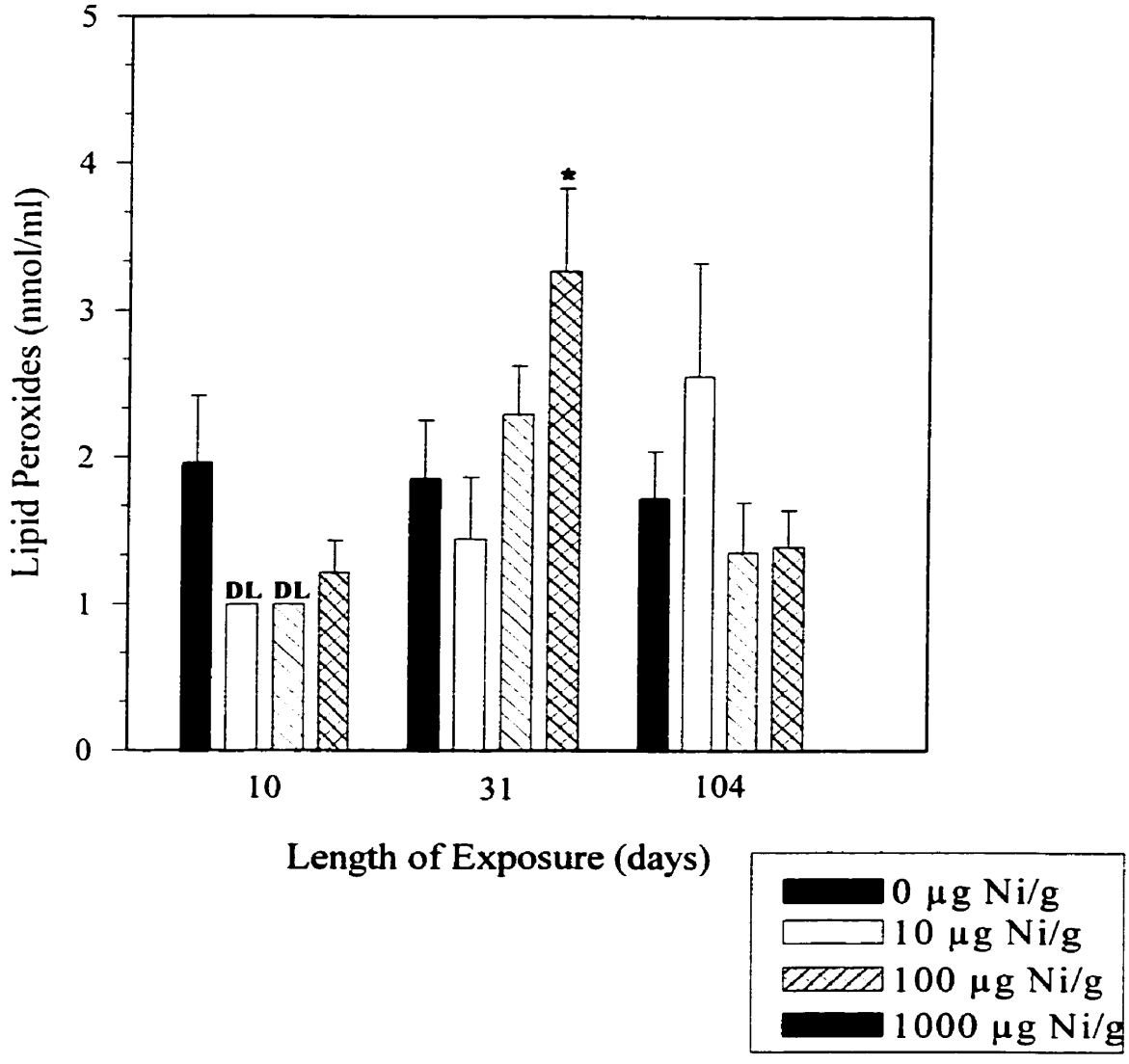

5
4
3
2
1
0
Lipid Peroxides (nmol/ml)
DL
DL
*
10
31
104
Length of Exposure (days)
0 µg Ni/g
10 µg Ni/g
100 µg Ni/g
1000 µg Ni/g

Figure 2: Posterior kidney Photomicrographs. a) Posterior kidney of a lake whitefish fed a control diet. Bar = 30μm. H&E stain. P1, 1st segment of the proximal tubule; P2, 2nd segment of the proximal tubule; D, distal tubule; G, glomerulus; H, hematopoietic tissue. b) Posterior kidney of a lake whitefish fed 1000 μg Ni/g for 104 days. Alterations, including necrotic tubular epithelial cells (NC), desquamated epithelial cells (EC), accumulation of cellular debris in lumen of tubules (DE), and degenerating tubules (U, segments unidentifiable), are observed. Bar = 30 μm. H&E stain. P2, 2nd segment of the proximal tubule. c) Glomerulus of a lake whitefish fed a control diet. Bar = 20 μm. H&E stain. G, glomerulus; C, capillary; E, erythrocyte, BC, Bowman's capsule; BS, Bowman's space. d) Accumulation of acellular debris (DE) in Bowman's space (BS) of a glomerulus of a lake whitefish fed 1000 μg Ni/g for 104 days. Bar = 20 μm. H&E stain. e) Distal tubule in kidney of a lake whitefish fed a control diet. Nuclei of epithelial cells are spherical and basally located (EC). Bar = 20 μm. H&E stain. D, Distal tubule; L, lymphocyte. f) Distal tubule in the kidney of a lake whitefish fed 1000 μg Ni/g for 10 days. Desquamated necrotic epithelial cells (NC) accumulate in the lumen of the tubule. Bar = 20 μm. H&E stain. D, Distal tubule. g) Distal tubule in the kidney of a lake whitefish fed 1000 μg Ni/g for 104 d. Bar = 20 μm. H&E stain. Alterations, including necrotic tubular epithelial cells (NC), and accumulation of cellular debris in the lumen of tubules (DE), are observed. D, Distal tubule. h) Severely altered tubule (U, segment unidentifiable) in kidney of a lake whitefish fed 1000 μg Ni/g for 104 days. Bar = 20 μm. H&E stain. DH, depletion of hematopoietic tissue.

Figure 2

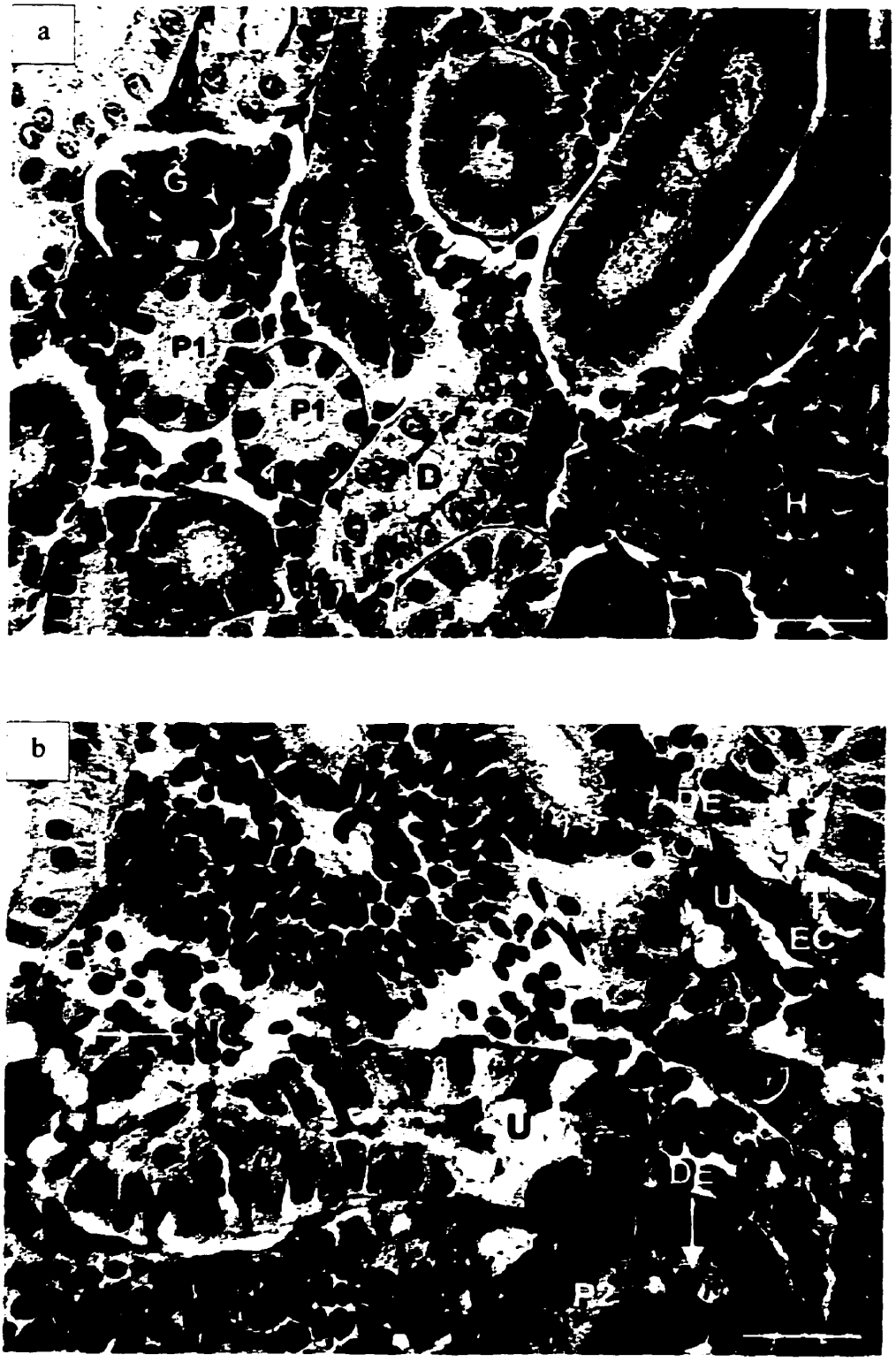


Figure 2

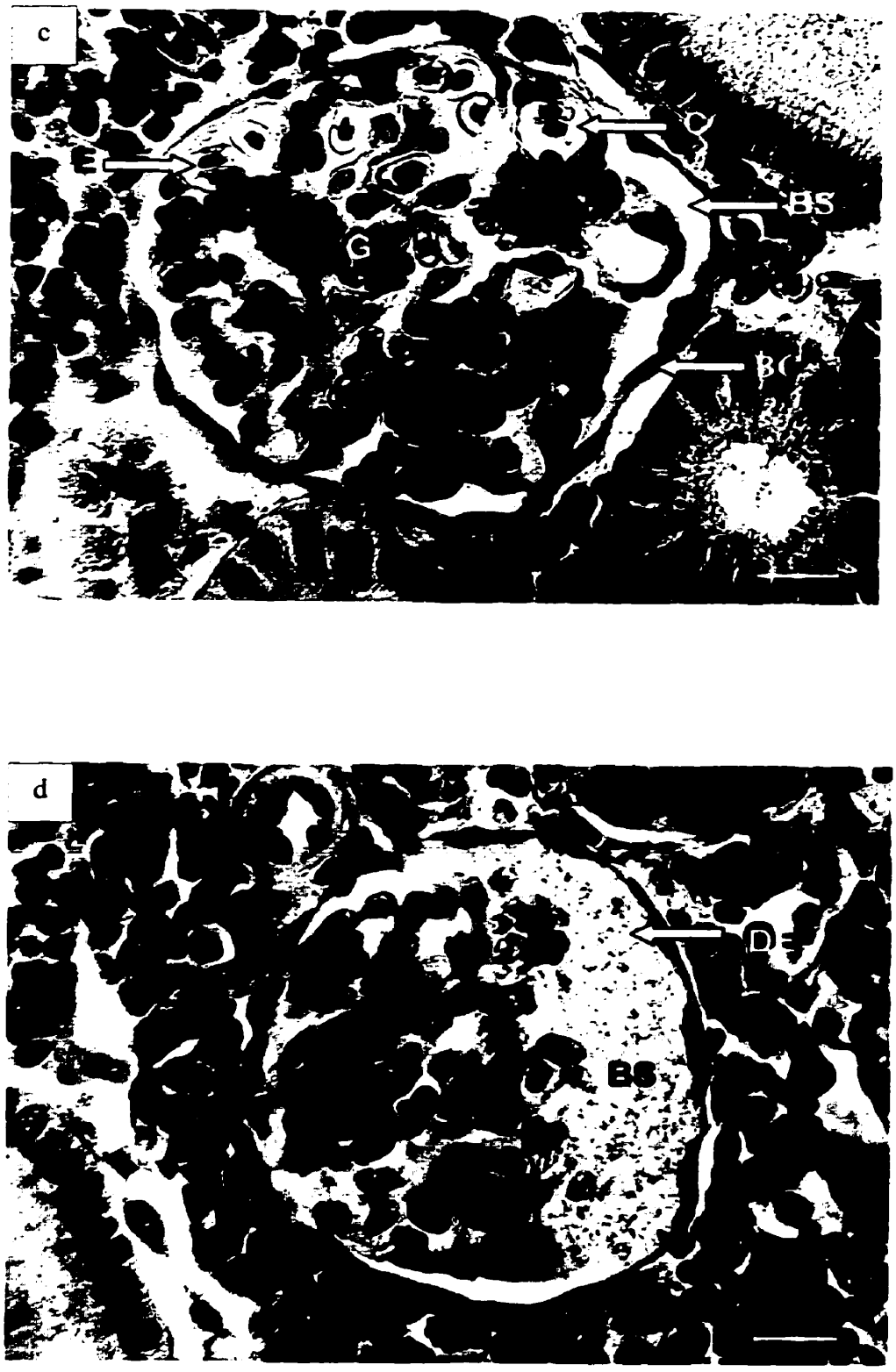


Figure 2

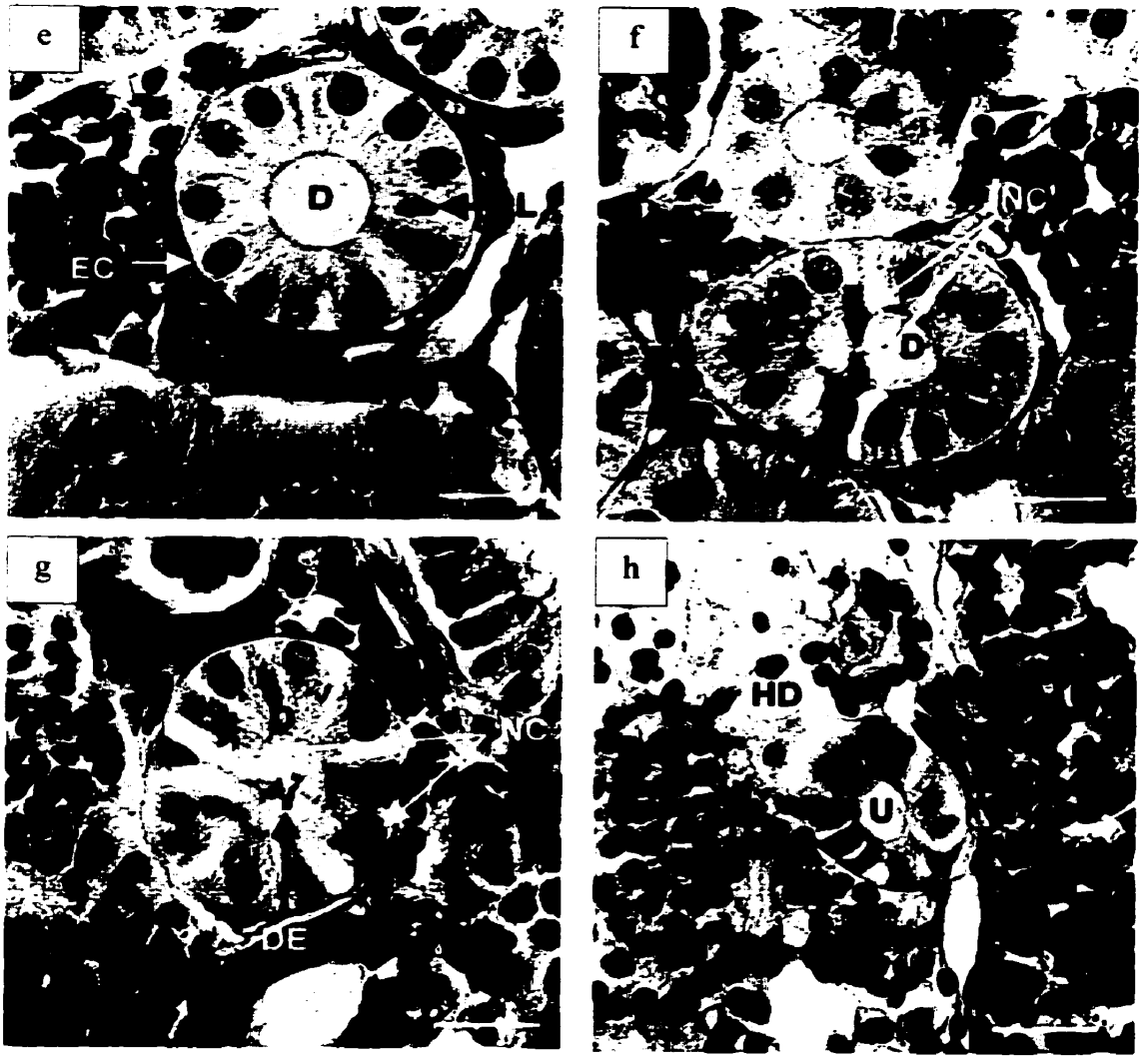


Figure 3. Frequency (%) of altered a) glomeruli, b) distal tubules, c) 1st segment of proximal tubules, and d) 2nd segment of proximal tubules in kidneys of lake whitefish fed diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days. Data are expressed as mean (± SE). Asterisks represent significant differences observed between control and treatment groups for each duration ($p<0.05$).

Figure 3

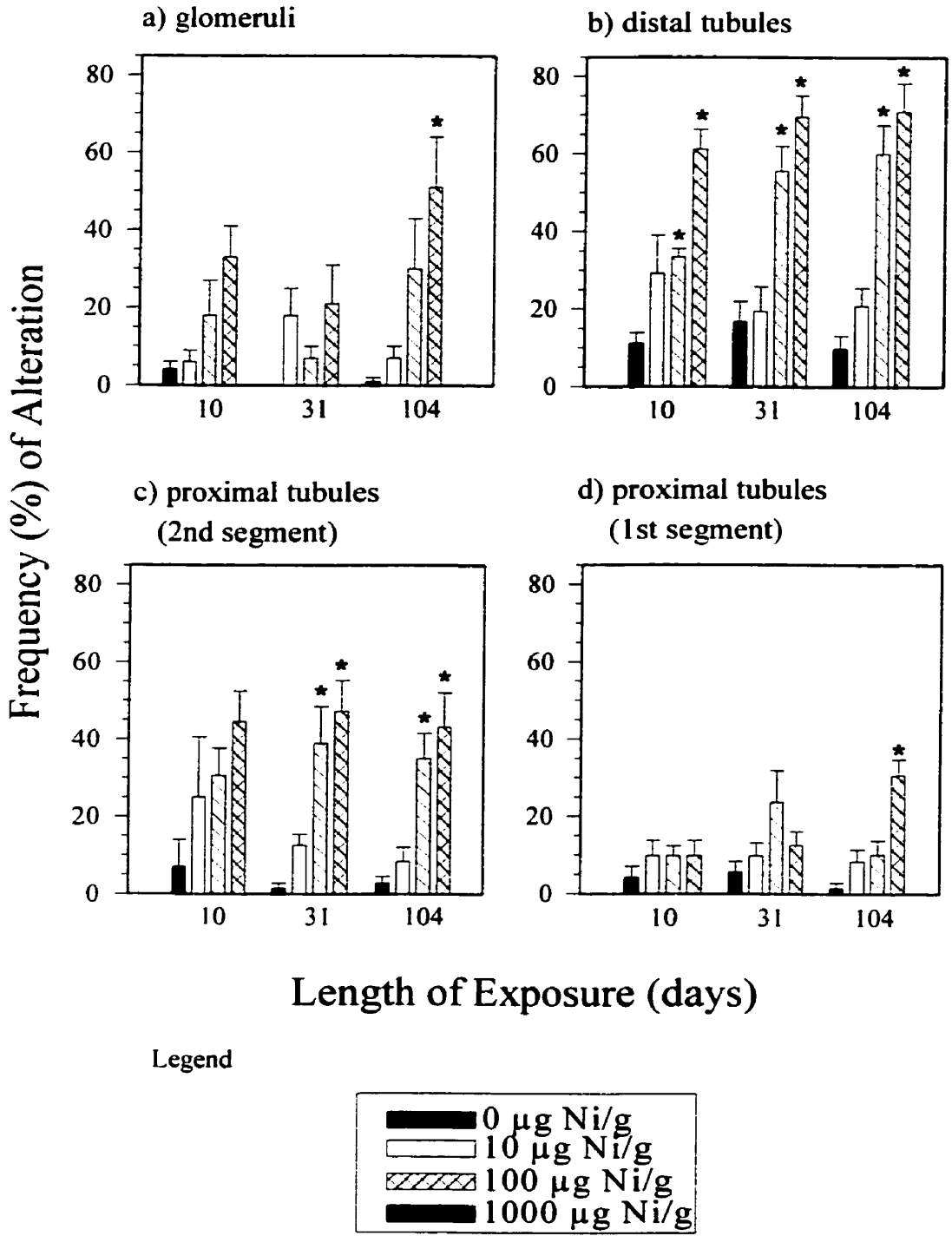

a) glomeruli
b) distal tubules
c) proximal tubules
(2nd segment)
d) proximal tubules
(1st segment)
Frequency (%) of Alteration
0
20
40
60
80
10
31
104
Length of Exposure (days)
Legend
0 µg Ni/g
10 µg Ni/g
100 µg Ni/g
1000 µg Ni/g

Figure 4. Frequency (%) of altered field of views in kidneys of lake whitefish fed diets containing 0, 10, 100, and 1000 μg Ni/g for 10, 31, and 104 days. An area measuring 355 x 230 μm was visible in each field of view. Data are expressed as mean (± SE). Asterisks represent significant differences observed between control and treatment groups for each duration ($p<0.05$).

Figure 4

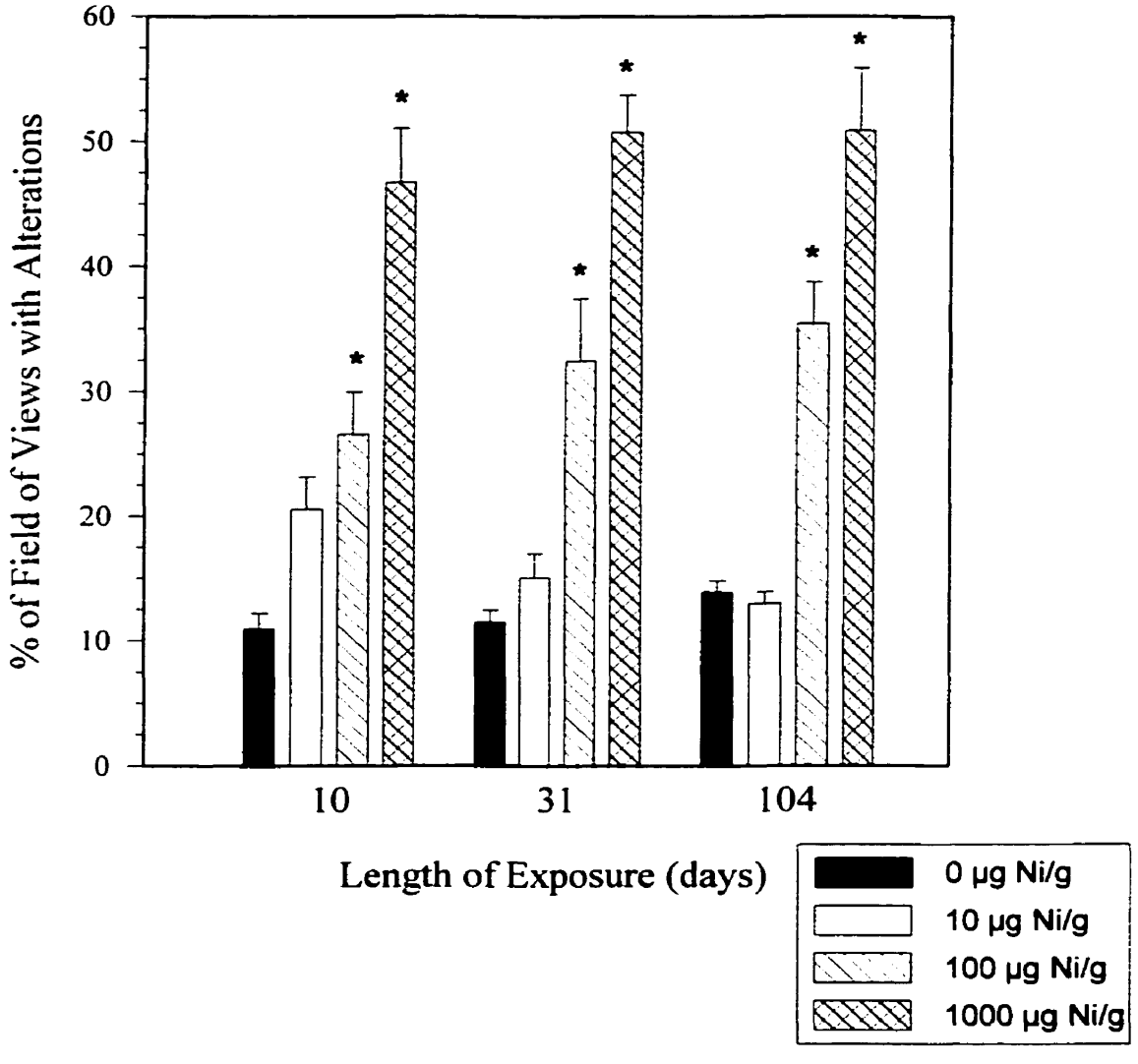
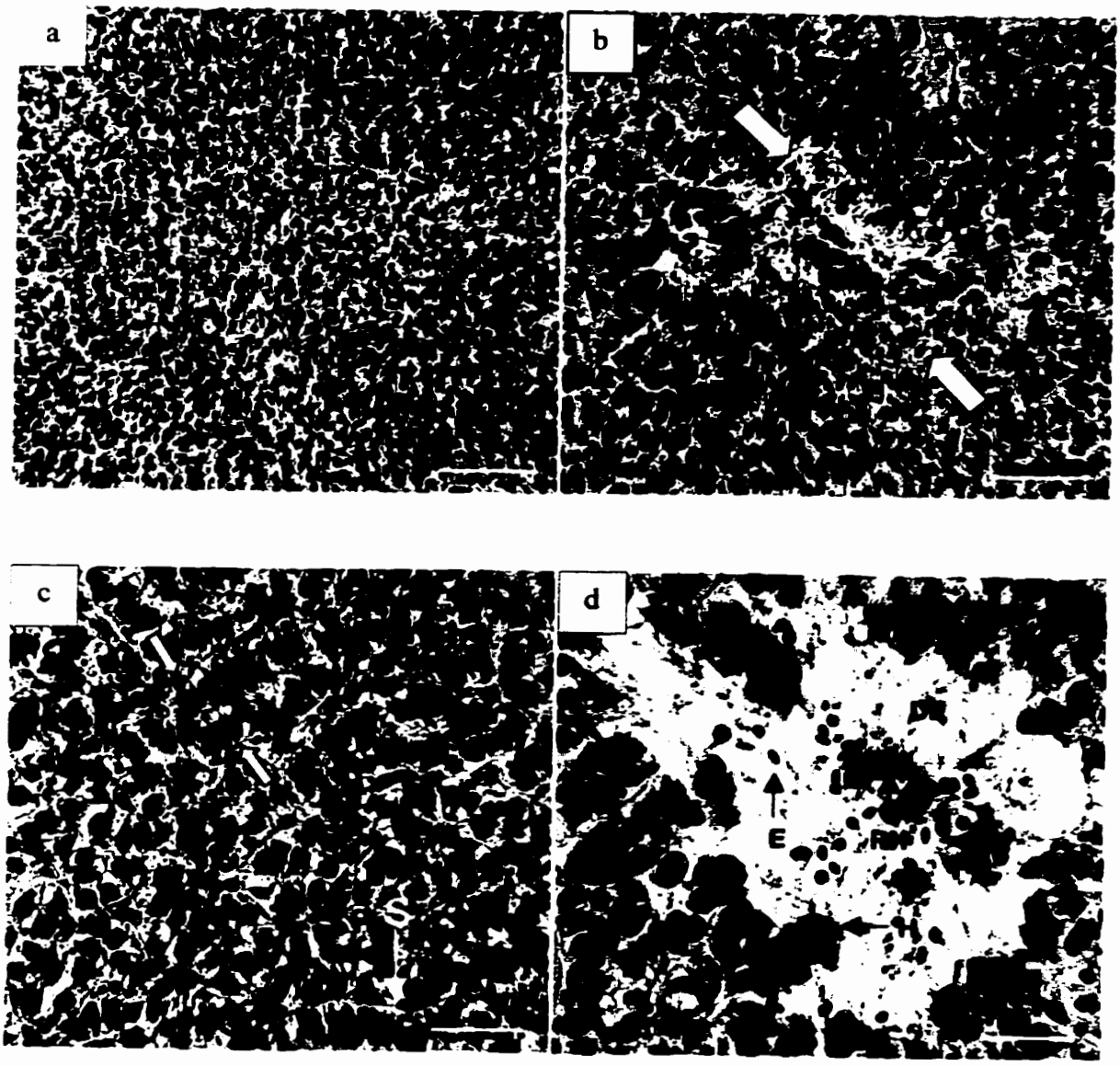

60
50
40
30
20
10
0
% of Field of Views with Alterations
*
10
31
104
Length of Exposure (days)
0 µg Ni/g
10 µg Ni/g
100 µg Ni/g
1000 µg Ni/g

Figure 5. Liver photomicrographs. a) Low magnification photomicrograph of the liver of a lake whitefish fed a control diet. Bar = 80 μm. H&E stain. b) Low magnification photomicrograph of a lesion (between arrows) in the liver of a lake whitefish fed 1000 μg Ni/g for 104 days. Bar = 80 μm. H&E stain. c) High magnification photomicrograph of the liver of a lake whitefish fed a control diet. Normal cellular architecture, with hepatocytes arranged in 2-cell thick cords (between arrows) that are separated by sinusoids (S) containing erythrocytes, is observed. Bar = 30 μm. H&E stain. d) High magnification photomicrograph of a lesion in the liver of a lake whitefish fed 1000 μg Ni/g for 104 days. An area of dissolution, with ruptured hepatocytes (RH), cellular debris (DE), and erythrocytes (E), is surrounded by hepatocytes with darkened nuclei and eosinophilic cytoplasm (H). Bar = 30 μm. H&E stain.

Figure 5



GENERAL CONCLUSIONS

Chapter One

The accumulation, distribution, and toxicity of dietary Ni in lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*).

This preliminary experiment was used to determine which diet concentrations, diet type, and fish species to use in the long-term experiment. The findings of this study were as follows:

- Diets
    - Diets selected for the long-term experiments will contain 0, 10, 100, and 1000 µg Ni/g because lake trout and lake whitefish readily consumed diets containing 1000 µg Ni/g, but refused diets containing 10000 µg Ni/g.
    - Brine shrimp will not be incorporated into the diets used in the long-term exposure because it was not observed to increase the consumption of the high dose diets.

- Selection of fish species
    - Lake whitefish were selected for use in the long-term experiments because of the following observations:
        - lake whitefish consumed the high dose diet for a longer period of time
        - increases in Ni concentrations in all lake whitefish tissues, except intestine, were associated with increases in the dose of Ni administered

- o lake whitefish fed Ni-contaminated diets accumulated Ni at concentrations similar to those observed in natural populations of whitefish residing in Ni-contaminated environments
- o lake whitefish fed 10000 µg Ni/g (prepared without shrimp) exhibited alterations in the endogenous concentrations of Cu and Zn in kidney and liver
- o lake whitefish sustained renal and hepatic lesions due to consumption of Ni-contaminated diets

Chapter Two

The accumulation and distribution of dietary Ni in lake whitefish (*Coregonus clupeaformis*).

- Fish fed 100 and 1000 µg Ni/g accumulated significant amounts of Ni in a majority of the tissues sampled.
- The highest Ni concentrations were observed in intestine and pyloric caeca of fish fed the high dose diet for 10 days. Protective mechanisms including increased excretion, desquamation of the mucosal layer, and/or controlled uptake may explain the decreased Ni accumulation observed in the intestine and pyloric caeca after 31 and 104 days of exposure.
- Ni accumulation, in stomach, kidney, liver, gill, skin, and scales, was dose and duration-dependent, with the highest concentrations observed in the kidney and scales.

- Tissues that best assess dietary Ni bioavailability in freshwater fish are the kidney and scales.
- Analyses of the tissues and contents of the gastrointestinal tract from natural populations of fish collected from contaminated areas are recommended to elucidate the route of exposure to Ni.

Chapter Three

The toxicology of dietary Ni in lake whitefish (*Coregonus clupeaformis*).

- Histopathological lesions in kidney and liver proved to be the most sensitive and reliable indicators of Ni exposure.
    - In liver of treated fish, areas of focal necrosis and altered bile ducts were observed.
    - Histological alterations were observed throughout the posterior kidneys of fish fed medium and high dose diets, in glomeruli, tubules, collecting ducts, and hematopoietic tissue. In whitefish kidneys, the percentage of altered D tubules, combined percentage of altered tubules (P1, P2, and D), and percentage of fields of views with alterations all increased with the dose and duration of exposure.
- Molecular responses, including metallothionein induction in intestine and production of lipid peroxides in plasma, may, with additional research, prove to be important indicators of Ni exposure in natural fish populations.

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